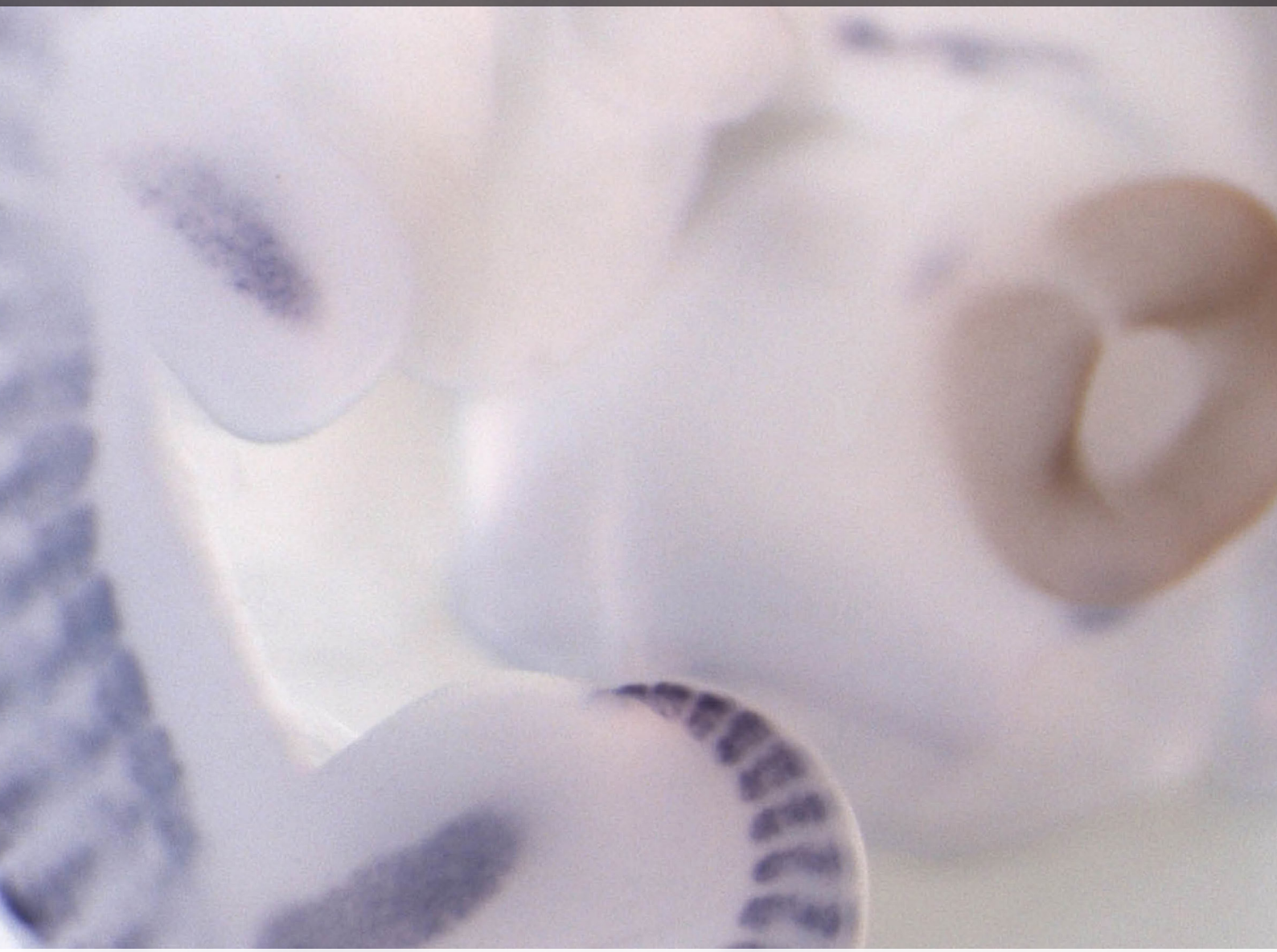
SIGNALLING PATHWAYS IN EMBRYONIC DEVELOPMENT

EDITED BY: Juan J. Sanz-Ezquerro, Andrea E. Münsterberg and Sigmar Stricker

PUBLISHED IN: Frontiers in Cell and Developmental Biology



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ISSN 1664-8714
ISBN 978-2-88945-346-7
DOI 10.3389/978-2-88945-346-7

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SIGNALLING PATHWAYS IN EMBRYONIC DEVELOPMENT

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Whole-mount in-situ hybridisation of a 4 day old chicken embryo for MyoD shows the developing muscles in the somites (striped pattern), the limbs and branchial arches.

Image by Sigmar Stricker.

The formation of a complex multicellular organism from a single cell is one of the most amazing processes of biology. Embryonic development is characterised by the careful regulation of cellular behaviours such that cells proliferate, migrate, differentiate and form tissues at the correct place and time. These processes are genetically controlled and depend both on the history of cells, their lineage, and on the activities of signalling pathways, which coordinate the cell interactions leading to organogenesis.

The aim of the Frontiers research topic "Signalling pathways in embryonic development" has been to provide a forum for experts in cell and developmental biology to share recent advances

in the field of signalling during embryonic development. Sixteen articles in a variety of formats are united in this Topic, offering a valuable collection for researchers looking for an update in the knowledge of signalling pathways operating during embryogenesis. The works, focused mainly on vertebrates, explore different aspects of this theme from cell communication to organ formation and have implications for areas as distant as evolution or pathology.

Understanding developmental signalling pathways is important for several reasons. It gives us information about basic mechanisms of cell function and interactions needed for morphogenesis and organogenesis. It uncovers the basis of congenital malformations, since errors at any step of cell signalling during development are a major cause of defects. This fundamental insight gives us clues to understand the mechanisms operating in evolution that explain diversity in form and function. And finally, it allows the identification of possible causes of disease in the adult organism (such as cancer or degenerative diseases) pinpointing possible targets for therapeutic approaches.

Citation: Sanz-Ezquerro, J. J., Münsterberg, A. E., Stricker, S., eds. (2017). Signalling Pathways in Embryonic Development. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-346-7

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frontiers in Cell and Developmental Biology

EDITORIAL
published: 31 August 2017
doi: 10.3389/fcell.2017.00076

Editorial: Signaling Pathways in Embryonic Development

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Keywords: signaling pathways, embryo development, mechanotransduction, notch signaling pathway, Wnt Signaling Pathway, Shh signaling

Editorial on the Research Topic

Signaling Pathways in Embryonic Development

The formation of a complex multicellular organism from a single cell is one of the most amazing processes of biology. Embryonic development is characterized by the careful regulation of cellular behaviors such that cells proliferate, migrate, differentiate, and form tissues at the correct place and time. These processes are genetically controlled and depend both on the history of cells, their lineage, and on the activities of signaling pathways, which coordinate the cell interactions leading to organogenesis.

A limited number of key signaling pathways—Fgf, Hedgehog, Wnt, TGFß, Notch among the most important—operate during development, acting repeatedly at different times and in different regions in the embryo and eliciting diverse cellular responses. This raises the question of how cells integrate all the information they receive and can respond in cell type-specific ways to the same signals. Classical concepts in embryology such as organizers (groups of cells producing instructive signals) and competence (ability of cells to respond) can now be analyzed in molecular terms. In recent years many advances have been made in identifying the signals acting during embryo development and understanding their properties and functions, which is equally of relevance for human pathology and evolution. An important discovery is the conservation of signals and mechanisms, not only in evolutionary terms (similar genes and signals acting in distant organisms), but also in the repeated use of the same signaling pathways at different times and places in the embryos. Moreover, many of those mechanisms are involved in adult tissue homeostasis and regeneration.

Understanding developmental signaling pathways is important for several reasons. It gives us information about basic mechanisms of cell function and interactions needed for morphogenesis and organogenesis. It uncovers the basis of congenital malformations, since errors at any step of cell signaling during development are a major cause of defects. Fundamental insight also gives us clues to understand the mechanisms operating in evolution that explain diversity in form and function. And finally, it allows the identification of possible causes of disease in the adult organism (such as cancer or degenerative diseases) pinpointing possible targets for therapeutic approaches.

In this context, the aim of the Frontiers research topic "Signaling pathways in embryonic development" has been to provide a forum for experts in cell and developmental biology to share recent advances in the field of signaling during embryonic development. Sixteen articles in a variety of formats are united in this Topic, offering a valuable collection for researchers looking for an update in the knowledge of signaling pathways operating during embryogenesis. The works, focused mainly on vertebrates, explore different aspects of this theme from cell communication to organ formation and have implications for areas as distant as evolution or pathology.

Among the signaling pathways with important and widespread roles in development is the Wnt pathway, comprising a family of ligands with homology to wingless in Drosophila. Wnts can bind

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Edited and reviewed by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *02 August 2017*
Accepted: *11 August 2017*
Published: *31 August 2017*

Citation:
Sanz-Ezquerro JJ, Münsterberg AE and Stricker S (2017) Editorial: Signaling Pathways in Embryonic Development. Front. Cell Dev. Biol. 5:76. doi: 10.3389/fcell.2017.00076

to multiple receptor complexes and trigger several downstream signaling cascades [including the so-called canonical WNT/β-catenin dependent signaling pathway, the non-canonical WNT/planar cell polarity (PCP), and the WNT/Ca^{2+} pathways], illustrating how the same signal can elicit diverse cellular responses depending on the cell type, context, and developmental timing. Fujimura reviews the role of canonical Wnt signaling in eye development, highlighting the important roles it plays in patterning of ocular tissue, differentiation of retinal pigment epithelium, and morphogenesis of the optic cup. Importantly, mis-regulation of the signaling cascade can lead to eye malformations and disease. Gentzel and Schambony review a group of core intracellular effectors of the Wnt pathway, disheveled (DVL) proteins, which comprise three members in vertebrates. Although all DVLs share a common basic function in Wnt signaling, the expression patterns, and functions of the different isoforms are not totally redundant and have also diverged between different species, suggesting they play specific roles depending on the tissue distribution and specific interactions. Again, mutations in DVL genes can cause human congenital disease, highlighting their important role in development. Additionally, Berger et al. review the role of PTK7 (protein tyrosine kinase 7, a transmembrane receptor) in the fine-tuning of the Wnt signaling network. Its functions in establishing cell polarity, regulation of cell movements, and migration are also essential for development and disease, particularly in cancer and metastasis.

Another important signaling pathway is Notch, a transmembrane protein that mediates juxtacrine cell-cell communication. Notch has many functions in organ formation and adult homeostasis, including cell determination and stem cell maintenance. Carrieri and Dale review the particularly well-studied function of Notch in somitogenesis and also present recent data on the role of FBXW7 protein in regulating the turnover of Notch intracellular domain (NICD, the effector of the pathway), in development and cancer. This relates to an often-overlooked essential point in signaling, which is the termination of activation and resetting of the components, allowing the cells to become competent again. Multiple mechanisms of regulation exist (positive and negative feedback loops) that allow a fine control of signaling pathways at different steps of the intracellular cascades.

Crosstalk between the limited numbers of signaling pathways is a mechanism that allows cells to respond differently to the same signal, producing the diverse cellular behaviors that are needed to build tissues and organs. A new example of this is provided by Bernatik et al. reporting on the role of the BMP antagonist Noggin in sensitizing cells and potentiating the activation of non-canonical Wnt signaling in skeletal development. They also provide evidence for a genetic interaction between these two pathways, which are involved in human congenital malformations.

The role of specific signaling pathways in the formation of particular organs is discussed in other articles. Díez del Corral and Morales review the multiple roles of Fgf signaling in the developing spinal cord. This important structure of the nervous system arises from neural derivatives of an early neuromesodermal population located at the caudal part of the embryo. Extension of this region is coupled to spinal cord formation and several essential processes such as neurogenesis, ventral patterning or neural crest specification are controlled by Fgf signaling. These embryonic functions of Fgfs could be related to its ability to promote regeneration in the injured spinal cord of adults.

Signalling pathways often converge on controlling the expression of transcription factors, which regulate cell fate specification. The integration of Notch signaling and bHLH transcription factors during inner ear development is analyzed by Gálvez et al. which also highlight that these same mechanisms are involved in hair cell regeneration, opening avenues for possible therapeutic approaches in hearing impairment. Ear development is also the topic reviewed by Magariños et al. They present evidence for a crucial role of autophagy, the regulated process of degradation, and recycling of cellular components, in vertebrate inner ear formation.

The limb is a classic model in embryology and some of the most important discoveries related to the roles of signaling pathways in pattern formation, growth, and differentiation have been made studying limb development. Tickle and Towers review the role of Shh in this process, a paradigm of how signals control and integrate tissue pattern and growth. They also discuss the implications of this important pathway for congenital malformations in humans and for the generation of limb morphological diversity during evolution. Montero et al. also treat this evolutionary aspect in their perspective article. They present a detailed analysis of Sox9 expression in developing digits of several species. This transcription factor, regulated by signaling pathways such as BMPs, Tgfßs, or Fgfs is involved in formation of the chondrogenic template of the skeleton. Differences in Sox9 expression patterns among species that have specific morphologies may reflect differences in signaling pathways controlling its expression. Also related to skeletal development, Amara et al. show that the effects of Calcium/Calmodulin dependent kinase II (CAMKII), an effector for Ca+2 -dependent signal transduction, in promoting chondrogenic differentiation seems to be specific for chicken embryos. This function is not observed in the mouse, thus highlighting the existence of differences in signaling functions and regulation among different species.

Integration of extrinsic and intrinsic regulatory cues is essential for organ formation. Dueñas et al. review the role of signals, transcription factors and cellular processes in the formation of the epicardium. This is the external-most layer of the heart that serves not only as the outer cover for this organ, but also seems to play a role in regeneration. Thus, understanding the basis of its development may have important therapeutic implications. Two articles deal with muscle development. Hernandez-Torres et al. review the role of Pitx2 in embryonic and adult myogenesis. A hierarchy of transcription factors controls skeletal muscle differentiation and Pitx2 plays an important role in the regulation of this process. Importantly, it also seems to be involved in the establishment and function of satellite

cells, the stem cells resident in adult muscle, thus opening new avenues for development of regenerative therapies. Additionally, Nassari et al. review the role of connective tissues in muscle development. Apart from the intrinsic molecular signals mentioned above, the interaction of muscle cells with surrounding tissues (bone, cartilage, tendon, and ligament) is critical for the correct assembly of the musculoskeletal system during development and for maintaining adult homeostasis.

An emerging theme in developmental biology is the control of tissue morphogenesis by physical forces (mechanotransduction). Valdivia et al. review the mechanical control of myotendinous junction formation and tendon differentiation, highlighting again the importance of the interplay between chemical and mechanical signaling during embryogenesis. In the same line, Stricker et al. provide a timely discussion reminding us that cells in embryos and adult organisms are not present in isolation, but embedded in extracellular matrices into complex tissues. Cells attach to the ECM and sense its mechanical properties. Typically, experimental *in vitro* conditions do not fully reproduce this environment, which is however critical for the physiological cellular responses to signaling cascades. The challenge for the future is to try and integrate as many interactions as possible when designing experiments.

We hope that the articles in this topic will be of interest to researchers working in development and cell biology, fuelling discussion on this area and opening new avenues for thinking and investigation.

AUTHOR CONTRIBUTIONS

JS was the Guest editor of this Research Topic, inviting co-editors AM and SS and working with them to define the subjects to be treated. They identified and invited leaders in specific research fields to contribute their work to the Research Topic. They acted as handling editors of manuscripts in the topic. JS wrote the Editorial with input from the other co-editors.

FUNDING

Research in AM laboratory is supported by BBSRC grants BB/N007034, BB/N002970 and BHF grant PG/15/77/31761. Research in JS laboratory is supported by grants SAF2013-45331-R and SAF2016-79792-R from spanish MINECO.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 02 June 2017
doi: 10.3389/fcell.2017.00058

The Multiple Roles of FGF Signaling in the Developing Spinal Cord

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During vertebrate embryonic development, the spinal cord is formed by the neural derivatives of a neuromesodermal population that is specified at early stages of development and which develops in concert with the caudal regression of the primitive streak. Several processes related to spinal cord specification and maturation are coupled to this caudal extension including neurogenesis, ventral patterning and neural crest specification and all of them seem to be crucially regulated by Fibroblast Growth Factor (FGF) signaling, which is prominently active in the neuromesodermal region and transiently in its derivatives. Here we review the role of FGF signaling in those processes, trying to separate its different functions and highlighting the interactions with other signaling pathways. Finally, these early functions of FGF signaling in spinal cord development may underlay partly its ability to promote regeneration in the lesioned spinal cord as well as its action promoting specific fates in neural stem cell cultures that may be used for therapeutical purposes.

Keywords: spinal cord, spinal cord injury, neuromesodermal progenitors, neural stem cells, patterning, neurogenesis, caudal extension, FGF

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Centro Nacional de Biotecnología, Spain

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *17 February 2017*
Accepted: *11 May 2017*
Published: *02 June 2017*

Citation:
Diez del Corral R and Morales AV (2017) The Multiple Roles of FGF Signaling in the Developing Spinal Cord. Front. Cell Dev. Biol. 5:58. doi: 10.3389/fcell.2017.00058

INTRODUCTION

The spinal cord is the most caudal part of the nervous system which is responsible for body motion, including locomotion, somatosensation and the control of basic functions of the autonomous nervous system. During development, in addition to the neurons that reside within the spinal cord, it provides neural crest cells for the formation of sensory ganglia, ganglia of the autonomous system and for the enteric nervous system. A fundamental aspect of spinal cord development is its relation to the organs and muscles it innervates. Thus, spinal cord development appears highly coordinated in space and time with the caudal extension that accompanies the development of the whole body.

The spinal cord cells of vertebrates derive from a region initially specified as neuromesodermal progenitors (NMP) with mixed neural and mesodermal characteristics (Wilson et al., 2009; Henrique et al., 2015; Row et al., 2016), with the exception of those forming the floor plate which in amniotes derive from the node. In chick and mouse, this corresponds to a region of the epiblast adjacent to the early node and the rostral primitive streak. From this population, some cells remain in the ectoderm layer and form most of the spinal cord, while others gastrulate through the primitive streak to become part of the paraxial mesoderm (Wilson et al., 2009; Henrique et al., 2015). Later, with the closure of the caudal neuropore, the NMP region remains in the tailbud from which the caudal spinal cord and mesodermal populations segregate. Overall, this constitutes an ongoing process that takes several days to generate the complete rostrocaudal axis. Different aspects of spinal cord development such as initiation of neurogenesis, ventral patterning and neural crest specification and migration are conditioned by this caudal axis elongation (**Figure 1**). This

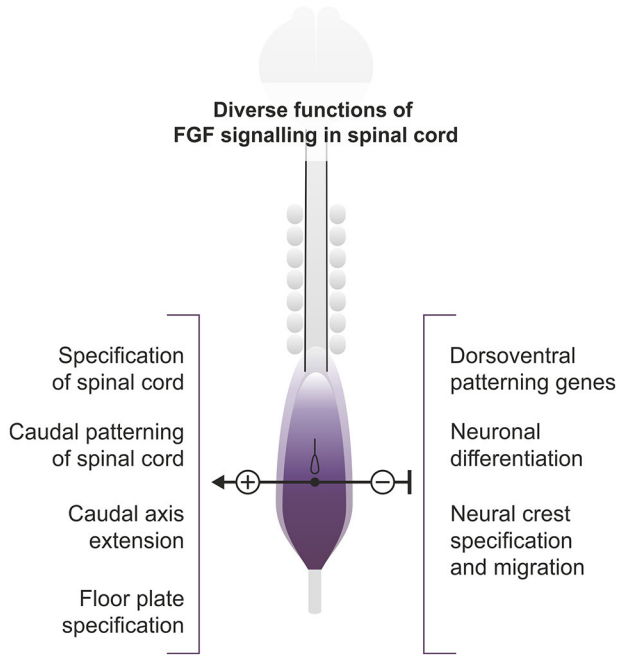

FIGURE 1 | Roles of FGF signaling in the extending spinal cord. Diagram representing a 7-somite stage chick embryo and showing the region of active caudal FGF signaling. The different processes discussed in the review either promoted (+) or inhibited (–) by FGF signaling are shown.

is a complex process involving several signaling pathways and gene networks in which the FGF signaling pathway stands as a crucial regulator, maintaining cells in an immature state until they are displaced to a region where they are no longer influenced by it.

FGF signaling acts in numerous stages and tissues during embryonic development and the use of experimental approaches designed to manipulate the FGF signaling pathways at specific stages and tissues has been fundamental to overcome its early roles in implantation, gastrulation, and neural induction. These include treatment of tissue explants with FGF factors or pharmacological antagonists, expression of pathway inhibitors or truncated FGFR proteins that function interfering with the normal function in the neural tube cells *in ovo* and the use of conditional mouse mutants specifically removing FGFs or FGFR in the NMP and its derivatives.

Here, we review the contribution of FGF signaling in the initial process of spinal cord specification and elongation and then we cover the initiation of neurogenesis, ventral patterning, and neural crest specification and migration. We make an effort to identify and separate the different steps in these highly interconnected networks governing spinal cord extension and associated events, focusing on the influence of FGF signaling on the neural tissue. In addition, we have selected some of the evidence supporting the use of FGF to promote regeneration of the lesioned adult spinal cord both acting on spinal cord cells *in vivo* as well as to promote expansion of neural stem cells *in vitro* and their differentiation toward specific neuronal fates for their use for regenerative purposes.

Note: Gene symbols are italicized in all species, but there are specie-specific differences. Thus, gene symbols for human and chick appear all in upper-case; for mouse and rat with only the first letter in upper-case and for fish, gene symbols appear with all letters in lower-case. In the case of protein symbols, they are not italicized and all letters are in upper-case, except in fishes where only the first letter is upper-case (http://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx). When referring to genes from several species they have been separated by a slash.

FGF SIGNALING PATHWAY: EXPRESSION OF COMPONENTS IN THE DEVELOPING SPINAL CORD

Let's first start with a brief introduction of the components of the FGF signaling pathway in the context of spinal cord development. As most signaling pathways, the FGF pathway includes ligands, receptors, modulators, intracellular transducers, and final effectors (Ornitz and Itoh, 2015). The only components exclusive for the pathway are the ligands (up to 23 FGFs have been described in vertebrates) and their receptors of the tyrosin kinase (RTK) type (FGFR1–4 in vertebrates). Other more general players, which are also used by other signaling pathways, such as the pathway inhibitors SPROUTY2, SEF, DUSP6, and the transcription factor effectors of the ETV family, are particularly associated to this pathway as the corresponding mRNAs are highly expressed in regions with high FGF activity and in particular in the caudal NMP region (Chotteau-Lelievre et al., 2001; Karabagli et al., 2002; Corson et al., 2003; Harduf et al., 2005; Lunn et al., 2007). Interestingly, they are themselves downstream targets of the pathway and are thus considered its readouts and have been the basis for the development of pathway activity reporters (Molina et al., 2007; Ekerot et al., 2008). However, as these downstream targets of the pathway are not exclusively activated by the FGF pathway, they do not constitute definitive readouts of the activity of the FGF pathway. The identification of cells where the pathway is truly active is still one of the main difficulties in the analysis of FGF function, as none of the intracellular cascades is specific for FGF signaling and the difference with other RTK pathways may be in the fine tuning of the signaling properties.

The three main intracellular cascades that can mediate the FGF signal are: the RAS-MAPK, the PI3K-AKT and the PLPCγ pathways (**Figure 2**). High levels of MAPK phosphorylation are detected in the NMPs and surrounding area and these depend on the activation of FGF pathway (Lunn et al., 2007). Moreover, most of the effects resulting from FGFR inhibition in this region can also be observed following the inhibition of MEK (MAPK Kinase), suggesting this is the main FGFR downstream pathway in this region (Diez del Corral et al., 2002; Delfino-Machin et al., 2005; Lunn et al., 2007; Martinez-Morales et al., 2011; Olivera-Martinez et al., 2012; Morales et al., 2016).

A gradient of AKT phosphorylation has also been described in the region surrounding the node with higher levels caudally (Dubrulle et al., 2001) but the exposure to PI3K inhibitors does

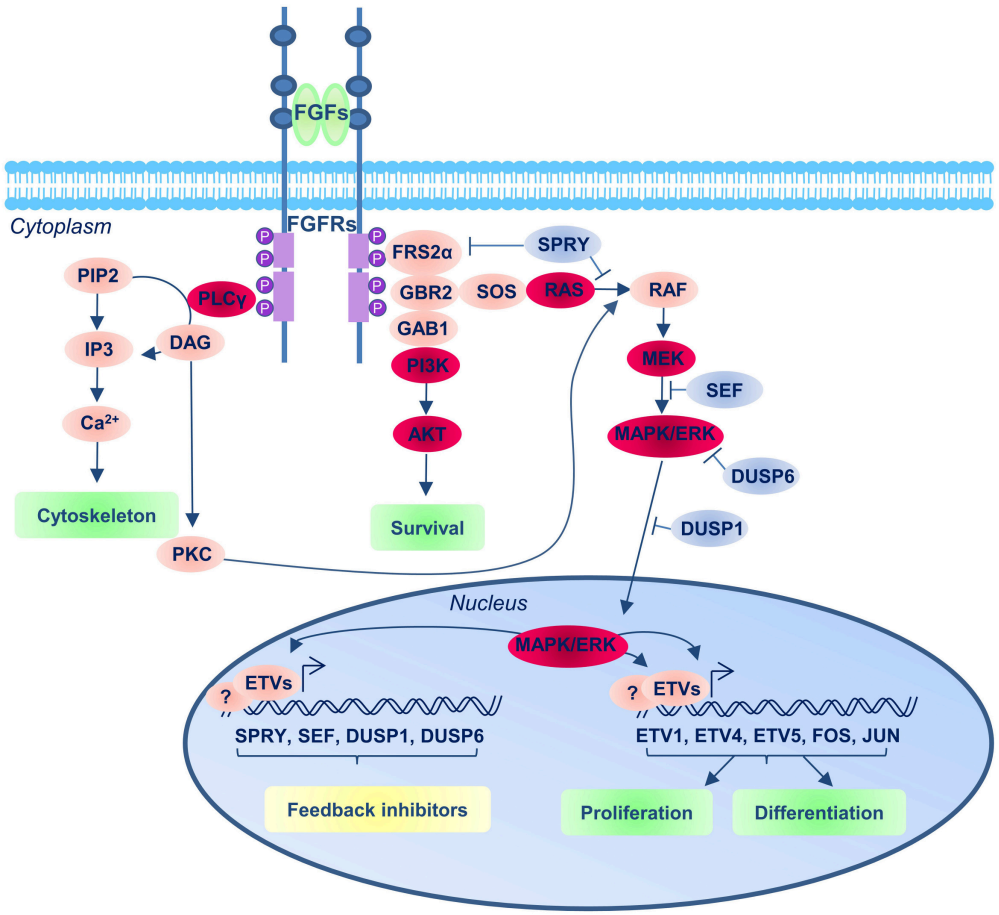

FIGURE 2 | FGF signaling pathway. The FGFRs consist of three extracellular immunoglobulin-type domains (D1–D3; blue balls in the receptor), a single-span trans-membrane domain and an intracellular split domain. FGFs interact with the D2 and D3 domains, and promote upon binding receptor dimerization and tyrosine kinase autophosphorylation of the FGFRs that results in the recruitment and assembly of signaling complexes. The main three downstream FGF/FGFR signaling complexes operating in the context of neural development are represented (the red balloons indicate the main components of the pathway): the Ras/MEK/MAPK/ERK; the PI3K/AKT and the PLCγ pathways. The blue balloons indicate the repressor regulators of the pathways.

not result in the same effects as blockade of FGFR signaling (Martinez-Morales et al., 2011) and thus the relevance of the AKT pathway in this context has not been addressed further.

The most comprehensive analysis of the expression patterns of FGF signaling related genes in spinal cord development has been performed in the chick. At the stages of chick spinal cord specification, several *FGFs*, including *FGF3*, *FGF4*, *FGF8*, *FGF13*, *FGF18*, are expressed in the caudal NMP region or surrounding tissues (Karabagli et al., 2002; Delfino-Machin et al., 2005). During later stages (during spinal cord elongation and including tailbud formation) *FGF3*, *FGF4*, *FGF13* and *FGF18* become restricted to the primitive streak while *FGF8* is more broadly expressed in the streak, the adjacent NMP region and the ingressing mesoderm (Karabagli et al., 2002; Delfino-Machin et al., 2005). Expression of *FGF8* is highly dynamic as those cells that progress from the NMP state to the spinal cord fate or from the presomitic mesoderm to the somitic mesoderm slowly downregulate their expression (**Figures 3A–C**). Expression of *FGF4* and *FGF8* in the NMP region (including caudal lateral epiblast and later, the tailbud) continues for several days but declines toward the final stages of somitogenesis and the cessation of axis elongation (Cunningham et al., 2011; Olivera-Martinez et al., 2012).

FGFR1-3 are initially present in the NMP zone (Karabagli et al., 2002; Lunn et al., 2007; Nishita et al., 2011), but later, only *FGFR1* remains throughout the neural tissue including the NMP region while *FGFR2* is absent there and becomes restricted to the neural tube, rostral to Hensen's node, and *FGFR3* restricts to the neural tube adjacent to somites (Karabagli et al., 2002; Lunn et al., 2007; Nishita et al., 2011).

Similar expression patterns have been described in mouse for those genes analyzed. In mouse, expression of *Fgf3*, *Fgf4*, *Fgf8*, *Fgf17*, and *Fgfr1* has been reported in and around the NMP region (Gofflot et al., 1997; Wahl et al., 2007; Anderson et al., 2016a). In zebrafish, in addition to *fgf4* and *fgf8*, *fgf17*, *fgf17b*, and *fgf24* (a zebrafish exclusive gene) have also been shown in or near the tailbud (Reifers et al., 2000; Draper et al., 2003; Cao et al., 2004; Akiyama et al., 2014).

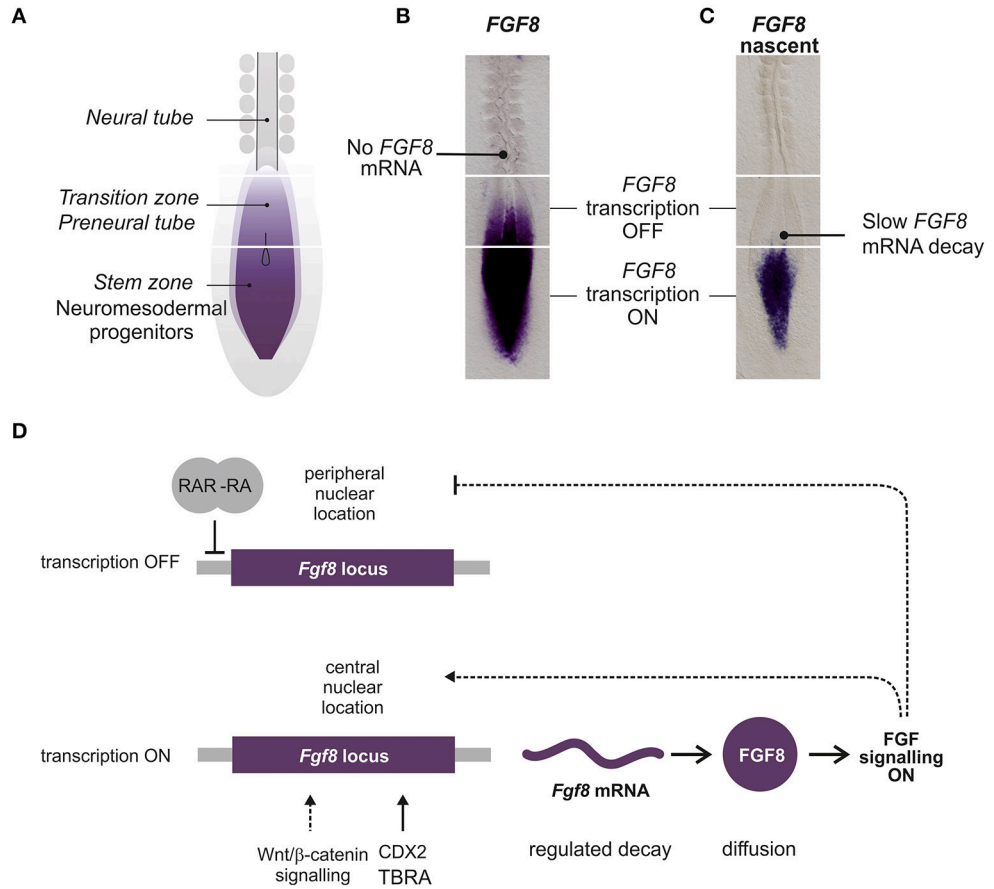

FIGURE 3 | Regulation of caudal *FGF8*/*Fgf8* expression during spinal cord extension. (A) Diagram representing the caudal part of a chick embryo where the different regions related to *FGF8* expression are labeled. **(B)** Chick embryo showing *FGF8* mRNA detected by *in situ* hybridization with a full length *FGF8* probe. **(C)** Embryo showing the nascent *FGF8* pre-mRNA detected by *in situ* hybridization with an intronic *FGF8* probe. **(D)** Diagram representing the main factors contributing to the expression of *Fgf8* in the caudal NMP. Lines represent possible direct interactions of transcription factors and dashed lines indirect relations.

Very little is known about the regulation of expression of FGFRs and FGFs. Most work has been done with *FGF8,* but the control of *FGF8* transcription in the spinal cord NMP region and its progressive downregulation coordinated with embryonic axial extension still constitutes an unsolved enigma (**Figure 3**). Three regions have been identified with respect to *FGF8* expression: the most caudal region where *FGF8* is actively transcribed (NMP), a more rostral region where transcription is stopped but transcripts remain (known as the transition zone or the preneural tube) and a third most rostral region, adjacent to the mesoderm ready to segment where transcripts are no longer detected (**Figures 3A–C**).

Several signaling pathways have been shown to influence *Fgf8* expression either promoting or decreasing *Fgf8* levels (**Figure 3D**). The WNT/β-Catenin pathway is active in the caudal region (Aulehla et al., 2003; Olivera-Martinez and Storey, 2007; Cunningham et al., 2011) and manipulation of the pathway has been shown to affect *Fgf8* expression. Reduced levels of *Fgf8* have been shown in the *Wnt3a* mouse mutant *vestigial tail* (Aulehla et al., 2003) and a further reduction is observed in double *Wnt3a/Wnt8a* mutants (Cunningham et al., 2015b). Furthermore, altering the levels of β-Catenin in the PSM promotes changes in *Fgf8* expression (Aulehla et al., 2008; Dunty et al., 2008). In fact, studies in mouse craniofacial development support a direct role for WNT in *Fgf8* regulation throughout a conserved Tcf/Lef site 2.8 kb upstream of *Fgf8* (Wang et al., 2011). However, no upregulation of *FGF8* by WNT has been observed in chick spinal cord suggesting a more indirect regulation of *FGF8* by the WNT in the context of caudal neural tube (Olivera-Martinez and Storey, 2007). Evidence has also been presented for the requirement of signals from the notochord. In particular, a reduced level of *FGF8* is observed in the absence of the notochord that can be rescued by SHH supplementation (Resende et al., 2010).

An autoregulatory mechanism of FGF activating *FGF8* has been suggested based on the ability of FGF8 to activate the transcriptional repressor *NKX1.2* (previously known as *SAX1;* Bertrand et al., 2000) which when overexpressed can in turn result in increased *FGF8* levels (Sasai et al., 2014). However, exposure of the neural explants or embryos to FGF does not result in activation of *FGF8* expression and inhibition of FGF signaling does not result in decreased *FGF8* or *Fgf8* levels in

chick and mouse, respectively (Harrison et al., 2011; Patel et al., 2013), raising the possibility that NKX1.2 may be involved in the stabilization of *FGF8*/*Fgf8* transcripts.

In addition, although a localized source of a regulator of *FGF8* caudal to Hensen's node has been ruled out (Dubrulle and Pourquie, 2004; Harrison et al., 2011), other caudally active pathways such the HMG-CoA reductase/mevalonate pathway (mediating steroid biogenesis) could be also playing a role in *FGF8* expression activation and maintenance (Olivera-Martinez et al., 2014).

Recent efforts for the characterization of the *Fgf8* gene regulatory region have led to the identification of several regions driving expression around the NMP region (Beermann et al., 2006; Marinic et al., 2013) and furthermore to the identification of CDX2 and TBRA as direct transcriptional activators (Amin et al., 2016). This could explain the decreased *Fgf8* levels that have been observed in *Cdx2* mutants (Savory et al., 2009) but additional activators may also be acting to regulate *Fgf8*, such as WNT/β–Catenin.

In addition to signals maintaining *FGF8*/*Fgf8* expression in the caudal precursor region, progressive downregulation of *FGF8*/*Fgf8* involves cessation of transcription in cells that exit the NMP region. Retinoic acid (RA; which is produced by somites and rostral presomitic mesoderm) has been shown to downregulate *FGF8*/*Fgf8* and reduction in RA signaling (in a vitamin A deprived quail model and in *Raldh2*$^{-/-}$ mutants) results in a rostral expansion of the *FGF8*/*Fgf8* expression domain (Diez del Corral et al., 2003; Molotkova et al., 2005; Vermot and Pourquie, 2005; Sirbu and Duester, 2006; Olivera-Martinez and Storey, 2007; Patel et al., 2013; Kumar and Duester, 2014; Cunningham et al., 2015a). This effect of RA has recently been attributed to direct binding of RA receptor (RAR) to an *RA response-element* (*RARE*) in the regulatory region in the *Fgf8* promoter (Kumar and Duester, 2014; Cunningham and Duester, 2015). This constitutes one of the few examples described where RAR bound to RA would repress gene transcription. Furthermore, additional studies show how NCOR repressors are required for RA repression of *Fgf8* (Kumar et al., 2016).

However, as the forced reduction in RA signaling only promotes a limited expansion of the *FGF8*/*Fgf8* domain, additional mechanisms of transcriptional repression must be involved. One possible theoretical mechanism proposed would involve a caudal diffusing signal transcribed in NMP cells that would repress both its own transcription as well as *FGF8*/*Fgf8* (Harrison et al., 2011). However, to date no such signal has been identified. Moreover, failure of *FGF8*/*Fgf8* to be expressed more anteriorly in spinal cord and somites could be due to a lack of transcriptional activators such as TBRA, CDX, and WNT expression.

Interestingly, the change in the transcriptional state of *Fgf8* locus from active to inactive is associated to a change in its nuclear position from a more central location in the NMP to a more peripheral position in neural tube cells (Patel et al., 2013). This location seems to be regulated by FGF signaling *per se* as inhibiting FGFR signaling results in a more peripheral location of *Fgf8* transcription in the caudal region. However, in spite of the change of location, transcription of *Fgf8* still occurs (Harrison et al., 2011; Patel et al., 2013) suggesting that location of the *Fgf8* locus to the periphery is required but is not sufficient for cessation of expression.

As mentioned above, analysis of active transcription by *in situ* hybridization using intronic *FGF8*/*Fgf8* probes has shown that the actively transcribing region is rather limited and that cessation of *FGF8*/*Fgf8* transcription seems to be abrupt (Dubrulle and Pourquie, 2004). However, the high stability of the transcript (which can perdure more than 5 h) is such that a gradient of *FGF8*/*Fgf8* transcripts can be generated. The mechanism accounting for this high *FGF8*/*Fgf8* stability however has not been further explored.

Final steps in the regulation of *FGF8*/*Fgf8* expression are the decrease in *FGF8*/*Fgf8* levels associated to the trunk to tail transition and the termination of transcription in the tailbud associated to the termination of axis elongation. Mouse embryos with mutations associated to a prolonged trunk extension *(Gdf11* loss of function mutants or overexpression of OCT4; Aires et al., 2016) show an abnormal increase in *Fgf8* expression in the tailbud region. In the chick, RA derived from the tailbud is also important for the correct termination of *FGF8* expression (Olivera-Martinez et al., 2012) while in the mouse other mechanisms seem to be responsible (Cunningham et al., 2011), as body axis extension continues for a much longer time to form the tail in mouse.

As FGFs are secreted factors, their distribution also depends on their diffusion and transport in the extracellular medium. This has been examined in detail in zebrafish embryos mostly in the context of gastrulation but this may be extended to other situations (reviewed in Bokel and Brand, 2013). There, the binding to heparan sulfates, important constituents of the extracellular matrix, is not only relevant for the activation of the receptor by the ligands but also has an influence on the spread of Fgfs (Yu et al., 2009). The shape of the gradient is also greatly influenced by degradation of Fgf8 that may be largely due to its endocytic removal (Scholpp and Brand, 2004).

Overall, a complex gene regulatory network is in place in the NMP region involving several interconnected signaling pathways that ensures that FGF signaling components are expressed at the appropriate levels for the control of a number of processes that take place as the neural tube extends to form the spinal cord. Given the temporally controlled exposure of cells to FGFs in and around the NMP region, the measurement of the activity of the pathway at the level of the receptor as well as at the different downstream components in fixed tissue and *in vivo*, as the axis extends caudally, would greatly improve our understanding of the coordination of morphogenetic movements and the control of tissue differentiation.

FGFs AND THE ESTABLISHMENT OF THE CAUDAL NEUROMESODERMAL PROGENITORS AND OF THE SPINAL CORD IDENTITY

It has recently become clear that the spinal cord derives progressively from the caudal NMP region which is specified

through FGF and WNT actions on sensitized epiblast cells around the primitive streak (Henrique et al., 2015). Specification of the NMP region is initiated at gastrulation stages (Muhr et al., 1999; Delfino-Machin et al., 2005; Nordstrom et al., 2006) within a region around the node and primitive streak characterized by expression of genes such as *NKX1.2, CDX1, CDX2, CDX4,* and *HOXB8* where high MAPK signaling levels are present. This coincidence is maintained during axis extension (Delfino-Machin et al., 2005; Lunn et al., 2007) and reflects the activity of FGF in the control of the expression of those genes (Storey et al., 1998; Muhr et al., 1999; Bel-Vialar et al., 2002; Delfino-Machin et al., 2005; Nordstrom et al., 2006; Sasai et al., 2014). Thus, in the chick embryo, blockade of FGF signaling with a dominant negative form of FGFR (DN-FGFR) and with pharmacological inhibitors results in downregulation of *NKX1.2* and *HOXB8 in vivo* and in explant cultures (Delfino-Machin et al., 2005). In double *Fgf4*; *Fgf8* conditional mutant mice, there is a decrease in *Wnt3a, Wnt5a, Cyp26a1, T-Bra* in the NMP region (Naiche et al., 2011; Boulet and Capecchi, 2012). Similarly, *Fgfr1* conditional mutants also show decreased levels of a number of NMP region genes, such as *Gbx2* and *Cyp26a1* (Wahl et al., 2007). All these results, from the current perspective that stresses the relevance of the NMP cells, suggest that FGF contributes in an important way to the specification of the NMP character in chick and mouse embryos, including genes expressed in NMP and its mesodermal derivatives (*T-BRA*) as well as those expressed in NMP and its neural derivatives (i.e., *NKX1.2*). In the same direction, in Xenopus and zebrafish embryos, expression of a dominant negative form of FGFR/Fgfr (DN-FGFR/DN-Fgfr) results in the loss of markers of NMP and its derivatives (Isaacs et al., 1994; Griffin et al., 1995; Holowacz and Sokol, 1999; Ota et al., 2009).

The NMP give rise to both spinal cord and mesodermal cells during an extended period of time and FGF levels also contribute to preserve the balance between the three cell types. For instance, double *Fgf4*; *Fgf8* conditional mutant mice where defective signaling is restricted to the NMP and its derivatives display dramatic reduction of the presomitic mesoderm markers *Tbx6* (Naiche et al., 2011; Boulet and Capecchi, 2012) and display ectopic neural tubes (Boulet and Capecchi, 2012) similar to the ones observed in *Fgfr* mutant chimeras (Ciruna et al., 1997). In chick, pharmacological inhibition of FGFR results in precocious and caudal expression of the neural tube specific gene *SOX1* (Stavridis et al., 2010). On the other hand, situations with excessive caudal FGF8 signaling such as the *Raldh2* mutant present an imbalanced NMP differentiation favoring mesodermal fate (Cunningham et al., 2015a).

This suggests a requirement of FGF signaling for the promotion of mesodermal or neuromesodermal vs. neural fates (Henrique et al., 2015). Most interestingly, FGF signaling has been shown recently to promote the expression of enzymes that drive the glycolytic metabolic state of the NMP region (Oginuma et al., 2017) that is in turn important for WNT signaling and for restraining the transition from a NMP state to a neural state (Oginuma et al., 2017). Later on, that glycolytic metabolic state in a gradient fashion also operates in presomitic mesoderm development (Bulusu et al., 2017).

In spite of FGF promotion of neuromesodermal and mesodermal fates, FGF signaling in combination with WNT signaling also appears to contribute to the activation and maintenance of the expression of the neural genes *SOX2* and *SOX3* through specific gene regulatory regions (Takemoto et al., 2006; Nishimura et al., 2012) and this might help to prevent the excess production of mesoderm precursors from the NMP (Yoshida et al., 2014).

The precise sequence of exposure of cells to FGF in combination with the other caudal signal WNT as well as the temporal dynamics within the cells may here determine whether cells are maintained in a NMP state, differentiate toward a mesodermal fate or toward a neural fate. This idea has been recently explored with experiments developing *in vitro* methods to generate a population of cells that co-express the NMP genes from mouse and human pluripotent stem cells by timed exposure to FGF2 in combination with WNTs (Gouti et al., 2014; Turner et al., 2014; reviewed in Henrique et al., 2015). This constitutes a good example of how the temporal exposure and competence to interpret FGF signals play an important role in specification of cell fates.

Once spinal cord cells leave the NMP region, FGF is not required for the maintenance of the spinal cord identity. Thus, the spinal cord specific homeobox transcription factor *HOXB8,* that initially requires FGF for its expression in the NMP (Delfino-Machin et al., 2005), remains actively expressed in spinal cord progenitors after the levels of FGF signaling have dropped during axis elongation.

The different mechanisms responsible for the role of FGF in specification of the NMP and then in the balance of mesodermal and neural derivatives may be related to the coactivity with other signals and/or to the temporal sequence of exposure and response of cells to FGF and other signals, as suggested by the cell culture experiments. All these crucial aspects certainly deserve now a thorough analysis within the developing embryo.

FGFs AND THE CONTROL OF SPINAL CORD CAUDAL EXTENSION

The most striking feature of embryos where FGF signaling has been diminished (once the early lethality is overcome) is the truncation of the caudal embryonic axis, observed in mouse, Xenopus and zebrafish. Mouse $Fgfr1^{-/-}$ embryonic chimeras cannot gastrulate properly and mutant cells tend to accumulate in the tail displaying a short axis (Ciruna et al., 1997; Ciruna and Rossant, 2001). Similarly, *Fgfr1* conditional mutant mice where defective signaling is restricted to the caudal NMP and derivatives (using a *TBra*- driven Cre-line), result in truncated axis at the level of sacral regions (Wahl et al., 2007). An even shorter axis is observed in the double *Fgf4; Fgf8* conditional knock-out (Naiche et al., 2011; Boulet and Capecchi, 2012; either using a TBra- or a Hoxb1-driven Cre-lines). A shortened tail is also apparent in the *Fgf3* null mutant embryos (Anderson et al., 2016a,b). Similarly, in Xenopus and zebrafish the overexpression of DN-FGFR/DN-Fgfr versions also result in truncated embryos (Griffin et al., 1995; Holowacz and Sokol, 1999) and in chick decreased elongation rates have been observed following blockade of FGFR (Benazeraf et al., 2010).

However, in all these situations the lack of FGF signaling affects specification of both mesodermal and neural derivatives and it is therefore not possible to assess whether the defect on elongation is a consequence of an alteration in gastrulation, in the specification of NMP, spinal cord or mesoderm, the result of abnormal motility in the mesoderm (Benazeraf et al., 2010) or whether there is a more specific requirement within the spinal cord population. Support for a more localized role of FGF signaling in spinal cord caudal extension came from analysis of cell distribution after electroporating a *DN-FGFR1* construct in chick NMP region (Mathis et al., 2001; therein referred to as node region). In control experiments, cells could either remain in the NMP region and continue the backward displacement or get incorporated into the neural tube. However, cells with decreased FGF signaling had an increased probability to get incorporated in the neural tube and thus would not be part of the caudally displaced NMP region suggesting some changes in cell adhesion properties of those cells, at least indirectly. In addition, a role of FGF in the maintenance of proliferating cells could also contribute to the extension of the axis (Mathis et al., 2001).

In presomitic mesoderm, axis extension has been shown to involve differential motility of cells along the rostrocaudal axis in a space constrained by lateral boundaries (possibly the lateral plate), with cells moving more in caudal presomitic mesoderm than in the rostral part. Interestingly, in that context, FGF signaling has been shown to promote cell motility (Benazeraf et al., 2010; Lawton et al., 2013). As mentioned before, FGF is required for the transcription of rate limiting enzymes responsible for the glycolytic metabolic state of the NMP that has been shown to be important for cell motility and axis elongation (Oginuma et al., 2017). The mechanism of control of cell motility is still not known but it has been proposed to be related to the ability of localized glycolytic activity to ensure rapid production of ATP for actin polymerization in the forming protrusions of motile cells (Oginuma et al., 2017). In other contexts, FGF has been shown to have chemotaxis properties (Yang et al., 2002) and this has been suggested as an additional mechanism that could in theory contribute to axis extension (Harrison et al., 2011). In any case, given that the spinal cord is composed by epithelial cells and not by mesenchymal cells (as it is the case for presomitic mesoderm) it is unlikely that the same morphogenetic mechanisms are responsible for its extension which may be a more passive process driven by mesoderm.

Recent work on the generation of a population with NMP properties by differentiation of mouse embryonic stem cells (mESCs) in adherent cell culture has shown that these cell aggregates also have the ability to elongate *in vitro* and that this elongation requires FGF signaling, providing an *in vitro* system where this function can be further examined (Turner et al., 2014). In conclusion, there are still many unknowns in relation to the cellular process of spinal cord extension. Most likely, the combination of *in vitro* culture systems together with imaging techniques (both *in vitro* and *in vivo*), the use of biosensors to investigate metabolism in developing embryos (such as the PYRATES mouse line, Bulusu et al., 2017) and *in silico* simulations will greatly contribute to the understanding of the important role of FGF signaling in spinal cord extension.

FGFs AND THE CONTROL OF CELL PROLIFERATION, CELL CYCLE EXIT AND NEURONAL DIFFERENTIATION

FGFs play important roles in cell survival and proliferation in many developmental contexts and in particular for neural stem cells and progenitors (Vaccarino et al., 1999; Storm et al., 2006; Maric et al., 2007). In the developing spinal cord, analysis of cell cycle exit (Sechrist and Bronner-Fraser, 1991) and of the early postmitotic marker *NeuroM* (Roztocil et al., 1997) revealed two regions with respect to cell proliferation. *NeuroM*$^+$ cells start to appear in the region flanked by somites while no *NeuroM*$^+$ cells are found in the more caudal region (the preneural tube) nor in the NMP region, coinciding with the region of influence of FGF signaling (Diez del Corral et al., 2002).

Exposure of the neural tube to FGF at a stage when some cells are already exiting the cell cycle can impair the generation of new *NeuroM* expressing cells (Diez del Corral et al., 2002) and by that way, the onset of neurogenesis. By following the fate of neural progenitors using time lapse imaging, it has been possible to analyze the changes in the dynamics of progenitors associated to FGF exposure (Wilcock et al., 2007). Neural progenitors and stem cells can normally experience three modes of division to give rise to neurons (N) and progenitors and stem cells (P): self-expanding, PP (i.e., giving rise to 2 progenitors or stem cells); self-replacing, PN (i.e., giving rise to a progenitor and a neuron); and self-consuming, NN (i.e., giving rise to 2 neurons). Previous studies in the developing cortex and spinal cord suggest that different modes are associated with different cell cycle duration times, with neuron generating divisions (PN or NN) characterized by a longer cell cycle than PP divisions (Takahashi et al., 1995; Calegari and Huttner, 2003; Calegari et al., 2005; Wilcock et al., 2007).

Upon exposure to FGFs, progenitors only go through PP divisions while no PN nor NN divisions could be observed (Wilcock et al., 2007). These FGF induced PP divisions exhibited the typical short PP cell cycle length while no changes in the range of cleavage plane orientation were observed. Interestingly, a subpopulation of cells was found dividing without contacting the apical membrane and with very short cell cycle times (Wilcock et al., 2007). These data support a role for FGF in the maintenance of cells characterized by a rapid cell cycle that can only generate further progenitors. Interestingly, within the embryo, shorter cell cycle lengths are observed in the region exposed to FGF with respect to the rostral neural tube (Olivera-Martinez et al., 2014) and several cell cycle genes are differentially expressed in the caudal vs. more rostral region and could be regulated by FGF (Lobjois et al., 2004; Olivera-Martinez et al., 2014). One example is *CYCLIN D2*, a cell cycle regulator specifically expressed in the chicken caudal neural plate that can be activated by and requires FGF signaling (Lobjois et al., 2004; Molina and Pituello, in press).

Although exposure to FGF can impede neurogenesis, blockade of FGF signal in explants is not sufficient to drive premature expression of the postmitotic and neurogenesis marker *NeuroM* (Diez del Corral et al., 2002). However, as discussed above, cells subject to interference with FGF signaling in the embryo tend to prematurely leave the NMP region (Mathis

et al., 2001) where only proliferating cells are found and it remains to be assessed whether they have alterations in their type of division or cell cycle exit parameters.

A high level of aerobic glycolysis is known to facilitate cancer cell proliferation. Although no significant change in proliferation was observed by Oginuma et al. (2017) in embryos grown in the absence of glucose, more detailed analysis are required in order to determine a possible implication of the FGF dependent changes in metabolism in the control of proliferation during axis extension and more specifically within the spinal cord.

The contribution of FGF to the control of proliferation in the spinal cord discussed above is restricted to cells before or at the onset of neurogenesis and could be equivalent to the ability of FGF2 and FGF8 in the telencephalon to maintain the proliferative symmetrical PP divisions of neuroepithelial cells before the onset of neurogenesis (Raballo et al., 2000; Storm et al., 2006; Maric et al., 2007; Rash et al., 2013). Interestingly, the analysis of the telencephalon of mutant mouse embryos has revealed additional requirements for FGF signaling in proliferation of neurogenic lineages at different steps. At the start of telencephalon neurogenesis, neuroepithelial cells transform into radial glial cells, which divide asymmetrically to generate another radial glia and a postmitotic neuron or a basal progenitor (Gotz and Huttner, 2005) and this transition is promoted by FGF10 (Sahara and O'Leary, 2009). Finally, after neurogenesis has started, it has been demonstrated (using mutants for three FGF receptors) that FGF signaling is required to slow down the progression from radial glia to basal progenitors (Kang et al., 2009; Rash et al., 2011). Similar roles for FGF at later stages of spinal cord development remain to be explored (see below for functions during spinal cord adult neurogenesis).

In addition to a more direct action of FGF on the cell cycle, several FGF dependent pathways could mediate its influence on cell cycle exit and neuronal differentiation before the onset of neurogenesis in the spinal cord. FGF signaling is required for the expression of *DELTA-1*, an important component of the NOTCH signaling pathway involved in mutual inhibition in the NMP region and required to limit precocious cell cycle exit (Akai et al., 2005). Additionally, FGF signaling promotes *WNT8a* expression, which in turn prevents neuronal differentiation (Olivera-Martinez and Storey, 2007).

Manipulation of FGF signaling in chick embryo explants and the use of mouse mutants has shown that FGFs can reduce the levels of RA signaling, a neuronal differentiation promoter (reviewed in Diez del Corral and Morales, 2014) and this would also favor the maintenance of the progenitor state. Double *Fgf4; Fgf8* conditional mutant mouse embryos exhibit increased caudal *RARE-lacZ* reporter expression (Naiche et al., 2011). But, at what level could FGF act on the control of RA signaling? FGF4 and FGF8 can repress the gene encoding the RA-synthesizing enzyme *RALDH2* in the paraxial mesoderm (Diez del Corral et al., 2003). However, the contribution of this repression to the RA levels is probably partial since double *Fgf4; Fgf8* conditional mutant mouse embryos do not exhibit increased *Raldh2* expression (Boulet and Capecchi, 2012). FGF signaling is required for the caudal expression of the RA-degrading enzyme *Cyp26a1* (Wahl et al., 2007) and this could also contribute to the control of RA levels similarly to what has been described in the context of the hindbrain (Gonzalez-Quevedo et al., 2010). FGF4 and FGF8 can also downregulate *RARβ* receptor levels in the spinal cord (Olivera-Martinez and Storey, 2007) and this would affect the sensitivity to RA levels. This receptor gene depends on RA for its activation (Olivera-Martinez and Storey, 2007) and thus its downregulation by FGF could be due to upregulation of *Cyp26a1* but this has not been examined yet.

FGF signaling is also required to prevent precocious activation of *PAX6* and *IRX3* in chick and *Pax6* in mouse (Bertrand et al., 2000; Diez del Corral et al., 2003; Patel et al., 2013), two transcription factors which promote neuronal differentiation (de la Calle-Mustienes et al., 2002; Bel-Vialar et al., 2007). Thus, FGF seems to contribute to a rather complex network that controls proliferation before the onset of neurogenesis maintaining an undifferentiated state. However, open questions still remain: does FGF signaling act differentially on the process of proliferation within NMP and then for promotion of self-renewal of neural progenitors? Does it act differently in the spinal cord than in telencephalon progenitors where it has also been involved in the appearance of intermediate progenitors? What are the cell cycle components modulated by FGF signaling in all these processes?

FGFs AND PATTERNING OF SPINAL CORD ALONG THE ROSTRO-CAUDAL AXIS

Once the region of the neural plate giving rise to the spinal cord has been specified (in an FGF dependent way), FGF signaling has an additional role in the further regionalization of the spinal cord along the rostral-caudal axis. The spinal cord presents heterogeneity along the rostro-caudal axis responsible for differences in motor neuron subpopulations, interneuron distribution (Francius et al., 2013; Lai et al., 2016) or neural crest derivatives (Le Douarin et al., 2004). This regionalization, which has been mainly examined in motor neurons, is a consequence of the restricted rostro-caudal expression of *Hox* genes in progenitor cells and subsequently in the resulting postmitotic motor neurons (reviewed in Philippidou and Dasen, 2013).

Experiments in chick embryos have shown that exposure to FGF or electroporation of *FGFs* expressing constructs shifts rostrally the domain of expression of caudal *HOX* mRNAs (*HOXB6*, *HOXC6*, *HOXB7*, *HOXB8,* and *HOXA9-B9-C9*) in neural progenitors resulting in an increase in the protein levels of a subset of HOXB proteins (Bel-Vialar et al., 2002; Dasen et al., 2003). FGF signaling appears to act here by activating the transcription factor genes of the Cdx family, known to activate *HOX*/*Hox* gene expression, in particular *cdx2* and *cdx4* in zebrafish (Shimizu et al., 2006), *CDX1* and *CDX2* in the chick (Bel-Vialar et al., 2002), and *Cdx1*, *Cdx2,* and *Cdx4* in mouse (van den Akker et al., 2002; Amin et al., 2016). Exposure to FGF not only has consequences in the expression of genes in progenitors but also in the resulting motor neurons (Liu et al., 2001; Dasen et al., 2003). Explants of neural tissue fated to give rise to cervical spinal cord do not express HOXC6, HOXC8, HOXC9, or HOXC10 after culture but their exposure to increasing FGF levels results in progressive activation of the production of these

proteins suggesting that FGF works in a concentration dependent way. Considering that in the embryo, caudal cells are exposed to FGF for a longer period of time than rostral cells, but not necessarily to higher levels of FGF signaling, this concentration dependent effect has also been interpreted as an effect of the duration of exposure to the FGF morphogen. The mechanism to explain such concentration/time of exposure dependence is still not known but may involve the regulation of genes encoding transcription factors of the CDX family mentioned above.

The role of FGF signaling in this further caudalization, however, has not yet been ascertained by loss of function approaches and therefore, the extent of its contribution to patterning remains an open question. A possible contribution of FGF to rostro-caudal patterning of interneurons has also been suggested (Francius et al., 2013) but has not been explored yet.

FGFs AND VENTRAL PATTERN (INTERMEDIATE, VENTRAL AND FLOOR PLATE)

Another regionalization process where FGF signaling plays an essential role is the patterning of the spinal cord along the dorso-ventral (DV) axis which is fundamental for the assignment of neuronal subtype identities such as motor neurons and the different interneuron subtypes (reviewed in Gouti et al., 2015). Specific combinations of transcription factors of the homeodomain and bHLH families are expressed in restricted domains along the DV axis (reviewed in Le Dreau and Marti, 2012). In the ventral/intermediate neural tube this is regulated by the SHH morphogen. The graded distribution of SHH, produced in the ventral midline, results in a graded activation of the pathway and the expression of target genes (reviewed in Briscoe and Small, 2015). In addition, cross-repressive interactions between target genes occur to further delimit and ensure gene expression in the appropriate domains (Briscoe et al., 2000; Kutejova et al., 2016).

During spinal cord caudal extension, SHH is expressed in the node and along the derived notochord while in the neural tissue it is expressed in floor plate (FP) cells at the level of the somitic mesoderm. Thus, cells in the preneural tube (the transient spinal cord population derived from NMP and adjacent to presomitic mesoderm) are initially exposed to notochord derived SHH and express some SHH target genes such as *GLI1*, *PTCH1* and *PTCH2* suggesting that at least low SHH signaling is achieved (Diez del Corral et al., 2003; Morales et al., 2016). However, neural progenitors in the preneural tube do not display expression of the complete repertoire of ventral identity genes, suggesting that the pathway is being modulated in this region. A role for FGF signaling in the control of ventral patterning was first inferred from its ability to repress *PAX6*, a gene expressed in an intermediate domain in the neural tube (Bertrand et al., 2000). Since that observation, a more complex picture has emerged showing that FGF signaling is crucial for controlling the onset of SHH signaling and ventral patterning in the spinal cord (Diez del Corral et al., 2003; Morales et al., 2016) and for the early specification of the most ventral fate, the FP (Sasai et al., 2014; **Figure 4**).

Forced maintenance of FGF signaling in preneural tube tissue, impairs not only *PAX6* but also other ventral and intermediate patterning genes such as *NKX6.1*, *NKX6.2*, *IRX3,* and *FOXA2* (Bertrand et al., 2000; Diez del Corral et al., 2003; Novitch et al., 2003). Conversely, interference with FGF signaling in chick embryos results in precocious caudal activation of *PAX6* and *IRX3* (Bertrand et al., 2000; Diez del Corral et al., 2003) and in the dorsal expansion of ventral markers such as OLIG2 and NKX6.1 (Morales et al., 2016). Reduced FGF signaling in mouse embryos results in precocious caudal *Pax6* (Patel et al., 2013) and NKX6.1 expression as well as in alterations in the ventral patterning with an increase in the number of NKX6.1 expressing neural progenitor cells (Morales et al., 2016).

FGF would thus be repressing more or less indirectly the two types of SHH responding genes, ventral genes activated by SHH (*FOXA2* and *NKX6.1*) and intermediate genes repressed by SHH (*PAX6* and *IRX3*) (Briscoe et al., 2000). Repression of *PAX6* and *IRX3* and their mouse homologs seems to involve several mechanisms (**Figures 4B,C**). FGF signaling promotes chromatin compaction and peripheral nuclear position around the mouse *Pax6* and *Irx3* loci, a chromatin organization associated to transcriptionally inactive loci (Patel et al., 2013). In addition, repression of these genes appears to be mediated by transcriptional repressor NKX1.2, transcriptionally activated by FGF signaling in NMP region (Storey et al., 1998; Bertrand et al., 2000; Sasai et al., 2014; **Figure 4C**).

A molecular mechanism that accounts for the effect of FGF on genes relying on SHH for their expression has been identified recently (**Figure 4C**; Morales et al., 2016). FGF can activate the expression of *PTCH2*, one of the SHH receptors that also acts as an inhibitor of the SHH pathway, and can thus restrain expression of SHH targets. Experiments in chick explants have shown that *PTCH2* is expressed in the preneural tube in a SHH and FGF dependent way indicating the existence of an enhanced feedback loop where SHH activates PTCH2 more efficiently in regions of high FGF signaling. This regulation also appears to be conserved in mouse as *Fgfr1* conditional mutant embryos show extremely reduced *Ptch2* levels (Morales et al., 2016). Surprisingly, however, the *Ptch2* gene seems to be largely dispensable as no obvious phenotype has been identified yet in the mouse mutant (Holtz et al., 2013). It is possible that its function is only apparent when the development of the embryo is challenged, for example when the elongation process is altered. If elongation is arrested, the high levels of PTCH2 in the spinal cord precursor may maintain the levels of SHH signaling low and ventral patterning on standby mode until the elongation is restored (**Figures 4D,E**). In fact, *Ptch2* is required to keep SHH signaling in check in situations of partial deficiency of the other member of the family, *Ptch1,* both in development and in tumorigenesis (Lee et al., 2006; Nieuwenhuis et al., 2006; Holtz et al., 2013; Zhulyn et al., 2015).

In addition, and probably as a result of its role on repressing ventral and intermediate genes, an important role for FGF on the specification of FP cells has been recently identified in chick

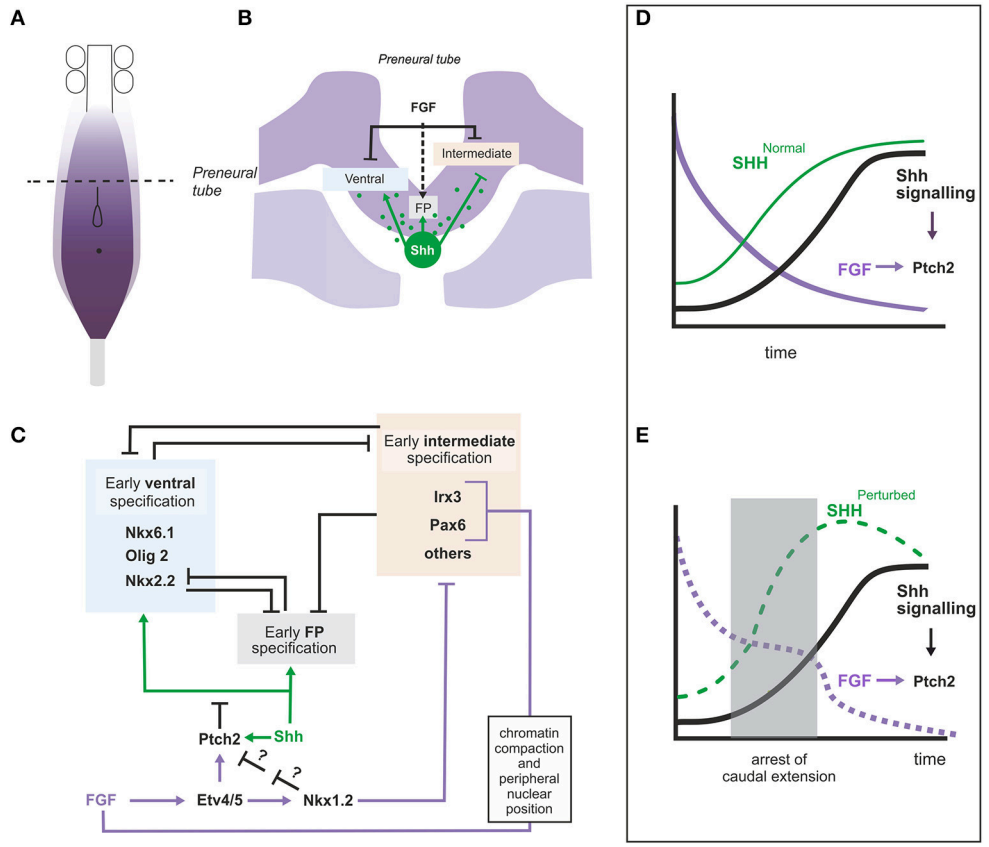

FIGURE 4 | Role of FGF signaling in the regionalization of the intermediate and ventral neural tube. (A) Diagram representing the caudal part of an embryo showing the region with active FGF signaling and the rostro-caudal level of the transverse section represented in **(B)**. **(B)** Transverse section at the level of the preneural tube showing the neural tissue and the underlying presomitic mesoderm and notochord. FGF8 (produced by neural and mesoderm tissues) and Shh (produced by notochord) are represented in purple and green respectively and their influence on intermediate, ventral and floorplate specification is shown. The dashed arrow indicates that FGF provides competence for floor plate specification. **(C)** Gene regulatory network relating FGF and Shh signaling in the pre-neural tube where the two signals coincide. Data from chick, mouse or both are included in this figure. **(D,E)** Graphs to illustrate the hypothetical role of the regulation of *Ptch2* by FGF during the initial establishment of the Shh signaling levels. The graphs represent the changing levels of SHH in time at a particular position (within the dorsoventral and rostrocaudal axis). **(D)** In embryos continuously extending their caudal axis, the levels of FGF (purple) at a particular position would decrease constantly while the levels of SHH (green, SHH^{Normal}) would increase until they reach their maximum. The levels of Shh signaling thus also increase progressively. **(E)** In embryos where elongation is arrested for some time (shaded area), FGF levels would remain constant during the arrested period, while the levels of SHH ($SHH^{Perturbed}$) would accumulate more rapidly due to the decreased amount of tissue generated through which SHH could diffuse (or be transported). If Shh signaling was dependent exclusively on SHH levels, the level of the signaling would also increase to levels higher than normal and this may result in the irreversible activation of its targets. However, the ability of FGF to activate *Ptch2* and thus downregulate the Shh pathway could serve to limit Shh signaling levels to normal values. FGF signaling levels (decaying in time) are shown in purple.

(Sasai et al., 2014). FP territory, characterized by expression of the ARX1 protein, is induced by the highest levels of SHH that are only achieved in the cells closest to its source and also requires transient FGF exposure (Sasai et al., 2014). Here again, NKX1.2 plays an important role, providing competence to respond to high SHH levels and drive ARX1 expression. Given the repressive interactions between FP specific genes and the ventral and intermediate patterning genes (Cho et al., 2014; Kutejova et al., 2016), one important function for FGF signaling and NKX1.2 here would be to ensure that a region free of expression of non-floor plate factors such as PAX6, IRX3 and NKX2.2 is established in the future FP region (Sasai et al., 2014). Nevertheless, the details of the gene regulatory network are still not elucidated as expression of ARX1 (and other definitive floor plate markers) is only apparent well after the FGF signaling levels have decayed. Here again, the system may be highly redundant as no obvious alterations in the FP have been reported in *Nkx1.2* mutant mice (Simon and Lufkin, 2003), raising the possibility that the related *Nkx1.1* gene could be also playing a role.

Antagonism of the FGF signaling pathway with the RA pathway is also important in the context of ventral patterning as RA is required for expression of several intermediate and ventral genes (chick *NKX6.1*, *IRX3*, *PAX6*, *OLIG2*; Diez del Corral et al., 2003; Novitch et al., 2003; Diez del Corral and Morales, 2014 and mouse *Nkx6.1*, *Pax6,* and *Olig2*; Molotkova et al., 2005). However, the temporal and quantitative contributions of both FGF and RA pathways in the modulation of ventral specification require a deeper analysis.

FGFs AND NEURAL CREST SPECIFICATION

At the most dorsal part of the spinal cord, the development of a specific cell population also requires the participation of FGF signaling: the neural crest cells (NCCs). The neural crest is formed by a transient population of multipotent cells that arise from the dorsal neural tube. Once specified, NCCs undergo a process of epithelium to mesenchyme transition (EMT) that confers NCCs the ability to delaminate and migrate away from the dorsal neural tube, giving rise to NCC derivatives that include craniofacial skeleton, the peripheral nervous system (sensory neurons and glia, sympathetic neurons) and melanocytes, amongst others (Le Douarin and Kalcheim, 1999).

The process of neural crest formation implies the orchestration of a complex gene regulatory network. This involves signaling pathways and transcription factors that are responsible for the sequence of early induction of the NCC during gastrulation; the specification of the neural plate border; the expression of *bona fide* NCC transcription factors and the regulation of numerous downstream effectors involved in EMT, cell adhesion, and cell cycle control, amongst others (Morales et al., 2005; Sauka-Spengler and Bronner-Fraser, 2008). First, parallel to the induction and patterning of the neural plate that generates the central nervous system, at the border between the neural ectoderm and the non-neural ectoderm, the NCCs are specified through a series of steps controlled by FGF, WNT, and BMP signaling pathways (reviewed in Saint-Jeannet and Moody, 2014).

Transient exposure to FGF has been shown to allow neural tube cells to activate NCC markers in response to BMP (Sasai et al., 2014). This seems related to the ability of FGF to repress *PAX6* and *IRX3*, two intermediate neural tube genes which can repress the NCC marker *SNAIL* (Sasai et al., 2014). It has been proposed that the repression of IRX3 and PAX6 by FGF, acting through activation of NKX1.2, is required for the early establishment of a territory competent to NCC specification (see the ventral patterning section for a further discussion on possible mechanisms for FGF regulation of PAX6 and IRX3). However, in FGF deficient conditions impaired NCC specification *in vivo* has not been reported yet. On the contrary, forced reduction of FGF signaling allowed neuroepithelial cells to prematurely initiate the expression of the early NCC specifier *SNAIL2* at caudal levels (Martinez-Morales et al., 2011). This indicates that dorsal neuroepithelial progenitors in the caudal neural tube are maintained in an uncommitted non-NCC state in presence of strong FGF/MAPK signaling pathway (Martinez-Morales et al., 2011). Thus, in the elongating neural tube, as the dorsal neuroepithelial progenitors are progressively exposed to decreasing FGF signaling levels, they initiate the expression of neural crest specifier genes *SNAIL2* and *FOXD3*.

Interestingly, upon reduction of FGF signaling, when those prematurely *SNAIL2* expressing NCCs initiate the expression of other NCCs specifiers such as *FOXD3*, *SOX5*, and *SOX10* they prematurely start EMT from the neural tube at mid-rostral PSM levels. Essentially, the regulated decrease in FGF signaling is primary responsible for the control of the initiation of NCC specification in the trunk, and as a consequence of that, it controls the timing of EMT and emigration. Subsequent development of trunk NCCs is highly dependent on the development of adjacent somites, which impose a segmented migration and organization to the trunk NCCs and to the derived peripheral nervous system (Sela-Donenfeld and Kalcheim, 1999). Considering that FGF signaling is important both for segmentation of the mesoderm and for the neural crest specification it would constitute an important mechanism of coordination of both tissues.

As it has been described above, FGF and RA signaling can act as opposite gradients, each one negatively regulating the activity of the other. In the context of NCC development, RA signaling produced by the somites does not appear to promote their specification but does trigger the EMT of already specified NCCs (Martinez-Morales et al., 2011). FGF and RA signaling control the timing of EMT and emigration in part through modulation of elements of the BMP and WNT signaling pathways, important signaling cascades operating in the dorsal neural tube (Sela-Donenfeld and Kalcheim, 1999; Burstyn-Cohen et al., 2004). Whereas, RA signaling triggers the initiation of *WNT1* expression in the dorsal neural tube at levels where the NCCs are already specified, FGF signaling prevents the premature expression of *WNT1* (Martinez-Morales et al., 2011).

Moreover, recently it has been established that another FGF ligand, FGF3, coming from the caudal presomitic mesoderm provides another level of regulation of BMP signaling in the spinal cord at tailbud stages. *Fgf3* mutant embryos exhibit axis truncation, increase in neuroepithelial proliferation, delay in neural tube closure and premature neural crest formation (Anderson et al., 2016a). The removal of one copy of NOGGIN, a BMP antagonist, in *Fgf3* mutants, exacerbated all the *Fgf3* phenotypes including premature neural crest specification. Conversely, genetically decreasing BMP signaling in *Fgf3* mutants, via loss of BMP receptor activity, ameliorates morphological defects (Anderson et al., 2016a).

In summary, the data discussed in this section show that there is a limited time window during which the onset of the NCC emigration can be modulated, once those cells have acquired the expression of the essential gene network of the NCC specification program. That window coincides with the region where FGF and RA gradients collide. This FGF function constitutes another example of a general FGF role in controlling the onset of differentiation of cell types as they are generated at the tail end, during trunk axial elongation. Again, the molecular mechanism that allows the cells to interpret and execute that temporal window imposed by FGF signaling is far from understood and remains an important open question within the developmental biology field.

FGFs AND NEURAL STEM MAINTENANCE IN THE ADULT SPINAL CORD

As we have discussed, during the development of the nervous system, the generation of hundreds of subtypes of neurons and glial cells relies upon the relatively fast production, amplification, specification, and differentiation of a pool of neural progenitors

and neural stem cells (NSCs). Surprisingly, this strategy is retained to some extent in niches in the adult nervous system throughout lifetime under physiological conditions to generate specific subtypes of neural cells in limited numbers.

Adult NSCs are maintained into adulthood in two main niches, the ventricular-subventricular zone (V-SVZ) adjacent to the lateral ventricles and the subgranular zone (SGZ) in the hippocampus (reviewed in Fuentealba et al., 2012; Christian et al., 2014). Nevertheless, cells with neural stem cell properties can be isolated from most regions of the adult central nervous system, including, for example, the spinal cord (Weiss et al., 1996; Shihabuddin et al., 1997).

In the adult spinal cord, the cells with neural stem cell properties are the ependymal cells (Johansson et al., 1999; Meletis et al., 2008; Barnabe-Heider et al., 2010; Pfenninger et al., 2011). They rarely proliferate under physiological conditions and they mostly give rise to ependymal progeny *in vivo*. It is unclear which signals are responsible for maintaining this population of ependymal cells. However, in other neurogenic niches such as the SGZ of the hippocampus dentate gyrus (DG), the specific deletion of all the FGF receptors that are expressed in DG (*Fgfr1*, *Fgfr2*, and *Fgfr3*) in adult precursor cells has shown that, FGF signaling is required for neural stem-cell maintenance while an activated FGF receptor expressed in all precursors can increase the number of neurons produced (Kang and Hebert, 2015). The requirement for FGF receptors in maintaining stem but not progenitor cells in the adult hippocampus is reminiscent of their role in maintaining cortical radial glial stem cells during development (Kang et al., 2009).

In spite of the limited expansion of spinal cord ependymal stem cells under normal physiological conditions, their proliferation is dramatically increased after spinal cord injury, giving rise to scar-forming astrocytes as well as to a small population of remyelinating oligodendrocytes (Johansson et al., 1999; Meletis et al., 2008; Barnabe-Heider et al., 2010). More importantly, the ependymal derived astrocytes are essential for repairing the lesions because if their formation is inhibited, the lesions grow deeper over time and a higher number of axonal tracts are lost (Sabelstrom et al., 2013).

The application of FGF2 has been shown to promote functional recovery after spinal cord injury (SCI) in rodents (Lee et al., 1999; Rabchevsky et al., 1999; Yan et al., 2000; Kim et al., 2006). In SCI the recovery is thought to be due to FGF promoting the proliferation of spinal cord neural stem and progenitor cells expressing PAX6, NESTIN, and SOX2 (Shihabuddin et al., 1997; Goldshmit et al., 2014), promoting neuronal survival (Teng et al., 1998, 1999), angiogenesis (Kang et al., 2013), and causing a reduction in injury volume (Lee et al., 1999; Rabchevsky et al., 1999). In addition, FGF2 may reduce glial scar formation and astrogliosis after SCI in the mouse model (Goldshmit et al., 2014). In this situation, FGF2 influences glial cell activation, generating a proregenerative radial/progenitor-like state rather than reactive astrocytes that form scar tissue that are inhibitory to axonal regeneration. It is unclear if these proliferating astrocytes could be derived from the neural stem ependymal cells.

FGF2 also reduces the inflammatory response, as it causes the reduction in macrophage infiltration and cytokine levels (Goldshmit et al., 2014). The reduction in macrophage infiltration may be due to the ability of FGF-2 to reduce the leakiness of the blood-spinal cord barrier after SCI (Kang et al., 2010). Moreover, in combination with transplanting specific cells (Meijs et al., 2004; Kuo et al., 2011; Guzen et al., 2012; Lu et al., 2012) or with special scaffold forming hydrogels FGF1 and FGF2 can provide a proregenerative effect and may have clinical applications in the treatment of SCI (Chen et al., 2015). In fact, FGF1 is currently in clinical trials in human patients with cervical SCI (Wu et al., 2011) and more recently also in combination with special devices and rehabilitation in patients with thoracic SCI (clinical trial, NIH reference NCT02490501).

Since SCI has multiple factors that determine the progress of the injury, a combinatorial therapeutic approach including FGF will most likely be required for the most effective treatment of SCI (reviewed in Siddiqui et al., 2015; Ahuja et al., 2016).

FGFs PROMOTING NEUROGENESIS IN A DISH

As a complementary approach and as a way to overcome the limited capacity for self-repair of the mammalian nervous system, efforts are being made to boost the repair process by transplanting exogenous cells into sites of injury (Rosser et al., 2007). FGFs can be used to generate, expand, and differentiate neurons *in vitro* and therefore have a major role to play in such cell replacement therapies.

First, FGF2 together with EGF has been extensively used to promote proliferation and self-renewal of NSCs *in vitro* (Kilpatrick and Bartlett, 1993; Gage et al., 1995; Gritti et al., 1996; Qian et al., 1997; Nelson and Svendsen, 2006). FGF2 converts embryonic stem cells into neural stem cells characterized by rapid self-renewing and the potential to generate neurons, astrocytes, and oligodendrocytes. This acquired tripotent neural stem cell state, which does not exist *in vivo*, provide high proliferative capacity and glial differentiation potential to the treated cells (Palmer et al., 1999; Laywell et al., 2000; Zhang et al., 2001; Gabay et al., 2003; Hack et al., 2004; Pollard et al., 2008). Several studies then showed that FGF2 ventralizes cultured rodent NSCs/NPCs of dorsal origin and induces oligodendrocytes from NSCs derived from regions where oligodendrocytes are not present (Gabay et al., 2003).

FGF2 has also been proved to be involved in neuronal subtype specification, as it has been shown that *in-vitro*-expanded human fetal forebrain-derived NSCs can generate cholinergic neurons with spinal motor neuron properties when treated with FGF2 within a specific time window (Jordan et al., 2009). Moreover, ESC-derived motor neurons, grown using a differentiation program that relies on endogenous embryoid body-derived WNTS, FGFs, and HH signaling, and then grafted isochronically into chick spinal cord, settle in appropriate columnar domains and select axonal trajectories with a fidelity that matches that of their *in vivo* generated counterparts (Peljto et al., 2010). Under those differentiation conditions, it is not clear if increasing FGF levels would increase motor neuron yields without sacrificing the columnar and motor pool subtype diversity achieved.

In the last few years induced pluripotent stem cells (iPSCs) have provided a platform for studying basic human development and disease mechanisms and hold great potential for future cell therapies (Murry and Keller, 2008). Nevertheless, biomedical application of iPSCs depends on the availability of robust cell expansion and differentiation protocols. A recent example is the use of FGFR inhibitor (SU5402) that promoted iPSCs to commit to a NCC cell fate that express specific genes, including *PAX3*, *SLUG*, *TFAP-2α*, and *TWIST1* (Jaroonwitchawan et al., 2016).

FGF is also required for the specification of cell types outside the embryonic spinal cord such as the midbrain dopaminergic neurons (Ye et al., 1998). Human pluripotent stem cells have also been successfully converted into dopaminergic neurons using a novel floor plate-based strategy that involves the use of SHH and WNT agonists together with FGF8 and these are efficiently engrafted *in vivo* using rat, mouse and monkey models (Kriks et al., 2011). This could be promising for the development of cell-based therapies in Parkinson's disease.

Finally, it also important to consider the oncogenic risk associated to the mitogenic potential of cells treated with FGFs in transplantation experiments. As a recent example, human cord blood-derived iPSCs have been differentiated into dopaminergic neurons using either FGF2 or BMP/TGF-β inhibitor for neural induction. After transplantation in hemiparkinsonian rats *in vivo*, proliferation still occurred in FGF2-derived grafts (but not in BMP inhibitor treated grafts), resulting in tumor-like growth (Effenberg et al., 2015). Similarly, those effects have also been described for neurospheres derived from hIPSCs and transplanted into spinal cord injured mice (Nori et al., 2015).

FUTURE DIRECTIONS AND CHALLENGES

This review highlights the multiple steps in spinal cord development that are regulated by FGF signaling, which may be viewed as a sensor of caudal elongation serving to coordinate different aspects of spinal cord maturation to each other, to adjacent mesoderm and to axial elongation. Further analysis of FGF signaling deficiency in mouse would help ascertain the extent of its contribution to floor plate formation, early neurogenesis, rostro-caudal patterning and neural crest development.

The molecular mechanisms that link FGF signaling specifically to the different functions are still not fully identified but for most of its functions, specific transcriptional targets downstream of the pathway have been proposed. It has been shown that FGF influences transcription by changing the phosphorylation state of transcription factors such as those of the ETV family. The analysis of the regulatory regions of the proposed targets will confirm which of them are more directly regulated. FGF also has an influence on chromatin compaction and nuclear positioning of specific gene loci (Patel et al., 2013) and this may be due, at least in part to the ability of FGF to regulate chromatin modifiers such as histone deacetylase 1 (HDAC1) (Olivera-Martinez et al., 2014).

The detailed regulation of the pathway including the intracellular dynamics of the MAPK pathway with its positive and negative feedbacks (Lake et al., 2016) as well as the involvement of the other FGFR dependent cascades (AKT, PKC) in some of the processes described here also remains largely unexplored. The understanding of the mechanisms responsible for the maintenance of FGF8 and FGF4, the principal ligands in this context, in the NMP and adjacent regions and their progressive downregulation would provide a better insight into axis elongation.

So far, the majority of the literature relies on static views of the expression of ligands and pathway components at different developmental stages. However, it is clear that those are highly dynamic and thus the development of reliable biosensors to measure FGF activity *in vivo* would help to address fundamental questions such as the mechanisms underlying the temporal changes in the response of NMP and its derivatives to FGF.

Throughout the review, we have focused on the similarities that exist in the different vertebrate species but it would also be interesting to understand how FGF functions may have diverged to accommodate the different modes of spinal cord formation (Steventon and Martinez Arias, in press). Equally interesting would be to study the emergence, during chordate evolution, of a function of caudal FGF on development of the caudal neural tube. FGF signaling has been described in the tailbud of the amphioxus cephalochordate embryo and, although only a limited role in somitogenesis has been described (Bertrand et al., 2015), it would be interesting to assess its requirement in spinal cord development.

Some of the functions of FGF described in the development of the spinal cord may also contribute to the maintenance of the ependymal neurogenic niche present in the adult spinal cord and to the functional recovery after SCI shown in rodents and currently under study in humans. Furthermore, the role of FGFs in the maintenance and expansion of neural progenitors as well as their promotion of specific fates *in vitro* supports their therapeutical potential in regenerative biomedicine. The advances in understanding the detailed mechanism underlying FGF function during the development of the central nervous system, and in particular of the spinal cord, should serve to selectively potentiate some of its functions.

AUTHOR CONTRIBUTIONS

RD and AM jointly conceived, organized and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Gil Costa (Champalimaud Research, Champalimaud Centre for the Unknown, Lisbon, Portugal) for illustrations in **Figures 1**, **3**, **4**. We acknowledge support of our work by a grant from the Spanish MINECO (BFU2014-57494-R) and from Champalimaud Foudation (to RD), and support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

REFERENCES

Ahuja, C. S., Martin, A. R., and Fehlings, M. (2016). Recent advances in managing a spinal cord injury secondary to trauma. *F1000Res* 5:1017. doi: 10.12688/f1000research.7586.1

Aires, R., Jurberg, A. D., Leal, F., Novoa, A., Cohn, M. J., and Mallo, M. (2016). Oct4 is a key regulator of vertebrate trunk length diversity. *Dev. Cell* 38, 262–274. doi: 10.1016/j.devcel.2016.06.021

Akai, J., Halley, P. A., and Storey, K. G. (2005). FGF-dependent Notch signaling maintains the spinal cord stem zone. *Genes Dev.* 19, 2877–2887. doi: 10.1101/gad.357705

Akiyama, R., Masuda, M., Tsuge, S., Bessho, Y., and Matsui, T. (2014). An anterior limit of FGF/Erk signal activity marks the earliest future somite boundary in zebrafish. *Development* 141, 1104–1109. doi: 10.1242/dev.098905

Amin, S., Neijts, R., Simmini, S., van Rooijen, C., Tan, S. C., Kester, L., et al. (2016). Cdx and T brachyury co-activate growth signaling in the embryonic axial progenitor niche. *Cell Rep.* 17, 3165–3177. doi: 10.1016/j.celrep.2016.11.069

Anderson, M. J., Schimmang, T., and Lewandoski, M. (2016a). An FGF3-BMP signaling axis regulates caudal neural tube closure, neural crest specification and anterior-posterior axis extension. *PLoS Genet.* 12:e1006018. doi: 10.1371/journal.pgen.1006018

Anderson, M. J., Southon, E., Tessarollo, L., and Lewandoski, M. (2016b). Fgf3-Fgf4-cis: a new mouse line for studying Fgf functions during mouse development. *Genesis* 54, 91–98. doi: 10.1002/dvg.22913

Aulehla, A., Wiegraebe, W., Baubet, V., Wahl, M. B., Deng, C., Taketo, M., et al. (2008). A β-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. *Nat. Cell Biol.* 10, 186–193. doi: 10.1038/ncb1679

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B., et al. (2003). *Wnt3a* plays a major role in the segmentation clock controlling somitogenesis. *Dev. Cell* 4, 395–406. doi: 10.1016/S1534-5807(03)00055-8

Barnabe-Heider, F., Goritz, C., Sabelstrom, H., Takebayashi, H., Pfrieger, F. W., Meletis, K., et al. (2010). Origin of new glial cells in intact and injured adult spinal cord. *Cell Stem Cell* 7, 470–482. doi: 10.1016/j.stem.2010.07.014

Beermann, F., Kaloulis, K., Hofmann, D., Murisier, F., Bucher, P., and Trumpp, A. (2006). Identification of evolutionarily conserved regulatory elements in the mouse Fgf8 locus. *Genesis* 44, 1–6. doi: 10.1002/gene.20177

Bel-Vialar, S., Itasaki, N., and Krumlauf, R. (2002). Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. *Development* 129, 5103–5115.

Bel-Vialar, S., Medevielle, F., and Pituello, F. (2007). The on/off of Pax6 controls the tempo of neuronal differentiation in the developing spinal cord. *Dev. Biol.* 305, 659–673. doi: 10.1016/j.ydbio.2007.02.012

Benazeraf, B., Francois, P., Baker, R. E., Denans, N., Little, C. D., and Pourquie, O. (2010). A random cell motility gradient downstream of FGF controls elongation of an amniote embryo. *Nature* 466, 248–252. doi: 10.1038/nature09151

Bertrand, N., Medevielle, F., and Pituello, F. (2000). FGF signalling controls the timing of Pax6 activation in the neural tube. *Development* 127, 4837–4843.

Bertrand, S., Aldea, D., Oulion, S., Subirana, L., de Lera, A. R., Somorjai, I., et al. (2015). Evolution of the role of RA and FGF signals in the control of somitogenesis in chordates. *PLoS ONE* 10:e0136587. doi: 10.1371/journal.pone.0136587

Bokel, C., and Brand, M. (2013). Generation and interpretation of FGF morphogen gradients in vertebrates. *Curr. Opin. Genet. Dev.* 23, 415–422. doi: 10.1016/j.gde.2013.03.002

Boulet, A. M., and Capecchi, M. R. (2012). Signaling by FGF4 and FGF8 is required for axial elongation of the mouse embryo. *Dev. Biol.* 371, 235–245. doi: 10.1016/j.ydbio.2012.08.017

Briscoe, J., Pierani, A., Jessell, T. M., and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* 101, 435–445. doi: 10.1016/S0092-8674(00)80853-3

Briscoe, J., and Small, S. (2015). Morphogen rules: design principles of gradient-mediated embryo patterning. *Development* 142, 3996–4009. doi: 10.1242/dev.129452

Bulusu, V., Prior, N., Snaebjornsson, M. T., Kuehne, A., Sonnen, K. F., Kress, J., et al. (2017). Spatiotemporal analysis of a glycolytic activity gradient linked to mouse embryo mesoderm development. *Dev Cell* 40, 331–341. e334. doi: 10.1016/j.devcel.2017.01.015

Burstyn-Cohen, T., Stanleigh, J., Sela-Donenfeld, D., and Kalcheim, C. (2004). Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition. *Development* 131, 5327–5339. doi: 10.1242/dev.01424

Calegari, F., Haubensak, W., Haffner, C., and Huttner, W. B. (2005). Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. *J. Neurosci.* 25, 6533–6538. doi: 10.1523/JNEUROSCI.0778-05.2005

Calegari, F., and Huttner, W. B. (2003). An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. *J. Cell Sci.* 116(Pt 24), 4947–4955. doi: 10.1242/jcs.00825

Cao, Y., Zhao, J., Sun, Z., Zhao, Z., Postlethwait, J., and Meng, A. (2004). fgf17b, a novel member of Fgf family, helps patterning zebrafish embryos. *Dev. Biol.* 271, 130–143. doi: 10.1016/j.ydbio.2004.03.032

Chen, B., He, J., Yang, H., Zhang, Q., Zhang, L., Zhang, X., et al. (2015). Repair of spinal cord injury by implantation of bFGF-incorporated HEMA-MOETACL hydrogel in rats. *Sci. Rep.* 5:9017. doi: 10.1038/srep09017

Cho, G., Lim, Y., Cho, I. T., Simonet, J. C., and Golden, J. A. (2014). Arx together with FoxA2, regulates Shh floor plate expression. *Dev. Biol.* 393, 137–148. doi: 10.1016/j.ydbio.2014.06.012

Chotteau-Lelievre, A., Dolle, P., Peronne, V., Coutte, L., de Launoit, Y., and Desbiens, X. (2001). Expression patterns of the Ets transcription factors from the PEA3 group during early stages of mouse development. *Mech. Dev.* 108, 191–195. doi: 10.1016/S0925-4773(01)00480-4

Christian, K. M., Song, H., and Ming, G. L. (2014). Functions and dysfunctions of adult hippocampal neurogenesis. *Annu. Rev. Neurosci.* 37, 243–262. doi: 10.1146/annurev-neuro-071013-014134

Ciruna, B. G., Schwartz, L., Harpal, K., Yamaguchi, T. P., and Rossant, J. (1997). Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development* 124, 2829–2841.

Ciruna, B., and Rossant, J. (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev. Cell* 1, 37–49. doi: 10.1016/S1534-5807(01)00017-X

Corson, L. B., Yamanaka, Y., Lai, K. M., and Rossant, J. (2003). Spatial and temporal patterns of ERK signaling during mouse embryogenesis. *Development* 130, 4527–4537. doi: 10.1242/dev.00669

Cunningham, T. J., Brade, T., Sandell, L. L., Lewandoski, M., Trainor, P. A., Colas, A., et al. (2015a). Retinoic acid activity in undifferentiated neural progenitors is sufficient to fulfill its role in restricting Fgf8 expression for somitogenesis. *PLoS ONE* 10:e0137894. doi: 10.1371/journal.pone.0137894

Cunningham, T. J., and Duester, G. (2015). Mechanisms of retinoic acid signalling and its roles in organ and limb development. *Nat. Rev. Mol. Cell Biol.* 16, 110–123. doi: 10.1038/nrm3932

Cunningham, T. J., Kumar, S., Yamaguchi, T. P., and Duester, G. (2015b). Wnt8a and Wnt3a cooperate in the axial stem cell niche to promote mammalian body axis extension. *Dev. Dyn.* 244, 797–807. doi: 10.1002/dvdy.24275

Cunningham, T. J., Zhao, X., and Duester, G. (2011). Uncoupling of retinoic acid signaling from tailbud development before termination of body axis extension. *Genesis* 49, 776–783. doi: 10.1002/dvg.20763

Dasen, J. S., Liu, J. P., and Jessell, T. M. (2003). Motor neuron columnar fate imposed by sequential phases of Hox-c activity. *Nature* 425, 926–933. doi: 10.1038/nature02051

de la Calle-Mustienes, E., Glavic, A., Modolell, J., and Gomez-Skarmeta, J. L. (2002). Xiro homeoproteins coordinate cell cycle exit and primary neuron formation by upregulating neuronal-fate repressors and downregulating the cell-cycle inhibitor *XGadd45-γ*. *Mech. Dev.* 119, 69–80. doi: 10.1016/S0925-4773(02)00296-4

Delfino-Machin, M., Lunn, J. S., Breitkreuz, D. N., Akai, J., and Storey, K. G. (2005). Specification and maintenance of the spinal cord stem zone. *Development* 132, 4273–4283. doi: 10.1242/dev.02009

Diez del Corral, R., Breitkreuz, D. N., and Storey, K. G. (2002). Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. *Development* 129, 1681–1691.

Diez del Corral, R., and Morales, A. V. (2014). Retinoic acid signaling during early spinal cord development. *J. Dev. Biol.* 2, 174–197. doi: 10.3390/jdb2030174

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M., and Storey, K. (2003). Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. *Neuron* 40, 65–79. doi: 10.1016/S0896-6273(03)00565-8

Draper, B. W., Stock, D. W., and Kimmel, C. B. (2003). Zebrafish fgf24 functions with fgf8 to promote posterior mesodermal development. *Development* 130, 4639–4654. doi: 10.1242/dev.00671

Dubrulle, J., McGrew, M. J., and Pourquie, O. (2001). FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal *Hox* gene activation. *Cell* 106, 219–232. doi: 10.1016/S0092-8674(01)00437-8

Dubrulle, J., and Pourquie, O. (2004). fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* 427, 419–422. doi: 10.1038/nature02216

Dunty, W. C. Jr., Biris, K. K., Chalamalasetty, R. B., Taketo, M. M., Lewandoski, M., and Yamaguchi, T. P. (2008). Wnt3a/beta-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation. *Development* 135, 85–94. doi: 10.1242/dev.009266

Effenberg, A., Stanslowsky, N., Klein, A., Wesemann, M., Haase, A., Martin, U., et al. (2015). Striatal transplantation of human dopaminergic neurons differentiated from induced pluripotent stem cells derived from umbilical cord blood using lentiviral reprogramming. *Cell Transplant.* 24, 2099–2112. doi: 10.3727/096368914X685591

Ekerot, M., Stavridis, M. P., Delavaine, L., Mitchell, M. P., Staples, C., Owens, D. M., et al. (2008). Negative-feedback regulation of FGF signalling by DUSP6/MKP-3 is driven by ERK1/2 and mediated by Ets factor binding to a conserved site within the DUSP6/MKP-3 gene promoter. *Biochem. J.* 412, 287–298. doi: 10.1042/BJ20071512

Francius, C., Harris, A., Rucchin, V., Hendricks, T. J., Stam, F. J., Barber, M., et al. (2013). Identification of multiple subsets of ventral interneurons and differential distribution along the rostrocaudal axis of the developing spinal cord. *PLoS ONE* 8:e70325. doi: 10.1371/journal.pone.0070325

Fuentealba, L. C., Obernier, K., and Alvarez-Buylla, A. (2012). Adult neural stem cells bridge their niche. *Cell Stem Cell* 10, 698–708. doi: 10.1016/j.stem.2012.05.012

Gabay, L., Lowell, S., Rubin, L. L., and Anderson, D. J. (2003). Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells *in vitro*. *Neuron* 40, 485–499. doi: 10.1016/S0896-6273(03)00637-8

Gage, F. H., Ray, J., and Fisher, L. J. (1995). Isolation, characterization, and use of stem cells from the CNS. *Annu. Rev. Neurosci.* 18, 159–192. doi: 10.1146/annurev.ne.18.030195.001111

Gofflot, F., Hall, M., and Morriss-Kay, G. M. (1997). Genetic patterning of the developing mouse tail at the time of posterior neuropore closure. *Dev. Dyn.* 210, 431–445. doi: 10.1002/(SICI)1097-0177(199712)210:4<431::AID-AJA7>3.0.CO;2-H

Goldshmit, Y., Frisca, F., Pinto, A. R., Pebay, A., Tang, J. K., Siegel, A. L., et al. (2014). Fgf2 improves functional recovery-decreasing gliosis and increasing radial glia and neural progenitor cells after spinal cord injury. *Brain Behav.* 4, 187–200. doi: 10.1002/brb3.172

Gonzalez-Quevedo, R., Lee, Y., Poss, K. D., and Wilkinson, D. G. (2010). Neuronal regulation of the spatial patterning of neurogenesis. *Dev. Cell* 18, 136–147. doi: 10.1016/j.devcel.2009.11.010

Gotz, M., and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788. doi: 10.1038/nrm1739

Gouti, M., Metzis, V., and Briscoe, J. (2015). The route to spinal cord cell types: a tale of signals and switches. *Trends Genet.* 31, 282–289. doi: 10.1016/j.tig.2015.03.001

Gouti, M., Tsakiridis, A., Wymeersch, F. J., Huang, Y., Kleinjung, J., Wilson, V., et al. (2014). *In vitro* generation of neuromesodermal progenitors reveals distinct roles for wnt signalling in the specification of spinal cord and paraxial mesoderm identity. *PLoS Biol.* 12:e1001937. doi: 10.1371/journal.pbio.1001937

Griffin, K., Patient, R., and Holder, N. (1995). Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. *Development* 121, 2983–2994.

Gritti, A., Parati, E. A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., et al. (1996). Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci.* 16, 1091–1100.

Guzen, F. P., Soares, J. G., de Freitas, L. M., Cavalcanti, J. R., Oliveira, F. G., Araujo, J. F., et al. (2012). Sciatic nerve grafting and inoculation of FGF-2 promotes improvement of motor behavior and fiber regrowth in rats with spinal cord transection. *Restor. Neurol. Neurosci.* 30, 265–275. doi: 10.3233/RNN-2012-110184

Hack, M. A., Sugimori, M., Lundberg, C., Nakafuku, M., and Gotz, M. (2004). Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol. Cell. Neurosci.* 25, 664–678. doi: 10.1016/j.mcn.2003.12.012

Harduf, H., Halperin, E., Reshef, R., and Ron, D. (2005). Sef is synexpressed with FGFs during chick embryogenesis and its expression is differentially regulated by FGFs in the developing limb. *Dev. Dyn.* 233, 301–312. doi: 10.1002/dvdy.20364

Harrison, N. C., Diez del Corral, R., and Vasiev, B. (2011). Coordination of cell differentiation and migration in mathematical models of caudal embryonic axis extension. *PLoS ONE* 6:e22700. doi: 10.1371/journal.pone.0022700

Henrique, D., Abranches, E., Verrier, L., and Storey, K. G. (2015). Neuromesodermal progenitors and the making of the spinal cord. *Development* 142, 2864–2875. doi: 10.1242/dev.119768

Holowacz, T., and Sokol, S. (1999). FGF is required for posterior neural patterning but not for neural induction. *Dev. Biol.* 205, 296–308. doi: 10.1006/dbio.1998.9108

Holtz, A. M., Peterson, K. A., Nishi, Y., Morin, S., Song, J. Y., Charron, F., et al. (2013). Essential role for ligand-dependent feedback antagonism of vertebrate hedgehog signaling by PTCH1, PTCH2 and HHIP1 during neural patterning. *Development* 140, 3423–3434. doi: 10.1242/dev.095083

Isaacs, H. V., Pownall, M. E., and Slack, J. M. (1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J.* 13, 4469–4481

Jaroonwitchawan, T., Muangchan, P., and Noisa, P. (2016). Inhibition of FGF signaling accelerates neural crest cell differentiation of human pluripotent stem cells. *Biochem. Biophys. Res. Commun.* 481, 176–181. doi: 10.1016/j.bbrc.2016.10.147

Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U., and Frisen, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96, 25–34.

Jordan, P. M., Ojeda, L. D., Thonhoff, J. R., Gao, J., Boehning, D., Yu, Y., et al. (2009). Generation of spinal motor neurons from human fetal brain-derived neural stem cells: role of basic fibroblast growth factor. *J. Neurosci. Res.* 87, 318–332. doi: 10.1002/jnr.21856

Kang, C. E., Baumann, M. D., Tator, C. H., and Shoichet, M. S. (2013). Localized and sustained delivery of fibroblast growth factor-2 from a nanoparticle-hydrogel composite for treatment of spinal cord injury. *Cells Tissues Organs* 197, 55–63. doi: 10.1159/000339589

Kang, C. E., Clarkson, R., Tator, C. H., Yeung, I. W., and Shoichet, M. S. (2010). Spinal cord blood flow and blood vessel permeability measured by dynamic computed tomography imaging in rats after localized delivery of fibroblast growth factor. *J. Neurotrauma* 27, 2041–2053. doi: 10.1089/neu.2010.1345

Kang, W., and Hebert, J. M. (2015). FGF signaling is necessary for neurogenesis in young mice and sufficient to reverse its decline in old mice. *J. Neurosci.* 35, 10217–10223. doi: 10.1523/JNEUROSCI.1469-15.2015

Kang, W., Wong, L. C., Shi, S. H., and Hebert, J. M. (2009). The transition from radial glial to intermediate progenitor cell is inhibited by FGF signaling during corticogenesis. *J. Neurosci.* 29, 14571–14580. doi: 10.1523/JNEUROSCI.3844-09.2009

Karabagli, H., Karabagli, P., Ladher, R. K., and Schoenwolf, G. C. (2002). Comparison of the expression patterns of several fibroblast growth factors during chick gastrulation and neurulation. *Anat. Embryol.* 205, 365–370. doi: 10.1007/s00429-002-0264-7

Kilpatrick, T. J., and Bartlett, P. F. (1993). Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. *Neuron* 10, 255–265.

Kim, K. N., Oh, S. H., Lee, K. H., and Yoon, D. H. (2006). Effect of human mesenchymal stem cell transplantation combined with growth factor infusion in the repair of injured spinal cord. *Acta Neurochir. Suppl.* 99, 133–136. doi: 10.1007/978-3-211-35205-2_25

Kriks, S., Shim, J. W., Piao, J., Ganat, Y. M., Wakeman, D. R., Xie, Z., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480, 547–551. doi: 10.1038/nature10648

Kumar, S., Cunningham, T. J., and Duester, G. (2016). Nuclear receptor corepressors Ncor1 and Ncor2 (Smrt) are required for retinoic acid-dependent repression of Fgf8 during somitogenesis. *Dev. Biol.* 418, 204–215. doi: 10.1016/j.ydbio.2016.08.005

Kumar, S., and Duester, G. (2014). Retinoic acid controls body axis extension by directly repressing Fgf8 transcription. *Development* 141, 2972–2977. doi: 10.1242/dev.112367

Kuo, H. S., Tsai, M. J., Huang, M. C., Chiu, C. W., Tsai, C. Y., Lee, M. J., et al. (2011). Acid fibroblast growth factor and peripheral nerve grafts regulate Th2 cytokine expression, macrophage activation, polyamine synthesis, and neurotrophin expression in transected rat spinal cords. *J. Neurosci.* 31, 4137–4147. doi: 10.1523/JNEUROSCI.2592-10.2011

Kutejova, E., Sasai, N., Shah, A., Gouti, M., and Briscoe, J. (2016). Neural progenitors adopt specific identities by directly repressing all alternative progenitor transcriptional programs. *Dev. Cell* 36, 639–653. doi: 10.1016/j.devcel.2016.02.013

Lai, H. C., Seal, R. P., and Johnson, J. E. (2016). Making sense out of spinal cord somatosensory development. *Development* 143, 3434–3448. doi: 10.1242/dev.139592

Lake, D., Correa, S. A., and Muller, J. (2016). Negative feedback regulation of the ERK1/2 MAPK pathway. *Cell. Mol. Life Sci.* 73, 4397–4413. doi: 10.1007/s00018-016-2297-8

Lawton, A. K., Nandi, A., Stulberg, M. J., Dray, N., Sneddon, M. W., Pontius, W., et al. (2013). Regulated tissue fluidity steers zebrafish body elongation. *Development* 140, 573–582. doi: 10.1242/dev.090381

Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C., and Steindler, D. A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13883–13888. doi: 10.1073/pnas.250471697

Le Douarin, N. M., Creuzet, S., Couly, G., and Dupin, E. (2004). Neural crest cell plasticity and its limits. *Development* 131, 4637–4650. doi: 10.1242/dev.01350

Le Douarin, N. M., and Kalcheim, C. (1999). *The Neural Crest*. Cambridge, UK: Cambridge University Press.

Le Dreau, G., and Marti, E. (2012). Dorsal-ventral patterning of the neural tube: a tale of three signals. *Dev. Neurobiol.* 72, 1471–1481. doi: 10.1002/dneu.22015

Lee, T. T., Green, B. A., Dietrich, W. D., and Yezierski, R. P. (1999). Neuroprotective effects of basic fibroblast growth factor following spinal cord contusion injury in the rat. *J. Neurotrauma* 16, 347–356. doi: 10.1089/neu.1999.16.347

Lee, Y., Miller, H. L., Russell, H. R., Boyd, K., Curran, T., and McKinnon, P. J. (2006). Patched2 modulates tumorigenesis in patched1 heterozygous mice. *Cancer Res.* 66, 6964–6971. doi: 10.1158/0008-5472.CAN-06-0505

Liu, J.-P., Laufer, E., and Jessell, T. M. (2001). Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron* 32, 997–1012. doi: 10.1016/S0896-6273(01)00544-X

Lobjois, V., Benazeraf, B., Bertrand, N., Medevielle, F., and Pituello, F. (2004). Specific regulation of cyclins D1 and D2 by FGF and Shh signaling coordinates cell cycle progression, patterning, and differentiation during early steps of spinal cord development. *Dev. Biol.* 273, 195–209. doi: 10.1016/j.ydbio.2004.05.031

Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., et al. (2012). Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. *Cell* 150, 1264–1273. doi: 10.1016/j.cell.2012.08.020

Lunn, J. S., Fishwick, K. J., Halley, P. A., and Storey, K. G. (2007). A spatial and temporal map of FGF/Erk1/2 activity and response repertoires in the early chick embryo. *Dev. Biol.* 302, 536–552. doi: 10.1016/j.ydbio.2006.10.014

Maric, D., Fiorio Pla, A., Chang, Y. H., and Barker, J. L. (2007). Self-renewing and differentiating properties of cortical neural stem cells are selectively regulated by basic fibroblast growth factor (FGF) signaling via specific FGF receptors. *J. Neurosci.* 27, 1836–1852. doi: 10.1523/JNEUROSCI.5141-06.2007

Marinic, M., Aktas, T., Ruf, S., and Spitz, F. (2013). An integrated holo-enhancer unit defines tissue and gene specificity of the Fgf8 regulatory landscape. *Dev. Cell* 24, 530–542. doi: 10.1016/j.devcel.2013.01.025

Martinez-Morales, P. L., Diez del Corral, R., Olivera-Martinez, I., Quiroga, A. C., Das, R. M., Barbas, J. A., et al. (2011). FGF and retinoic acid activity gradients control the timing of neural crest cell emigration in the trunk. *J. Cell Biol.* 194, 489–503. doi: 10.1083/jcb.201011077

Mathis, L., Kulesa, P. M., and Fraser, S. E. (2001). FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nat. Cell Biol.* 3, 559–566. doi: 10.1038/35078535

Meijs, M. F., Timmers, L., Pearse, D. D., Tresco, P. A., Bates, M. L., Joosten, E. A., et al. (2004). Basic fibroblast growth factor promotes neuronal survival but not behavioral recovery in the transected and Schwann cell implanted rat thoracic spinal cord. *J. Neurotrauma* 21, 1415–1430. doi: 10.1089/neu.2004.21.1415

Meletis, K., Barnabe-Heider, F., Carlen, M., Evergren, E., Tomilin, N., Shupliakov, O., et al. (2008). Spinal cord injury reveals multilineage differentiation of ependymal cells. *PLoS Biol.* 6:e182. doi: 10.1371/journal.pbio.0060182

Molina, A., and Pituello, F. (in press). Playing with the cell cycle to build the spinal cord. *Dev. Biol.* doi: 10.1016/j.ydbio.2016.12.022

Molina, G. A., Watkins, S. C., and Tsang, M. (2007). Generation of FGF reporter transgenic zebrafish and their utility in chemical screens. *BMC Dev. Biol.* 7:62. doi: 10.1186/1471-213X-7-62

Molotkova, N., Molotkov, A., Sirbu, I. O., and Duester, G. (2005). Requirement of mesodermal retinoic acid generated by Raldh2 for posterior neural transformation. *Mech. Dev.* 122, 145–155. doi: 10.1016/j.mod.2004.10.008

Morales, A. V., Barbas, J. A., and Nieto, M. A. (2005). How to become neural crest: from segregation to delamination. *Semin. Cell Dev. Biol.* 16, 655–662. doi: 10.1016/j.semcdb.2005.06.003

Morales, A. V., Espeso-Gil, S., Ocana, I., Nieto-Lopez, F., Calleja, E., Bovolenta, P., et al. (2016). FGF signaling enhances a sonic hedgehog negative feedback loop at the initiation of spinal cord ventral patterning. *Dev. Neurobiol.* 76, 956–971. doi: 10.1002/dneu.22368

Muhr, J., Graziano, E., Wilson, S., Jessell, T. M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. *Neuron* 23, 689–702.

Murry, C. E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132, 661–680. doi: 10.1016/j.cell.2008.02.008

Naiche, L. A., Holder, N., and Lewandoski, M. (2011). FGF4 and FGF8 comprise the wavefront activity that controls somitogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4018–4023. doi: 10.1073/pnas.1007417108

Nelson, A. D., and Svendsen, C. N. (2006). Low concentrations of extracellular FGF-2 are sufficient but not essential for neurogenesis from human neural progenitor cells. *Mol. Cell. Neurosci.* 33, 29–35. doi: 10.1016/j.mcn.2006.06.003

Nieuwenhuis, E., Motoyama, J., Barnfield, P. C., Yoshikawa, Y., Zhang, X., Mo, R., et al. (2006). Mice with a targeted mutation of patched2 are viable but develop alopecia and epidermal hyperplasia. *Mol. Cell. Biol.* 26, 6609–6622. doi: 10.1128/MCB.00295-06

Nishimura, N., Kamimura, Y., Ishida, Y., Takemoto, T., Kondoh, H., and Uchikawa, M. (2012). A systematic survey and characterization of enhancers that regulate Sox3 in neuro-sensory development in comparison with Sox2 enhancers. *Biology* 1, 714–735. doi: 10.3390/biology1030714

Nishita, J., Ohta, S., Bleyl, S. B., and Schoenwolf, G. C. (2011). Detection of isoform-specific fibroblast growth factor receptors by whole-mount *in situ* hybridization in early chick embryos. *Dev. Dyn.* 240, 1537–1547. doi: 10.1002/dvdy.22616

Nordstrom, U., Maier, E., Jessell, T. M., and Edlund, T. (2006). An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. *PLoS Biol.* 4:e252. doi: 10.1371/journal.pbio.0040252

Nori, S., Okada, Y., Nishimura, S., Sasaki, T., Itakura, G., Kobayashi, Y., et al. (2015). Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: oncogenic transformation with epithelial-mesenchymal transition. *Stem Cell Reports* 4, 360–373. doi: 10.1016/j.stemcr.2015.01.006

Novitch, B. G., Wichterle, H., Jessell, T. M., and Sockanathan, S. (2003). A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* 40, 81–95. doi: 10.1016/j.neuron.2003.08.006

Oginuma, M., Moncuquet, P., Xiong, F., Karoly, E., Chal, J., Guevorkian, K., et al. (2017). A gradient of glycolytic activity coordinates FGF and Wnt signaling during elongation of the body axis in amniote embryos. *Dev. Cell* 40, 342–353, e310. doi: 10.1016/j.devcel.2017.02.001

Olivera-Martinez, I., Harada, H., Halley, P. A., and Storey, K. G. (2012). Loss of FGF-dependent mesoderm identity and rise of endogenous retinoid signalling determine cessation of body axis elongation. *PLoS Biol.* 10:e1001415. doi: 10.1371/journal.pbio.1001415

Olivera-Martinez, I., Schurch, N., Li, R. A., Song, J., Halley, P. A., Das, R. M., et al. (2014). Major transcriptome re-organisation and abrupt changes in signalling, cell cycle and chromatin regulation at neural differentiation *in vivo*. *Development* 141, 3266–3276. doi: 10.1242/dev.112623

Olivera-Martinez, I., and Storey, K. G. (2007). Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension. *Development* 134, 2125–2135. doi: 10.1242/dev.000216

Ornitz, D. M., and Itoh, N. (2015). The fibroblast growth factor signaling pathway. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 215–266. doi: 10.1002/wdev.176

Ota, S., Tonou-Fujimori, N., and Yamasu, K. (2009). The roles of the FGF signal in zebrafish embryos analyzed using constitutive activation and dominant-negative suppression of different FGF receptors. *Mech. Dev.* 126, 1–17. doi: 10.1016/j.mod.2008.10.008

Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F., and Gage, F. H. (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* 19, 8487–8497.

Patel, N. S., Rhinn, M., Semprich, C. I., Halley, P. A., Dolle, P., Bickmore, W. A., et al. (2013). FGF signalling regulates chromatin organisation during neural differentiation via mechanisms that can be uncoupled from transcription. *PLoS Genet.* 9:e1003614. doi: 10.1371/journal.pgen.1003614

Peljto, M., Dasen, J. S., Mazzoni, E. O., Jessell, T. M., and Wichterle, H. (2010). Functional diversity of ESC-derived motor neuron subtypes revealed through intraspinal transplantation. *Cell Stem Cell* 7, 355–366. doi: 10.1016/j.stem.2010.07.013

Pfenninger, C. V., Steinhoff, C., Hertwig, F., and Nuber, U. A. (2011). Prospectively isolated CD133/CD24-positive ependymal cells from the adult spinal cord and lateral ventricle wall differ in their long-term *in vitro* self-renewal and *in vivo* gene expression. *Glia* 59, 68–81. doi: 10.1002/glia.21077

Philippidou, P., and Dasen, J. S. (2013). Hox genes: choreographers in neural development, architects of circuit organization. *Neuron* 80, 12–34. doi: 10.1016/j.neuron.2013.09.020

Pollard, S. M., Wallbank, R., Tomlinson, S., Grotewold, L., and Smith, A. (2008). Fibroblast growth factor induces a neural stem cell phenotype in foetal forebrain progenitors and during embryonic stem cell differentiation. *Mol. Cell. Neurosci.* 38, 393–403. doi: 10.1016/j.mcn.2008.03.012

Qian, X., Davis, A. A., Goderie, S. K., and Temple, S. (1997). FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. *Neuron* 18, 81–93.

Raballo, R., Rhee, J., Lyn-Cook, R., Leckman, J. F., Schwartz, M. L., and Vaccarino, F. M. (2000). Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *J. Neurosci.* 20, 5012–5023.

Rabchevsky, A. G., Fugaccia, I., Fletcher-Turner, A., Blades, D. A., Mattson, M. P., and Scheff, S. W. (1999). Basic fibroblast growth factor (bFGF) enhances tissue sparing and functional recovery following moderate spinal cord injury. *J. Neurotrauma* 16, 817–830. doi: 10.1089/neu.1999.16.817

Rash, B. G., Lim, H. D., Breunig, J. J., and Vaccarino, F. M. (2011). FGF signaling expands embryonic cortical surface area by regulating Notch-dependent neurogenesis. *J. Neurosci.* 31, 15604–15617. doi: 10.1523/JNEUROSCI.4439-11.2011

Rash, B. G., Tomasi, S., Lim, H. D., Suh, C. Y., and Vaccarino, F. M. (2013). Cortical gyrification induced by fibroblast growth factor 2 in the mouse brain. *J. Neurosci.* 33, 10802–10814. doi: 10.1523/JNEUROSCI.3621-12.2013

Reifers, F., Adams, J., Mason, I. J., Schulte-Merker, S., and Brand, M. (2000). Overlapping and distinct functions provided by *fgf17*, a new zebrafish member of the Fgf8/17/18 subgroup of Fgfs. *Mech. Dev.* 99, 39–49. doi: 10.1016/S0925-4773(00)00475-5

Resende, T. P., Ferreira, M., Teillet, M. A., Tavares, A. T., Andrade, R. P., and Palmeirim, I. (2010). Sonic hedgehog in temporal control of somite formation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12907–12912. doi: 10.1073/pnas.1000979107

Rosser, A. E., Zietlow, R., and Dunnett, S. B. (2007). Stem cell transplantation for neurodegenerative diseases. *Curr. Opin. Neurol.* 20, 688–692. doi: 10.1097/WCO.0b013e3282f132fc

Row, R. H., Tsotras, S. R., Goto, H., and Martin, B. L. (2016). The zebrafish tailbud contains two independent populations of midline progenitor cells that maintain long-term germ layer plasticity and differentiate in response to local signaling cues. *Development* 143, 244–254. doi: 10.1242/dev.129015

Roztocil, T., Matter-Sadzinski, L., Alliod, C., Ballivet, M., and Matter, J. M. (1997). NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. *Development* 124, 3263–3272.

Sabelstrom, H., Stenudd, M., Reu, P., Dias, D. O., Elfineh, M., Zdunek, S., et al. (2013). Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice. *Science* 342, 637–640. doi: 10.1126/science.1242576

Sahara, S., and O'Leary, D. D. (2009). Fgf10 regulates transition period of cortical stem cell differentiation to radial glia controlling generation of neurons and basal progenitors. *Neuron* 63, 48–62. doi: 10.1016/j.neuron.2009.06.006

Saint-Jeannet, J. P., and Moody, S. A. (2014). Establishing the pre-placodal region and breaking it into placodes with distinct identities. *Dev. Biol.* 389, 13–27. doi: 10.1016/j.ydbio.2014.02.011

Sasai, N., Kutejova, E., and Briscoe, J. (2014). Integration of signals along orthogonal axes of the vertebrate neural tube controls progenitor competence and increases cell diversity. *PLoS Biol.* 12:e1001907. doi: 10.1371/journal.pbio.1001907

Sauka-Spengler, T., and Bronner-Fraser, M. (2008). Evolution of the neural crest viewed from a gene regulatory perspective. *Genesis* 46, 673–682. doi: 10.1002/dvg.20436

Savory, J. G., Bouchard, N., Pierre, V., Rijli, F. M., De Repentigny, Y., Kothary, R., et al. (2009). Cdx2 regulation of posterior development through non-Hox targets. *Development* 136, 4099–4110. doi: 10.1242/dev.041582

Scholpp, S., and Brand, M. (2004). Endocytosis controls spreading and effective signaling range of Fgf8 protein. *Curr. Biol.* 14, 1834–1841. doi: 10.1016/j.cub.2004.09.084

Sechrist, J., and Bronner-Fraser, M. (1991). Birth and differentiation of reticular neurons in the chick hindbrain: ontogeny of the first neuronal population. *Neuron* 7, 947–963.

Sela-Donenfeld, D., and Kalcheim, C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development* 126, 4749–4762.

Shihabuddin, L. S., Ray, J., and Gage, F. H. (1997). FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp. Neurol.* 148, 577–586. doi: 10.1006/exnr.1997.6697

Shimizu, T., Bae, Y. K., and Hibi, M. (2006). Cdx-Hox code controls competence for responding to Fgfs and retinoic acid in zebrafish neural tissue. *Development* 133, 4709–4719. doi: 10.1242/dev.02660

Siddiqui, A. M., Khazaei, M., and Fehlings, M. G. (2015). Translating mechanisms of neuroprotection, regeneration, and repair to treatment of spinal cord injury. *Prog. Brain Res.* 218, 15–54. doi: 10.1016/bs.pbr.2014.12.007

Simon, R., and Lufkin, T. (2003). Postnatal lethality in mice lacking the Sax2 homeobox gene homologous to Drosophila S59/slouch: evidence for positive and negative autoregulation. *Mol. Cell Biol.* 23, 9046–9060. doi: 10.1128/MCB.23.24.9046-9060.2003

Sirbu, I. O., and Duester, G. (2006). Retinoic-acid signalling in node ectoderm and posterior neural plate directs left-right patterning of somitic mesoderm. *Nat. Cell Biol.* 8, 271–277. doi: 10.1038/ncb1374

Stavridis, M. P., Collins, B. J., and Storey, K. G. (2010). Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation. *Development* 137, 881–890. doi: 10.1242/dev.043117

Steventon, B., and Martinez Arias, A. (in press). Evo-engineering and the cellular and molecular origins of the vertebrate spinal cord. *Dev. Biol.* doi: 10.1016/j.ydbio.2017.01.021

Storey, K. G., Goriely, A., Sargent, C. M., Brown, J. M., Burns, H. D., Abud, H. M., et al. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. *Development* 125, 473–484.

Storm, E. E., Garel, S., Borello, U., Hebert, J. M., Martinez, S., McConnell, S. K., et al. (2006). Dose-dependent functions of Fgf8 in regulating telencephalic patterning centers. *Development* 133, 1831–1844. doi: 10.1242/dev.02324

Takahashi, T., Nowakowski, R. S., and Caviness, V. S. Jr. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J. Neurosci.* 15, 6046–6057.

Takemoto, T., Uchikawa, M., Kamachi, Y., and Kondoh, H. (2006). Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. *Development* 133, 297–306. doi: 10.1242/dev.02196

Teng, Y. D., Mocchetti, I., Taveira-DaSilva, A. M., Gillis, R. A., and Wrathall, J. R. (1999). Basic fibroblast growth factor increases long-term survival of spinal

motor neurons and improves respiratory function after experimental spinal cord injury. *J. Neurosci.* 19, 7037–7047.

Teng, Y. D., Mocchetti, I., and Wrathall, J. R. (1998). Basic and acidic fibroblast growth factors protect spinal motor neurones *in vivo* after experimental spinal cord injury. *Eur. J. Neurosci.* 10, 798–802.

Turner, D. A., Hayward, P. C., Baillie-Johnson, P., Rue, P., Broome, R., Faunes, F., et al. (2014). Wnt/beta-catenin and FGF signalling direct the specification and maintenance of a neuromesodermal axial progenitor in ensembles of mouse embryonic stem cells. *Development* 141, 4243–4253. doi: 10.1242/dev.112979

Vaccarino, F. M., Schwartz, M. L., Raballo, R., Nilsen, J., Rhee, J., Zhou, M., et al. (1999). Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. *Nat. Neurosci.* 2, 246–253. doi: 10.1038/6350

van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B. I., et al. (2002). Cdx1 and Cdx2 have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* 129, 2181–2193.

Vermot, J., and Pourquie, O. (2005). Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature* 435, 215–220. doi: 10.1038/nature03488

Wahl, M. B., Deng, C., Lewandoski, M., and Pourquie, O. (2007). FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. *Development* 134, 4033–4041. doi: 10.1242/dev.009167

Wang, Y., Song, L., and Zhou, C. J. (2011). The canonical Wnt/beta-catenin signaling pathway regulates Fgf signaling for early facial development. *Dev. Biol.* 349, 250–260. doi: 10.1016/j.ydbio.2010.11.004

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A. C., et al. (1996). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J. Neurosci.* 16, 7599–7609.

Wilcock, A. C., Swedlow, J. R., and Storey, K. G. (2007). Mitotic spindle orientation distinguishes stem cell and terminal modes of neuron production in the early spinal cord. *Development* 134, 1943–1954. doi: 10.1242/dev.002519

Wilson, V., Olivera-Martinez, I., and Storey, K. G. (2009). Stem cells, signals and vertebrate body axis extension. *Development* 136, 1591–1604. doi: 10.1242/dev.021246

Wu, J. C., Huang, W. C., Chen, Y. C., Tu, T. H., Tsai, Y. A., Huang, S. F., et al. (2011). Acidic fibroblast growth factor for repair of human spinal cord injury: a clinical trial. *J. Neurosurg. Spine* 15, 216–227. doi: 10.3171/2011.4.SPINE10404

Yan, H. Q., Yu, J., Kline, A. E., Letart, P., Jenkins, L. W., Marion, D. W., et al. (2000). Evaluation of combined fibroblast growth factor-2 and moderate hypothermia therapy in traumatically brain injured rats. *Brain Res.* 887, 134–143.

Yang, X., Dormann, D., Munsterberg, A. E., and Weijer, C. J. (2002). Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8. *Dev. Cell* 3, 425–437. doi: 10.1016/S1534-5807(02)00256-3

Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A., and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* 93, 755–766.

Yoshida, M., Uchikawa, M., Rizzoti, K., Lovell-Badge, R., Takemoto, T., and Kondoh, H. (2014). Regulation of mesodermal precursor production by low-level expression of B1 Sox genes in the caudal lateral epiblast. *Mech. Dev.* 132, 59–68. doi: 10.1016/j.mod.2014.01.003

Yu, S. R., Burkhardt, M., Nowak, M., Ries, J., Petrasek, Z., Scholpp, S., et al. (2009). Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules. *Nature* 461, 533–536. doi: 10.1038/nature08391

Zhang, S. C., Wernig, M., Duncan, I. D., Brustle, O., and Thomson, J. A. (2001). *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* 19, 1129–1133. doi: 10.1038/nbt1201-1129

Zhulyn, O., Nieuwenhuis, E., Liu, Y. C., Angers, S., and Hui, C. C. (2015). Ptch2 shares overlapping functions with Ptch1 in Smo regulation and limb development. *Dev. Biol.* 397, 191–202. doi: 10.1016/j.ydbio.2014.10.023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 18 January 2017
doi: 10.3389/fcell.2016.00151

Turn It Down a Notch

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In the developing vertebrate embryo, segmentation initiates through the formation of repeated segments, or somites, on either side of the posterior neural tube along the anterior to posterior axis. The periodicity of somitogenesis is regulated by a molecular oscillator, the segmentation clock, driving cyclic gene expression in the unsegmented paraxial mesoderm, from which somites derive. Three signaling pathways underlie the molecular mechanism of the oscillator: Wnt, FGF, and Notch. In particular, Notch has been demonstrated to be an essential piece in the intricate somitogenesis regulation puzzle. Notch is required to synchronize oscillations between neighboring cells, and is moreover necessary for somite formation and clock gene oscillations. Following ligand activation, the Notch receptor is cleaved to liberate the active intracellular domain (NICD) and during somitogenesis NICD itself is produced and degraded in a cyclical manner, requiring tightly regulated, and coordinated turnover. It was recently shown that the pace of the segmentation clock is exquisitely sensitive to levels/stability of NICD. In this review, we focus on what is known about the mechanisms regulating NICD turnover, crucial to the activity of the pathway in all developmental contexts. To date, the regulation of NICD stability has been attributed to phosphorylation of the PEST domain which serves to recruit the SCF/Sel10/FBXW7 E3 ubiquitin ligase complex involved in NICD turnover. We will describe the pathophysiological relevance of NICD-FBXW7 interaction, whose defects have been linked to leukemia and a variety of solid cancers.

Keywords: somitogenesis, embryonic development, signalling pathway, notch, FBXW7

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Spanish National Research Council, Spain

Reviewed by:
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Marc Vooijs, Maastricht University, Netherlands
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *22 September 2016*
Accepted: *16 December 2016*
Published: *18 January 2017*

Citation:
Carrieri FA and Dale JK (2017) Turn It Down a Notch. Front. Cell Dev. Biol. 4:151. doi: 10.3389/fcell.2016.00151

INTRODUCTION

The formation of a segmented body plan is a conserved feature of embryogenesis for all vertebrate species. This process leads to the formation of transient embryonic segments, called somites. Somites are precursors of vertebrae and ribs, associated skeletal muscles, and some dermis (Christ et al., 2007). Their formation is regulated by a molecular oscillator called the segmentation clock (Gibb et al., 2010; Oates et al., 2012; Benazeraf and Pourquie, 2013). Aberrations in this mechanism lead to human developmental disorders, such as spondylocostal dysostosis (Pourquie, 2011; Eckalbar et al., 2012). Some of these malformations originate from defects in Notch signaling, suggesting that this pathway is essential in controlling and regulating vertebrate segmentation.

This review aims to give a general overview of the importance of the Notch signaling pathway in the segmentation clock in addition to a description of our current understanding of the Notch pathway, particularly focusing on the turnover and regulation of the Notch intracellular domain.

SOMITOGENESIS

Somitogenesis has been the topic of several outstanding reviews (Pourquie, 2001; Maroto et al., 2012; Oates et al., 2012; Benazeraf and Pourquie, 2013; Hubaud and Pourquie, 2014; Bailey and Dale, 2015), thus we will provide a general overview.

Early in development, segmentation initiates through the formation of repeated segments, or somites (Christ et al., 2007; Gibb et al., 2010). Somitogenesis is a cyclical and gradual process such that somites are sequentially pinched off in pairs from the anterior end of two rods of paraxial mesoderm, the presomitic mesoderm (PSM), lying on either side of the caudal neural tube (Gossler and De Angelis, 1998; Cambray and Wilson, 2007; Dequeant and Pourquie, 2008; Gibb et al., 2010; Maroto et al., 2012). The PSM is continuously replenished with progenitor cells located initially in both the epiblast adjacent to the primitive streak and the rostral primitive streak and later in the tail bud (Iimura et al., 2007; Gomez and Pourquie, 2009; Henrique et al., 2015), and thus the presomitic mesoderm preserves its length (Dequeant and Pourquie, 2008; **Figure 1A**).

The periodicity of this segmentation process is different from species to species: 30 min in zebrafish (Schroter et al., 2008), 90 min in chicken (Palmeirim et al., 1997), 2 h in mice (Tam, 1981), 6–8 h in human (William et al., 2007). Similarly, the total number of somites is a characteristic feature of each species: 31 pairs in zebrafish, 50 somite pairs in chicken, 65 in mice, and about 500 in some snakes.

The regulation of the periodicity of somitogenesis is governed by the segmentation clock, a molecular oscillator (Palmeirim et al., 1997) whose existence was first proposed in theoretical models such as the "*Clock and Wavefront model*" (Cooke and Zeeman, 1976). According to the model, a wavefront of maturation sweeps along the body axis concomitant with extension of the trunk and tail, governing maturation of the PSM to become somites. This positional information gradient within the PSM interacts with a smooth cellular oscillator (the clock), driving cells to oscillate between a permissive and a non-permissive state. Segmentation of the PSM only occurs when the maturation wavefront reaches a group of cells in a specific "permissive" clock phase (Cooke and Zeeman, 1976).

Over the last 20 years the theoretical "*Clock and Wavefront model*" has received significant experimental support. The wavefront of maturation is thought to rely on the intersecting gradients and cross-regulatory activities of three signal pathways, namely a caudo-rostral gradient of FGF and Wnt and rostro-caudal gradient of retinoic acid (RA). The determination front marks the point of intersection of these gradients, where the next prospective somite boundary will form (**Figure 1B**). These cross-regulatory activities thereby regulate somite size. The activity of Wnt and FGF also controls cell maturation in the PSM. These roles have been reviewed elsewhere, thus will not be covered here (Aulehla et al., 2003; Dubrulle and Pourquie, 2004; Wahl et al., 2007; Aulehla and Pourquie, 2010; Hubaud and Pourquie, 2014).

It is well established that the rhythmicity of somitogenesis is regulated by the segmentation clock driving cyclic and dynamic expression of "clock genes" in the PSM, with a periodicity that matches somite formation. This feature is conserved among a variety of vertebrate species (Jiang et al., 2000; Cinquin, 2007; Dequeant and Pourquie, 2008; Gomez et al., 2008; Ozbudak and Lewis, 2008; Krol et al., 2011). The clock genes are components of the Notch, Wnt, and FGF pathways (Aulehla et al., 2003; Dequeant and Pourquie, 2008; Yabe and Takada, 2016), playing a reciprocal regulatory role in oscillatory gene expression (reviewed in Gibb et al., 2010; Maroto et al., 2012).

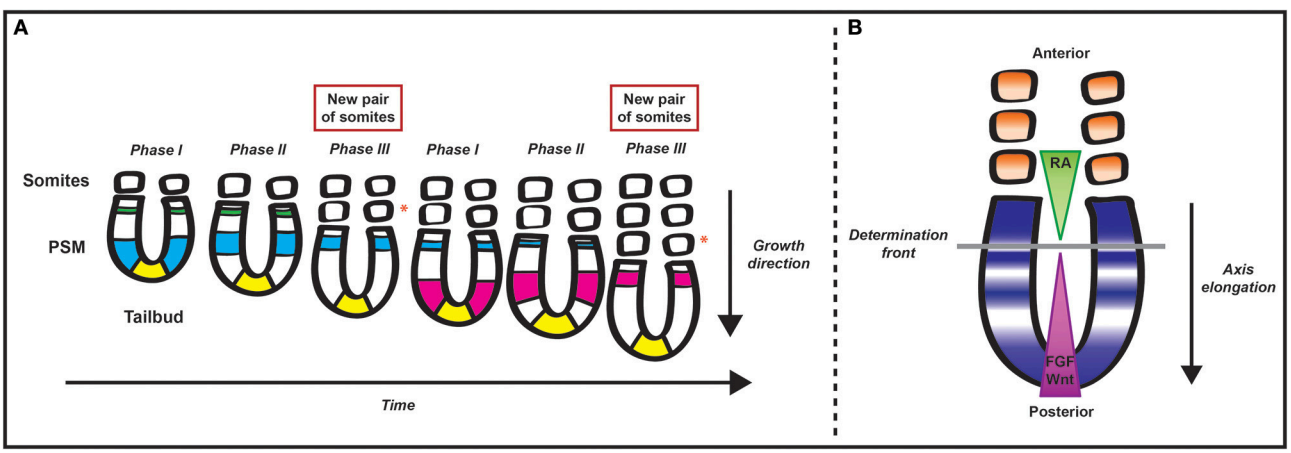

FIGURE 1 | Schematic representation of somitogenesis and the segmentation clock. (A) Pairs of somites bud off from the rostral end of the presomitic mesoderm (PSM) progressively during early development. The tail bud, a site of gastrulation that lies at the posterior end of the embryo, continuously "replenishes" the posterior end of the PSM with progenitor cells. The periodicity of segmentation is regulated by a molecular oscillator that drives cyclic gene expression from the posterior to the anterior tip of the PSM. The different colors represent domains of clock gene expression in different cycles. As time progresses in each cycle, the domain of clock gene expression shifts anteriorly while narrowing until it reaches the anterior limit of the PSM. The periodicity of this cyclic gene expression matches that of somite formation. An orange asterisk lies adjacent to each of the new pairs of somites formed in the time series—the first pair is formed after the blue wave of clock gene expression traverses the PSM and the second pair is formed after the pink wave of clock genes expression traverses the PSM from the tail bud to the anterior limit of the tissue. **(B)** Two mutually opposing gradients of retinoic acid (RA) and FGF/Wnt regulate the maturation wavefront within the paraxial mesoderm. Due to somite formation anteriorly and gastrulation at the caudal end of the PSM, cells within the PSM become progressively more anteriorly displaced, and, as a result, they are exposed to progressively lower levels of FGF/Wnt. There is a position within the PSM, termed the determination front, where cells are released from the effect of FGF and can respond to the segmentation clock and RA, embarking on their segmentation programme.

While the specific genes which oscillate may vary among species, the most highly represented pathway among the clock genes is the Notch (Krol et al., 2011).

Stemming from the observation that the proteins encoded by clock genes are predominantly unstable negative regulators of the pathway that activates them, it is believed that oscillatory gene expression relies on negative feedback loops of these unstable regulators, such as the two Notch target clock genes, *Hes7*, and *Lunatic Fringe* (Lfng) (Bessho et al., 2001a,b, 2003; Cole et al., 2002; Hirata et al., 2002; Dale et al., 2003; Serth et al., 2003; Kageyama et al., 2012; Okubo et al., 2012). It is particularly interesting that blocking *Lfng* oscillations disturbs somitogenesis in the thoracic and lumbar areas but not in more posterior areas of the embryo (Shifley et al., 2008), implying the role of Notch signaling in segmentation is not uniform along the axis.

In addition to negative feedback, oscillatory gene expression in the PSM also invokes positive feedback; Notch signaling regulates dynamic expression of *Notch1* itself, whereas Wnt regulates dynamic expression of *Dll1* (Bone et al., 2014).

As the most highly conserved pathway involved in the segmentation clock, a wealth of studies have focused on elucidating the fundamental role of Notch in somitogenesis and in the segmentation clock mechanism (Barrantes et al., 1999; Jiang et al., 2000; Bessho et al., 2001b, 2003; Dale et al., 2003; Julich et al., 2005; McGrew et al., 2008; Hubaud and Pourquie, 2014; Wahi et al., 2014; Liao and Oates, 2016). Notch is clearly required to synchronize oscillations between neighboring cells (Jiang et al., 2000; Shimojo et al., 2016). A question that arises is whether oscillations are actually necessary for the segmentation process to occur or whether just non-oscillatory activity of the Notch pathway is sufficient. Mutant mice or fish lacking Notch components all display severe segmentation defects (Conlon et al., 1995; Barrantes et al., 1999; Jiang et al., 2000; Liao and Oates, 2016). For example, the lack of the obligate transcription factor *RBP-Jκ*, in mouse, leads to lethality before day E10.5 and only the first few cervical somites are formed (Oka et al., 1995). A pivotal study conducted by Ferjentsik et al. pointed out that Notch activity, *per se*, is indeed essential for somite formation. Mutating crucial Notch pathway components, or using a complementary pharmacological approach, they demonstrated that in mouse Notch activity is crucial for the oscillatory activity of all clock genes, and thus essential for the formation of a segmented body axis (Ferjentsik et al., 2009) (see also Huppert et al., 2005).

NOTCH SIGNALING PATHWAY

The Notch pathway is highly conserved among metazoans and mediates short range juxtacrine communication. The Notch locus was first cloned in *Drosophila* and it was found to encode a large single pass type I transmembrane protein (Wharton et al., 1985), whose epidermal growth factor (EGF) repeats mediate interaction with their canonical activators—two ligands, Delta, and Serrate, in the Delta-Serrate-Lag2 (DSL) family. *Drosophila* studies have contributed hugely to our current understanding of Notch (Artavanis-Tsakonas et al., 1999). The role of Notch in developmental processes of multicellular species has been extensively elucidated (Dumortier et al., 2005; Radtke et al., 2005; Aster, 2014). Notch signaling outcome mostly relies on the cellular context, and thus Notch affects stem cell maintenance, cell fate choice, cell differentiation, lineage progression, and apoptosis in a context-dependent fashion (Bray, 2006; Hori et al., 2013).

Despite its multiple roles and versatility, the Notch pathway is relatively simple and conserved across species (Artavanis-Tsakonas et al., 1999; Bray, 2006; Kopan and Ilagan, 2009). In mammals, there are four Notch receptors (NOTCH1-4) and five DSL ligands (JAG1-2 and Delta-like 1-3-4). Both receptors and ligands are single transmembrane proteins and thus to trigger the signaling cascade, cell-cell contact is required (D'souza et al., 2010; Andersson et al., 2011; Greenwald and Kovall, 2013).

The Notch receptor is typically comprised of: (i) 29–36 EFG-like repeats in its extracellular domain, involved in ligand interaction; (ii) three juxtamembrane repeats (Lin-12-Notch, LIN), required for extra-intracellular domain interaction (located within the Negative Regulatory Region (NRR); (iii) the intracellular region, including seven ankyrin (ANK) repeats flanked by a PEST [rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues] and a transactivation (TAD) domain (**Figure 2C**).

During its maturation, Notch undergoes ligand-independent cleavage by a furin-like convertase in the *trans*-Golgi (Artavanis-Tsakonas et al., 1999; Fiuza and Arias, 2007; Hori et al., 2013). This first cleavage (the S1) results in the production of a heterodimeric receptor comprised of a transmembrane/intracellular fragment non-covalently bound to the Notch extracellular domain (NECD). Notch is thus presented to the cell surface as a heterodimer. The non-activated Notch receptor is constitutively internalized, ubiquitinated by Itch/AIP4 (a member of the Nedd4 family of HECT domain E3 ubiquitin ligases), and thus targeted for lysosomal degradation (Chastagner et al., 2008; Moretti and Brou, 2013).

To ensure correct folding and activity, during synthesis and secretion in the Golgi, NECD undergoes O-linked glycosylation and fucosylation (Rana and Haltiwanger, 2011). These two modifications on the EGF repeats modulate Notch activity by modulating interaction with the Delta or Serrate ligands. The reaction is catalyzed by three Fringe homologs (Lunatic, Manic, and Radical Fringe), recognizing specific amino acids in individual EGF repeats (Rampal et al., 2005). *In vitro,* in the signal-receiving cell, all Fringes enhance Dll1-Notch1 interactions with comparable effects in both *trans*- and *cis*- (Lebon et al., 2014). Rfng also enhances *trans*- and *cis*-interactions between JAG1 and Notch1, but these interactions are weakened by Lfng and Mfng. By contrast, JAG1 activation of Notch2 is potentiated by Lfng, thereby expanding the ligand-receptor combinations that are differentially modified by the Fringe enzymes (Hicks et al., 2000). In the context of somitogenesis, Lfng is the only family member expressed in the PSM. In most systems, Lfng acts in the receiving-cell to potentiate receptor activation by Delta-like ligands while reducing activation by Jagged ligands (Hicks et al., 2000; Yang et al., 2005; Kato et al., 2010). However, it has been suggested that LFNG protein may synchronize clock oscillations between

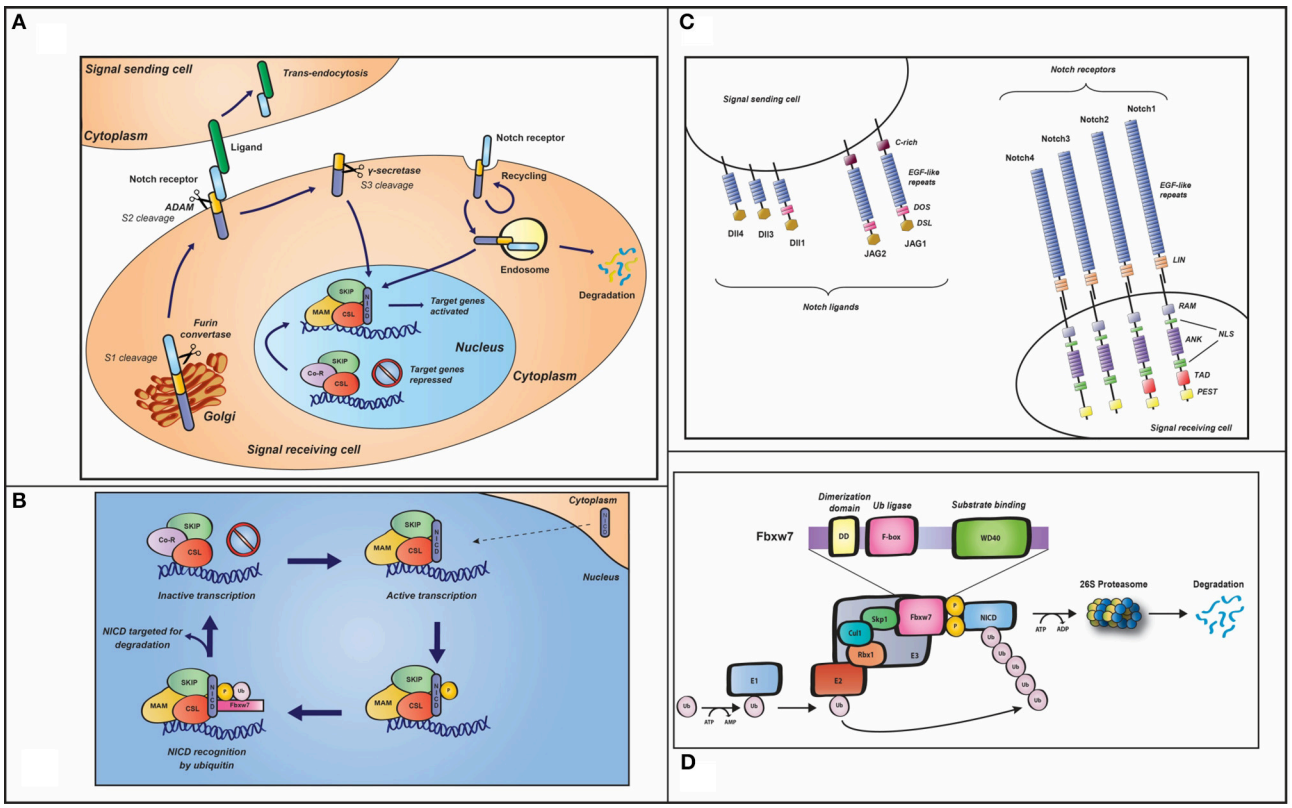

FIGURE 2 | (A) The Notch signaling pathway. In the Golgi, after being glycosylated by members of the Fringe family, pre-Notch is cleaved by a Furin-like convertase into the extracellular and intracellular domains (termed the S1 cleavage), resulting in a heterodimeric receptor with non-covalently associated domains that is transported to the plasma membrane. The Fringe modifications introduced in the Golgi affect sensitivity of the receptor on the signal-receiving cell to the DSL (Delta-Serrate-Lag2) ligands, in the signal-sending cell. Following ligand-receptor interaction, trans-endocytosis of the Notch extracellular domain, by the signal-sending cell, exposes the second so called S2 cleavage site, facilitating intramembranous cleavage by an ADAM (a disintegrin and metalloproteinase domain) protease, in the extracellular domain. S2 cleavage, in turns, exposes the S3 proteolytic cleavage site within the transmembrane domain, which is cleaved by the γ-secretase complex and liberates the intracellular domain of Notch (NICD), allowing it to translocate to the nucleus and thus activate transcription of target genes. In order to prevent inappropriate signaling from the pool of Notch that has not been activated by ligand, Notch receptor is continuously internalized into early endosomes and thus degraded. **(B)** Zoom-in into the nucleus of the signal-receiving cell **(A)**. Once released into the nucleus, NICD binds the DNA-binding protein CSL as well as the SKIP protein. The trimeric complex thus recruits Mastermind-like protein (MAM), which recruits additional co-activators (not shown), required for the transcriptional regulation of Notch target gene expression. Kinases, such as CDK8 and GSK3β, phosphorylate (p) NICD on its PEST domain, rendering it susceptible to recognition by Fbxw7 E3 ligase, leading to ubiquitination (Ub) and subsequent degradation by the proteasome. In the absence of NICD, CSL associates with transcriptional co-repressors blocking Notch target gene activation. Target genes are repressed until more NICD is produced to re-initiate a new cycle of target gene expression. **(C)** Notch ligands and receptors. In the signal-receiving cell, the four mammalian Notch receptors (Notch1-4) are represented. They are expressed on the cell surface as heterodimers and characterized by epidermal growth factor (EGF)-like and LIN repeats in their extracellular region. The intracellular domain includes an RBP-Jκ-associated molecule (RAM) domain, seven ankyrin (ANK) repeats, two nuclear localization signals (NLS), a transactivation (TAD) domain (lacking in Notch3 and Notch4), and a Proline-, Glutamate-, Serine- , and Threonine-rich (PEST) domain. The five Notch ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2) are represented on the surface of the signal-sending cell. Each ligand contains an EGF-like repeat region and a conserved domain DSL (Delta/Serrate/Lag). A conserved cysteine-rich (CR) domain is also present on Jagged1 and Jagged2. The DOS (Delta and OSM-11) domain, containing two atypical EGF repeats, is part of Dll1, JAG1 and JAG2 ligands. **(D)** SCF^{Fbxw7} E3 ubiquitin ligase complex representation. The upper part of the figure shows Fbxw7 domains: a conserved dimerization motif, which mediates dimerization of the SCF complex and thus facilitates ubiquitin conjugation; the F-box, which binds the SCF complex through Skp1; the WD40, containing three specific amino acid residues, which binds the phosphorylated substrate. In the bottom part of the figure the SCF^{Fbxw7} complex is schematically represented. In general, in the ubiquitin system, three enzymes are involved in the signaling cascade: the ubiquitin-activating E1, the ubiquitin-conjugating E2 and an E3 ubiquitin ligase. The first step is ATP-dependent and involves the binding of ubiquitin to E1. Ubiquitin is then activated and transferred to E2. The ubiquitin-E2 complex then interacts with a specific E3 (SCF^{Fbxw7}, refer to main text for a description), which recognizes the substrate (phosphorylated NICD, in this case) and facilitates transfer of the ubiquitin molecules to the substrate, leading to substrate degradation by the 26S proteasome.

neighboring cells by acting in the signal-sending cell to inhibit Notch1 activation by Dll1 (Okubo et al., 2012). Ligand binding in an adjacent cell triggers a second cleavage, mediated by the metalloprotease ADAM10 (A disintegrin and metalloprotease) at S2 site in the juxtamembrane extracellular domain, proximal to the Notch transmembrane domain (Mumm et al., 2000; Dyczynska et al., 2007; Bozkulak and Weinmaster, 2009; Gordon et al., 2009; Van Tetering et al., 2009; Weber et al., 2011; Groot et al., 2014). The cleaved NECD product, bound to the ligand, undergoes trans-endocytosis into the ligand-expressing

cell (Kramer, 2000; Parks et al., 2000; Meloty-Kapella et al., 2012). The second cleavage exposes the third cleavage site, S3, within the membrane-tethered Notch fragment, and is thus a rate-limiting step for the third and final cleavage (Brou et al., 2000; Mumm et al., 2000). Upon cleavage at the S3 site by a γ-secretase complex, the Notch intracellular domain (NICD) is then released (Schroeter et al., 1998) and translocates into the nucleus to activate transcription of target genes (**Figure 2A**). Notch can be activated in the endosomal pathway, independently of its ligands, through the activity of Deltex, a Ring-domain ubiquitin ligase that binds to NICD. However, it is unclear how the Deltex-activation mechanism relates to that of ligand-induced signaling.

Notch signaling does not require the use of second messengers. The activity is exclusively driven by nuclear concentration of NICD (Struhl and Adachi, 1998; Ehebauer et al., 2006). In the nucleus, NICD binds a bi-functional transcription factor CSL [CBF1, Su(H), Lag-1], a DNA binding complex Mastermind (MAM), and a variety of other co-activators involved in the transcriptional activation of Notch target gene expression (Fryer et al., 2004; Kopan and Ilagan, 2009; Hori et al., 2013). The transcriptional co-regulator SKIP (Ski-interaction protein) and the histone acetylase p300 are recruited concomitantly to the promoter region of target genes promoting the assembly of the initiation and elongation complexes (Zhou et al., 2000; Wallberg et al., 2002; Fryer et al., 2004; Bray, 2006; **Figure 2B**). MAM also engages kinases that phosphorylate NICD (Wu et al., 2000; Kitagawa et al., 2001; Nam et al., 2003; Fryer et al., 2004), a crucial step in the regulation of NICD stability and activity (Ingles-Esteve et al., 2001; Espinosa et al., 2003; Fryer et al., 2004; Jin et al., 2009). The domain targeted is the C-terminal PEST domain that is phosphorylated by the cyclin C cyclin-dependent kinase-8 complex (Cyc:CDK8) and glycogen synthase kinase 3β (GSK-3β) (Espinosa et al., 2003; Fryer et al., 2004; Jin et al., 2009).

FBXW7 AND ITS ROLE IN NICD TURNOVER

NICD phosphorylation leads to its ubiquitination, turnover, and degradation by the proteasome, defining the half-life of Notch signaling, allowing the cell once again to become ligand-competent and resetting the signaling for a new cycle of activation (Le Bras et al., 2011). In the prevailing model, the ubiquitin ligase involved is the SCF^{Fbxw7} [S phase kinase-associated protein 1 (SKP1)-Cullin 1 (CUL1)-F-box] protein complex (Wu et al., 2001; Tsunematsu et al., 2004; Crusio et al., 2010). SCF^{Fbxw7} is part of the RING-finger domain E3 family (Petroski and Deshaies, 2005). Briefly, Cullin 1 acts as a scaffold on which SKP1 and RBX1 subunits assemble. SKP1 is involved in the recruitment of F box proteins (FBXW7, in the case of NICD), and RBX1 recruits a cognate E2 (Hao et al., 2007; Skaar et al., 2013). Fbxw7 consists of three isoforms (α, β, and γ) generated by alternative splicing and the isoform α, shown to ubiquitinate NICD, is localized to the nucleus (Matsumoto et al., 2006; O'neil et al., 2007; Welcker and Clurman, 2008; Crusio et al., 2010). Two domains are functionally important in the FBXW7 protein: the F-box domain, binding SKP1 (Bai et al., 1996), and the seven WD40 repeats mediating recognition/binding to the target protein in a specific consensus phospho-motif, the Cdc4 phospho-degron (Thr-Pro-Pro-Xaa-Ser, in which Thr and Ser residues are phosphorylated; Koepp et al., 2001; Welcker et al., 2003; Hao et al., 2007; Skaar et al., 2013; **Figure 2D**). A number of these phospho-degrons have been identified in the NICD PEST domain. Intriguingly, an additional hNICD1-specific degron has recently been identified within the N-terminal region, distinct from the PEST domain that is not recognized by FBXW7 (Broadus et al., 2016). Moreover, the E3 ligase, Itch, promoting PEST domain-independent NICD1 degradation (Qiu et al., 2000), does not mediate NICD1 degradation through the N1-Box (Broadus et al., 2016).

NICD-FBXW7 INTERACTION

Given the importance of Notch signaling in cell fate determination and cell cycle progression, it is not surprising that aberrations in the pathway lead to cancers and other diseases (Roy et al., 2007; Simpson et al., 2011; Wang et al., 2011; Kamath et al., 2012; Bolos et al., 2013; Huang et al., 2013; Lobry et al., 2013). Moreover, the pleiotropic nature of the pathway means the various Notch receptors can act as tumor suppressors for example in epithelial tumors or as oncogenes in leukemia and a variety of solid cancers (Radtke and Raj, 2003; Miele et al., 2006; Lobry et al., 2014; Alketbi and Attoub, 2015; Habets et al., 2015; Bonyadi Rad et al., 2016). From this vast literature we will focus here on activating mutations in Notch1 which are predominantly located in the extracellular heterodimerization (HD) domain resulting in ligand-independent exposure of the S2 cleavage site (Malecki et al., 2006; Van Tetering et al., 2009), or in the PEST domain, leading to constitutive activation of the pathway through increased NICD stability or in FBXW7, in line with its fundamental role in restricting the signaling strength/duration of the Notch pathway (Oberg et al., 2001; Tetzlaff et al., 2004; O'neil et al., 2007; Thompson et al., 2007; Wang et al., 2012; Bolos et al., 2013). For instance, *Notch1* mutations occur in over 50% of both pediatric and adult T-cell acute lymphoblastic leukemia (T-ALL) cases (Malyukova et al., 2007; Erbilgin et al., 2010), while *Fbxw7* mutations are found in up to 20% of T-ALL cases (Baldus et al., 2009; Mullighan, 2009). Furthermore, *Notch1* mutations were found in diffuse large B-cell lymphoma (DLBCL), splenic marginal zone lymphoma (SMZL), Hadju-Cheney syndrome (Isidor et al., 2011; Simpson et al., 2011; Kiel et al., 2012), breast cancer (Wang et al., 2015), and in 12% of non-small-cell lung carcinomas (NSCLCs), of which half were in the PEST domain (Westhoff et al., 2009). In these conditions, Notch target genes are highly upregulated.

Considering the variety of pathological conditions associated with alterations of NICD and FBXW7, there is a limited understanding of the regulation of this interaction. Our current understanding stems from a study on Sel-10, the nematode homolog of Fbxw7, showing the two proteins bind directly to each other and FBXW7 negatively regulates

Notch signaling (Hubbard et al., 1997). Using cell models, human and murine homologs of Sel-10 were shown to play a key role in regulating Notch signaling by driving NICD to ubiquitin-proteasome mediated degradation (Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). NICD ubiquitination relies on the PEST domain. Studies on three NOTCH4 variants suggested that Sel-10 preferentially binds to phosphorylated forms of the C-terminal domain of NOTCH4 (Oberg et al., 2001; Wu et al., 2001). However, the NICD-Sel10 interaction has only been observed under overexpression conditions *in vitro*. It remains to be shown if this interaction occurs *in vivo*, if NICD interacts with any other E3 ligases, how this interaction is regulated and whether it is context-dependent. The *FBXW7* null mutant mice exhibit elevated levels of Notch4 intracellular domain and/or Notch1 intracellular domain alongside defects that are in alignment with a variety of roles identified for Notch in different developmental process such as cardiogenesis and vascular development. However, intriguingly, with respect to the segmentation clock, the absence of Fbxw7 seems to play a less major role in this process, at least according to the mutant phenotypes—although a detailed analysis has yet to be conducted (Tetzlaff et al., 2004; Tsunematsu et al., 2004). The results of these reports suggest that the mechanisms of NICD1 degradation during the somitogenesis process might actually rely on alternative (or redundant) mechanisms, highlighting again the need to further study alternative means of regulation of stability NICD1/degradation.

CONCLUSIONS

In this review we provided a general overview of the critical role of Notch signaling in regulating the segmentation clock involved in somitogenesis. Notch activity is based on stability and turnover of its intracellular domain, NICD. This stability is regulated by phosphorylation of the PEST domain, targeting NICD to proteasome degradation upon recognition by the E3 ligase FBXW7. Mutations in the PEST domain, leading to aberrations in NICD stability, are the underlying cause of a number of solid and non-solid cancers and different genetic disorders. Therefore, uncovering the finer details of Notch pathway regulation merits attention, particularly because a wider comprehension of this process would provide further insights into the mechanisms involved in the onset of Notch-related diseases.

AUTHOR CONTRIBUTIONS

FC and JD conceived the structure and content. FC wrote the initial draft document. FC designed and produced the figures. JD corrected and edited the document.

ACKNOWLEDGMENTS

We would like to thank Laura D'Ignazio, Lucas Morales Moya and Ioanna Mastromina for helpful and constructive feedback. This work was supported by a Wellcome Trust PhD studentship to FC and a Wellcome Trust Strategic award [097945/Z/11/Z].

REFERENCES

Alketbi, A., and Attoub, S. (2015). Notch signaling in cancer: rationale and strategies for targeting. *Curr. Cancer Drug Targets* 15, 364–374. doi: 10.2174/1568009615051507101133 53

Andersson, E. R., Sandberg, R., and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. *Development* 138, 3593–3612. doi: 10.1242/dev.063610

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* 284, 770–776. doi: 10.1126/science.284.5415.770

Aster, J. C. (2014). In brief: Notch signalling in health and disease. *J. Pathol.* 232, 1–3. doi: 10.1002/path.4291

Aulehla, A., and Pourquie, O. (2010). Signaling gradients during paraxial mesoderm development. *Cold Spring Harb. Perspect. Biol.* 2:a000869. doi: 10.1101/cshperspect.a000869

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B., et al. (2003). Wnt3A plays a major role in the segmentation clock controlling somitogenesis. *Dev. Cell* 4, 395–406. doi: 10.1016/S1534-5807(03)00055-8

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., et al. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* 86, 263–274. doi: 10.1016/S0092-8674(00)80098-7

Bailey, C., and Dale, K. (2015). "Somitogenesis in vertebrate development," in *eLS* (John Wiley & Sons, Ltd), 1–15. doi: 10.1002/9780470015902.a0003820.pub2

Baldus, C. D., Thibaut, J., Goekbuget, N., Stroux, A., Schlee, C., Mossner, M., et al. (2009). Prognostic implications of NOTCH1 and FBXW7 mutations in adult acute T-lymphoblastic leukemia. *Haematologica* 94, 1383–1390. doi: 10.3324/haematol.2008.005272

Barrantes, I. B., Elia, A. J., Wunsch, K., Hrabe De Angelis, M. H., Mak, T. W., Rossant, J., et al. (1999). Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse. *Curr. Biol.* 9, 470–480. doi: 10.1016/S0960-9822(99)80212-7

Benazeraf, B., and Pourquie, O. (2013). Formation and segmentation of the vertebrate body axis. *Annu. Rev. Cell Dev. Biol.* 29, 1–26. doi: 10.1146/annurev-cellbio-101011-155703

Bessho, Y., Hirata, H., Masamizu, Y., and Kageyama, R. (2003). Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. *Genes Dev.* 17, 1451–1456. doi: 10.1101/gad.1092303

Bessho, Y., Miyoshi, G., Sakata, R., and Kageyama, R. (2001a). Hes7: a bHLH-type repressor gene regulated by Notch and expressed in the presomitic mesoderm. *Genes Cells* 6, 175–185. doi: 10.1046/j.1365-2443.2001.00409.x

Bessho, Y., Sakata, R., Komatsu, S., Shiota, K., Yamada, S., and Kageyama, R. (2001b). Dynamic expression and essential functions of Hes7 in somite segmentation. *Genes Dev.* 15, 2642–2647. doi: 10.1101/gad.930601

Bolos, V., Mira, E., Martinez-Poveda, B., Luxan, G., Canamero, M., Martinez, A. C., et al. (2013). Notch activation stimulates migration of breast cancer cells and promotes tumor growth. *Breast Cancer Res.* 15:R54. doi: 10.1186/bcr3447

Bone, R. A., Bailey, C. S., Wiedermann, G., Ferjentsik, Z., Appleton, P. L., Murray, P. J., et al. (2014). Spatiotemporal oscillations of Notch1, Dll1 and NICD are coordinated across the mouse PSM. *Development* 141, 4806–4816. doi: 10.1242/dev.115535

Bonyadi Rad, E., Hammerlindl, H., Wels, C., Popper, U., Ravindran Menon, D., Breiteneder, H., et al. (2016). Notch4 signaling induces a mesenchymal-epithelial-like transition in melanoma cells to suppress malignant behaviors. *Cancer Res.* 76, 1690–1697. doi: 10.1158/0008-5472.CAN-15-1722

Bozkulak, E. C., and Weinmaster, G. (2009). Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling. *Mol. Cell. Biol.* 29, 5679–5695. doi: 10.1128/MCB.00406-09

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* 7, 678–689. doi: 10.1038/nrm2009

Broadus, M. R., Chen, T. W., Neitzel, L. R., Ng, V. H., Jodoin, J. N., Lee, L. A., et al. (2016). Identification of a paralog-specific notch1 intracellular domain degron. *Cell Rep.* 15, 1920–1929. doi: 10.1016/j.celrep.2016.04.070

Brou, C., Logeat, F., Gupta, N., Bessia, C., Lebail, O., Doedens, J. R., et al. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol. Cell* 5, 207–216. doi: 10.1016/S1097-2765(00)80417-7

Cambray, N., and Wilson, V. (2007). Two distinct sources for a population of maturing axial progenitors. *Development* 134, 2829–2840. doi: 10.1242/dev.02877

Chastagner, P., Israel, A., and Brou, C. (2008). AIP4/Itch regulates Notch receptor degradation in the absence of ligand. *PLoS ONE* 3:e2735. doi: 10.1371/journal.pone.0002735

Christ, B., Huang, R., and Scaal, M. (2007). Amniote somite derivatives. *Dev. Dyn.* 236, 2382–2396. doi: 10.1002/dvdy.21189

Cinquin, O. (2007). Understanding the somitogenesis clock: what's missing? *Mech. Dev.* 124, 501–517. doi: 10.1016/j.mod.2007.06.004

Cole, S. E., Levorse, J. M., Tilghman, S. M., and Vogt, T. F. (2002). Clock regulatory elements control cyclic expression of Lunatic fringe during somitogenesis. *Dev. Cell* 3, 75–84. doi: 10.1016/S1534-5807(02)00212-5

Conlon, R. A., Reaume, A. G., and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* 121, 1533–1545.

Cooke, J., and Zeeman, E. C. (1976). A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *J. Theor. Biol.* 58, 455–476. doi: 10.1016/S0022-5193(76)80131-2

Crusio, K. M., King, B., Reavie, L. B., and Aifantis, I. (2010). The ubiquitous nature of cancer: the role of the SCF(Fbw7) complex in development and transformation. *Oncogene* 29, 4865–4873. doi: 10.1038/onc.2010.222

Dale, J. K., Maroto, M., Dequeant, M. L., Malapert, P., McGrew, M., and Pourquie, O. (2003). Periodic notch inhibition by lunatic fringe underlies the chick segmentation clock. *Nature* 421, 275–278. doi: 10.1038/nature01244

Dequeant, M. L., and Pourquie, O. (2008). Segmental patterning of the vertebrate embryonic axis. *Nat. Rev. Genet.* 9, 370–382. doi: 10.1038/nrg2320

D'souza, B., Meloty-Kapella, L., and Weinmaster, G. (2010). Canonical and non-canonical Notch ligands. *Curr. Top. Dev. Biol.* 92, 73–129. doi: 10.1016/S0070-2153(10)92003-6

Dubrulle, J., and Pourquie, O. (2004). fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* 427, 419–422. doi: 10.1038/nature02216

Dumortier, A., Wilson, A., Macdonald, H. R., and Radtke, F. (2005). Paradigms of notch signaling in mammals. *Int. J. Hematol.* 82, 277–284. doi: 10.1532/IJH97.05099

Dyczynska, E., Sun, D., Yi, H., Sehara-Fujisawa, A., Blobel, C. P., and Zolkiewska, A. (2007). Proteolytic processing of delta-like 1 by ADAM proteases. *J. Biol. Chem.* 282, 436–444. doi: 10.1074/jbc.M605451200

Eckalbar, W. L., Fisher, R. E., Rawls, A., and Kusumi, K. (2012). Scoliosis and segmentation defects of the vertebrae. *Wiley Interdiscip. Rev. Dev. Biol.* 1, 401–423. doi: 10.1002/wdev.34

Ehebauer, M., Hayward, P., and Martinez-Arias, A. (2006). Notch signaling pathway. *Sci. STKE* 2006:cm7. doi: 10.1126/stke.3642006cm7

Erbilgin, Y., Sayitoglu, M., Hatirnaz, O., Dogru, O., Akcay, A., Tuysuz, G., et al. (2010). Prognostic significance of NOTCH1 and FBXW7 mutations in pediatric T-ALL. *Dis. Markers* 28, 353–360. doi: 10.1155/2010/740140

Espinosa, L., Ingles-Esteve, J., Aguilera, C., and Bigas, A. (2003). Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *J. Biol. Chem.* 278, 32227–32235. doi: 10.1074/jbc.M304001200

Ferjentsik, Z., Hayashi, S., Dale, J. K., Bessho, Y., Herreman, A., De Strooper, B., et al. (2009). Notch is a critical component of the mouse somitogenesis oscillator and is essential for the formation of the somites. *PLoS Genet.* 5:e1000662. doi: 10.1371/journal.pgen.1000662

Fiuza, U. M., and Arias, A. M. (2007). Cell and molecular biology of Notch. *J. Endocrinol.* 194, 459–474. doi: 10.1677/JOE-07-0242

Fryer, C. J., White, J. B., and Jones, K. A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol. Cell* 16, 509–520. doi: 10.1016/j.molcel.2004.10.014

Gibb, S., Maroto, M., and Dale, J. K. (2010). The segmentation clock mechanism moves up a notch. *Trends Cell Biol.* 20, 593–600. doi: 10.1016/j.tcb.2010.07.001

Gomez, C., Ozbudak, E. M., Wunderlich, J., Baumann, D., Lewis, J., and Pourquie, O. (2008). Control of segment number in vertebrate embryos. *Nature* 454, 335–339. doi: 10.1038/nature07020

Gomez, C., and Pourquie, O. (2009). Developmental control of segment numbers in vertebrates. *J. Exp. Zool. B Mol. Dev. Evol.* 312, 533–544. doi: 10.1002/jez.b.21305

Gordon, W. R., Vardar-Ulu, D., L'heureux, S., Ashworth, T., Malecki, M. J., Sanchez-Irizarry, C., et al. (2009). Effects of S1 cleavage on the structure, surface export, and signaling activity of human Notch1 and Notch2. *PLoS ONE* 4:e6613. doi: 10.1371/journal.pone.0006613

Gossler, A., and De Angelis, M. H. (1998). Somitogenesis. *Curr. Topics Dev. Biol.* 38, 225–287.

Greenwald, I., and Kovall, R. (2013). Notch signaling: genetics and structure. *WormBook* 1–28. doi: 10.1895/wormbook.1.10.2

Groot, A. J., Habets, R., Yahyanejad, S., Hodin, C. M., Reiss, K., Saftig, P., et al. (2014). Regulated proteolysis of NOTCH2 and NOTCH3 receptors by ADAM10 and presenilins. *Mol. Cell. Biol.* 34, 2822–2832. doi: 10.1128/MCB.00206-14

Gupta-Rossi, N., Le Bail, O., Gonen, H., Brou, C., Logeat, F., Six, E., et al. (2001). Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. *J. Biol. Chem.* 276, 34371–34378. doi: 10.1074/jbc.M101343200

Habets, R. A., Groot, A. J., Yahyanejad, S., Tiyanont, K., Blacklow, S. C., and Vooijs, M. (2015). Human NOTCH2 is resistant to ligand-independent activation by metalloprotease Adam17. *J. Biol. Chem.* 290, 14705–14716. doi: 10.1074/jbc.M115.643676

Hao, B., Oehlmann, S., Sowa, M. E., Harper, J. W., and Pavletich, N. P. (2007). Structure of a Fbw7-Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. *Mol. Cell* 26, 131–143. doi: 10.1016/j.molcel.2007.02.022

Henrique, D., Abranches, E., Verrier, L., and Storey, K. G. (2015). Neuromesodermal progenitors and the making of the spinal cord. *Development* 142, 2864–2875. doi: 10.1242/dev.119768

Hicks, C., Johnston, S. H., Disibio, G., Collazo, A., Vogt, T. F., and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat. Cell Biol.* 2, 515–520. doi: 10.1038/35019553

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., et al. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* 298, 840–843. doi: 10.1126/science.1074560

Hori, K., Sen, A., and Artavanis-Tsakonas, S. (2013). Notch signaling at a glance. *J. Cell Sci.* 126, 2135–2140. doi: 10.1242/jcs.127308

Huang, J., Song, H., Liu, B., Yu, B., Wang, R., and Chen, L. (2013). Expression of Notch-1 and its clinical significance in different histological subtypes of human lung adenocarcinoma. *J. Exp. Clin. Cancer Res.* 32:84. doi: 10.1186/1756-9966-32-84

Hubaud, A., and Pourquie, O. (2014). Signalling dynamics in vertebrate segmentation. *Nat. Rev. Mol. Cell Biol.* 15, 709–721. doi: 10.1038/nrm3891

Hubbard, E. J., Wu, G., Kitajewski, J., and Greenwald, I. (1997). sel-10, a negative regulator of lin-12 activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. *Genes Dev.* 11, 3182–3193. doi: 10.1101/gad.11.23.3182

Huppert, S. S., Ilagan, M. X., De Strooper, B., and Kopan, R. (2005). Analysis of Notch function in presomitic mesoderm suggests a gamma-secretase-independent role for presenilins in somite differentiation. *Dev. Cell* 8, 677–688. doi: 10.1016/j.devcel.2005.02.019

Iimura, T., Yang, X., Weijer, C. J., and Pourquie, O. (2007). Dual mode of paraxial mesoderm formation during chick gastrulation. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2744–2749. doi: 10.1073/pnas.0610997104

Ingles-Esteve, J., Espinosa, L., Milner, L. A., Caelles, C., and Bigas, A. (2001). Phosphorylation of Ser2078 modulates the Notch2 function in 32D cell differentiation. *J. Biol. Chem.* 276, 44873–44880. doi: 10.1074/jbc.M104703200

Isidor, B., Lindenbaum, P., Pichon, O., Bezieau, S., Dina, C., Jacquemont, S., et al. (2011). Truncating mutations in the last exon of NOTCH2 cause a rare skeletal disorder with osteoporosis. *Nat. Genet.* 43, 306–308. doi: 10.1038/ng.778

Jiang, Y. J., Aerne, B. L., Smithers, L., Haddon, C., Ish-Horowicz, D., and Lewis, J. (2000). Notch signalling and the synchronization of the somite segmentation clock. *Nature* 408, 475–479. doi: 10.1038/35044091

Jin, Y. H., Kim, H., Oh, M., Ki, H., and Kim, K. (2009). Regulation of Notch1/NICD and Hes1 expressions by GSK-3alpha/beta. *Mol. Cells* 27, 15–19. doi: 10.1007/s10059-009-0001-7

Julich, D., Hwee Lim, C., Round, J., Nicolaije, C., Schroeder, J., Davies, A., et al. (2005). beamter/deltaC and the role of Notch ligands in the zebrafish somite segmentation, hindbrain neurogenesis and hypochord differentiation. *Dev. Biol.* 286, 391–404. doi: 10.1016/j.ydbio.2005.06.040

Kageyama, R., Niwa, Y., Isomura, A., Gonzalez, A., and Harima, Y. (2012). Oscillatory gene expression and somitogenesis. *Wiley Interdiscip. Rev. Dev. Biol.* 1, 629–641. doi: 10.1002/wdev.46

Kamath, B. M., Bauer, R. C., Loomes, K. M., Chao, G., Gerfen, J., Hutchinson, A., et al. (2012). NOTCH2 mutations in Alagille syndrome. *J. Med. Genet.* 49, 138–144. doi: 10.1136/jmedgenet-2011-100544

Kato, T. M., Kawaguchi, A., Kosodo, Y., Niwa, H., and Matsuzaki, F. (2010). Lunatic fringe potentiates Notch signaling in the developing brain. *Mol. Cell. Neurosci.* 45, 12–25. doi: 10.1016/j.mcn.2010.05.004

Kiel, M. J., Velusamy, T., Betz, B. L., Zhao, L., Weigelin, H. G., Chiang, M. Y., et al. (2012). Whole-genome sequencing identifies recurrent somatic NOTCH2 mutations in splenic marginal zone lymphoma. *J. Exp. Med.* 209, 1553–1565. doi: 10.1084/jem.20120910

Kitagawa, M., Oyama, T., Kawashima, T., Yedvobnick, B., Kumar, A., Matsuno, K., et al. (2001). A human protein with sequence similarity to Drosophila mastermind coordinates the nuclear form of notch and a CSL protein to build a transcriptional activator complex on target promoters. *Mol. Cell. Biol.* 21, 4337–4346. doi: 10.1128/MCB.21.13.4337-4346.2001

Koepp, D. M., Schaefer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., et al. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* 294, 173–177. doi: 10.1126/science.1065203

Kopan, R., and Ilagan, M. X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 137, 216–233. doi: 10.1016/j.cell.2009.03.045

Kramer, H. (2000). RIPping notch apart: a new role for endocytosis in signal transduction? *Sci. STKE* 2000:pe1. doi: 10.1126/stke.2000.29.pe1

Krol, A. J., Roellig, D., Dequeant, M. L., Tassy, O., Glynn, E., Hattem, G., et al. (2011). Evolutionary plasticity of segmentation clock networks. *Development* 138, 2783–2792. doi: 10.1242/dev.063834

Lebon, L., Lee, T. V., Sprinzak, D., Jafar-Nejad, H., and Elowitz, M. B. (2014). Fringe proteins modulate Notch-ligand cis and trans interactions to specify signaling states. *Elife* 3:e02950. doi: 10.7554/eLife.02950

Le Bras, S., Loyer, N., and Le Borgne, R. (2011). The multiple facets of ubiquitination in the regulation of notch signaling pathway. *Traffic* 12, 149–161. doi: 10.1111/j.1600-0854.2010.01126.x

Liao, B. K., and Oates, A. C. (2016). Delta-notch signalling in segmentation. *Arthropod. Struct. Dev.* doi: 10.1016/j.asd.2016.11.007. [Epub ahead of print].

Lobry, C., Ntziachristos, P., Ndiaye-Lobry, D., Oh, P., Cimmino, L., Zhu, N., et al. (2013). Notch pathway activation targets AML-initiating cell homeostasis and differentiation. *J. Exp. Med.* 210, 301–319. doi: 10.1084/jem.20121484

Lobry, C., Oh, P., Mansour, M. R., Look, A. T., and Aifantis, I. (2014). Notch signaling: switching an oncogene to a tumor suppressor. *Blood* 123, 2451–2459. doi: 10.1182/blood-2013-08-355818

Malecki, M. J., Sanchez-Irizarry, C., Mitchell, J. L., Histen, G., Xu, M. L., Aster, J. C., et al. (2006). Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. *Mol. Cell. Biol.* 26, 4642–4651. doi: 10.1128/MCB.01655-05

Malyukova, A., Dohda, T., Von Der Lehr, N., Akhoondi, S., Corcoran, M., Heyman, M., et al. (2007). The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Res.* 67, 5611–5616. doi: 10.1158/0008-5472.CAN-06-4381

Maroto, M., Bone, R. A., and Dale, J. K. (2012). Somitogenesis. *Development* 139, 2453–2456. doi: 10.1242/dev.069310

Matsumoto, A., Onoyama, I., and Nakayama, K. I. (2006). Expression of mouse Fbxw7 isoforms is regulated in a cell cycle- or p53-dependent manner. *Biochem. Biophys. Res. Commun.* 350, 114–119. doi: 10.1016/j.bbrc.2006.09.003

McGrew, M. J., Sherman, A., Lillico, S. G., Ellard, F. M., Radcliffe, P. A., Gilhooley, H. J., et al. (2008). Localised axial progenitor cell populations in the avian tail bud are not committed to a posterior Hox identity. *Development* 135, 2289–2299. doi: 10.1242/dev.022020

Meloty-Kapella, L., Shergill, B., Kuon, J., Botvinick, E., and Weinmaster, G. (2012). Notch ligand endocytosis generates mechanical pulling force dependent on dynamin, epsins, and actin. *Dev. Cell* 22, 1299–1312. doi: 10.1016/j.devcel.2012.04.005

Miele, L., Golde, T., and Osborne, B. (2006). Notch signaling in cancer. *Curr. Mol. Med.* 6, 905–918. doi: 10.2174/156652406779010830

Moretti, J., and Brou, C. (2013). Ubiquitinations in the notch signaling pathway. *Int. J. Mol. Sci.* 14, 6359–6381. doi: 10.3390/ijms14036359

Mullighan, C. G. (2009). Mutations of NOTCH1, FBXW7, and prognosis in T-lineage acute lymphoblastic leukemia. *Haematologica* 94, 1338–1340. doi: 10.3324/haematol.2009.012047

Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., et al. (2000). A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Mol. Cell* 5, 197–206. doi: 10.1016/S1097-2765(00)80416-5

Nam, Y., Weng, A. P., Aster, J. C., and Blacklow, S. C. (2003). Structural requirements for assembly of the CSL.intracellular Notch1.Mastermind-like 1 transcriptional activation complex. *J. Biol. Chem.* 278, 21232–21239. doi: 10.1074/jbc.M301567200

Oates, A. C., Morelli, L. G., and Ares, S. (2012). Patterning embryos with oscillations: structure, function and dynamics of the vertebrate segmentation clock. *Development* 139, 625–639. doi: 10.1242/dev.063735

Oberg, C., Li, J. H., Pauley, A., Wolf, E., Gurney, M., and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian sel-10 homolog. *J. Biol. Chem.* 276, 35847–35853. doi: 10.1074/jbc.M103992200

Oka, C., Nakano, T., Wakeham, A., De La Pompa, J. L., Mori, C., Sakai, T., et al. (1995). Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* 121, 3291–3301.

Okubo, Y., Sugawara, T., Abe-Koduka, N., Kanno, J., Kimura, A., and Saga, Y. (2012). Lfng regulates the synchronized oscillation of the mouse segmentation clock via trans-repression of Notch signalling. *Nat. Commun.* 3:1141. doi: 10.1038/ncomms2133

O'neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., et al. (2007). FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to γ-secretase inhibitors. *J. Exp. Med.* 204, 1813–1824. doi: 10.1084/jem.20070876

Ozbudak, E. M., and Lewis, J. (2008). Notch signalling synchronizes the zebrafish segmentation clock but is not needed to create somite boundaries. *PLoS Genet.* 4:e15. doi: 10.1371/journal.pgen.0040015

Palmeirim, I., Henrique, D., Ish-Horowicz, D., and Pourquie, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* 91, 639–648. doi: 10.1016/S0092-8674(00)80451-1

Parks, A. L., Klueg, K. M., Stout, J. R., and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* 127, 1373–1385.

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* 6, 9–20. doi: 10.1038/nrm1547

Pourquie, O. (2001). Vertebrate somitogenesis. *Annu. Rev. Cell Dev. Biol.* 17, 311–350. doi: 10.1146/annurev.cellbio.17.1.311

Pourquie, O. (2011). Vertebrate segmentation: from cyclic gene networks to scoliosis. *Cell* 145, 650–663. doi: 10.1016/j.cell.2011.05.011

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., et al. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J. Biol. Chem.* 275, 35734–35737. doi: 10.1074/jbc.M007300200

Radtke, F., and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat. Rev. Cancer* 3, 756–767. doi: 10.1038/nrc1186

Radtke, F., Schweisguth, F., and Pear, W. (2005). The Notch 'gospel'. *EMBO Rep.* 6, 1120–1125. doi: 10.1038/sj.embor.7400585

Rampal, R., Li, A. S., Moloney, D. J., Georgiou, S. A., Luther, K. B., Nita-Lazar, A., et al. (2005). Lunatic fringe, manic fringe, and radical fringe recognize similar specificity determinants in O-fucosylated epidermal growth factor-like repeats. *J. Biol. Chem.* 280, 42454–42463. doi: 10.1074/jbc.M509552200

Rana, N. A., and Haltiwanger, R. S. (2011). Fringe benefits: functional and structural impacts of O-glycosylation on the extracellular domain of Notch receptors. *Curr. Opin. Struct. Biol.* 21, 583–589. doi: 10.1016/j.sbi.2011.08.008

Roy, M., Pear, W. S., and Aster, J. C. (2007). The multifaceted role of Notch in cancer. *Curr. Opin. Genet. Dev.* 17, 52–59. doi: 10.1016/j.gde.2006.12.001

Schroeter, E. H., Kisslinger, J. A., and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* 393, 382–386. doi: 10.1038/30756

Schroter, C., Herrgen, L., Cardona, A., Brouhard, G. J., Feldman, B., and Oates, A. C. (2008). Dynamics of zebrafish somitogenesis. *Dev. Dyn.* 237, 545–553. doi: 10.1002/dvdy.21458

Serth, K., Schuster-Gossler, K., Cordes, R., and Gossler, A. (2003). Transcriptional oscillation of lunatic fringe is essential for somitogenesis. *Genes Dev.* 17, 912–925. doi: 10.1101/gad.250603

Shifley, E. T., Vanhorn, K. M., Perez-Balaguer, A., Franklin, J. D., Weinstein, M., and Cole, S. E. (2008). Oscillatory lunatic fringe activity is crucial for segmentation of the anterior but not posterior skeleton. *Development* 135, 899–908. doi: 10.1242/dev.006742

Shimojo, H., Isomura, A., Ohtsuka, T., Kori, H., Miyachi, H., and Kageyama, R. (2016). Oscillatory control of Delta-like1 in cell interactions regulates dynamic gene expression and tissue morphogenesis. *Genes Dev.* 30, 102–116. doi: 10.1101/gad.270785.115

Simpson, M. A., Irving, M. D., Asilmaz, E., Gray, M. J., Dafou, D., Elmslie, F. V., et al. (2011). Mutations in NOTCH2 cause Hajdu-Cheney syndrome, a disorder of severe and progressive bone loss. *Nat. Genet.* 43, 303–305. doi: 10.1038/ng.779

Skaar, J. R., Pagan, J. K., and Pagano, M. (2013). Mechanisms and function of substrate recruitment by F-box proteins. *Nat. Rev. Mol. Cell Biol.* 14, 369–381. doi: 10.1038/nrm3582

Struhl, G., and Adachi, A. (1998). Nuclear access and action of notch *in vivo*. *Cell* 93, 649–660. doi: 10.1016/S0092-8674(00)81193-9

Tam, P. P. (1981). The control of somitogenesis in mouse embryos. *J. Embryol. Exp. Morphol.* 65(Suppl), 103–128.

Tetzlaff, M. T., Yu, W., Li, M., Zhang, P., Finegold, M., Mahon, K., et al. (2004). Defective cardiovascular development and elevated cyclin E and Notch proteins in mice lacking the Fbw7 F-box protein. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3338–3345. doi: 10.1073/pnas.0307875101

Thompson, B. J., Buonamici, S., Sulis, M. L., Palomero, T., Vilimas, T., Basso, G., et al. (2007). The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J. Exp. Med.* 204, 1825–1835. doi: 10.1084/jem.20070872

Tsunematsu, R., Nakayama, K., Oike, Y., Nishiyama, M., Ishida, N., Hatakeyama, S., et al. (2004). Mouse Fbw7/Sel-10/Cdc4 is required for notch degradation during vascular development. *J. Biol. Chem.* 279, 9417–9423. doi: 10.1074/jbc.M312337200

Van Tetering, G., Van Diest, P., Verlaan, I., Van Der Wall, E., Kopan, R., and Vooijs, M. (2009). Metalloprotease ADAM10 is required for Notch1 site 2 cleavage. *J. Biol. Chem.* 284, 31018–31027. doi: 10.1074/jbc.M109.006775

Wahi, K., Bochter, M. S., and Cole, S. E. (2014). The many roles of Notch signaling during vertebrate somitogenesis. *Semin. Cell Dev. Biol.* 49, 68–75. doi: 10.1016/j.semcdb.2014.11.010

Wahl, M. B., Deng, C., Lewandoski, M., and Pourquie, O. (2007). FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. *Development* 134, 4033–4041. doi: 10.1242/dev.009167

Wallberg, A. E., Pedersen, K., Lendahl, U., and Roeder, R. G. (2002). p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains *in vitro*. *Mol. Cell. Biol.* 22, 7812–7819. doi: 10.1128/MCB.22.22.7812-7819.2002

Wang, K., Zhang, Q., Li, D., Ching, K., Zhang, C., Zheng, X., et al. (2015). PEST domain mutations in Notch receptors comprise an oncogenic driver segment in triple-negative breast cancer sensitive to a gamma-secretase inhibitor. *Clin. Cancer Res.* 21, 1487–1496. doi: 10.1158/1078-0432.CCR-14-1348

Wang, N. J., Sanborn, Z., Arnett, K. L., Bayston, L. J., Liao, W., Proby, C. M., et al. (2011). Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17761–17766. doi: 10.1073/pnas.1114669108

Wang, Z., Inuzuka, H., Fukushima, H., Wan, L., Gao, D., Shaik, S., et al. (2012). Emerging roles of the FBW7 tumour suppressor in stem cell differentiation. *EMBO Rep.* 13, 36–43. doi: 10.1038/embor.2011.231

Weber, S., Niessen, M. T., Prox, J., Lullmann-Rauch, R., Schmitz, A., Schwanbeck, R., et al. (2011). The disintegrin/metalloproteinase Adam10 is essential for epidermal integrity and Notch-mediated signaling. *Development* 138, 495–505. doi: 10.1242/dev.055210

Welcker, M., and Clurman, B. E. (2008). FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat. Rev. Cancer* 8, 83–93. doi: 10.1038/nrc2290

Welcker, M., Singer, J., Loeb, K. R., Grim, J., Bloecher, A., Gurien-West, M., et al. (2003). Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Mol. Cell* 12, 381–392. doi: 10.1016/S1097-2765(03)00287-9

Westhoff, B., Colaluca, I. N., D'ario, G., Donzelli, M., Tosoni, D., Volorio, S., et al. (2009). Alterations of the Notch pathway in lung cancer. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22293–22298. doi: 10.1073/pnas.0907781106

Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* 43, 567–581. doi: 10.1016/0092-8674(85)90229-6

William, D. A., Saitta, B., Gibson, J. D., Traas, J., Markov, V., Gonzalez, D. M., et al. (2007). Identification of oscillatory genes in somitogenesis from functional genomic analysis of a human mesenchymal stem cell model. *Dev. Biol.* 305, 172–186. doi: 10.1016/j.ydbio.2007.02.007

Wu, G. Y., Lyapina, S., Das, I., Li, J. H., Gurney, M., Pauley, A., et al. (2001). SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol. Cell. Biol.* 21, 7403–7415. doi: 10.1128/MCB.21.21.7403-7415.2001

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26, 484–489. doi: 10.1038/82644

Yabe, T., and Takada, S. (2016). Molecular mechanism for cyclic generation of somites: Lessons from mice and zebrafish. *Dev. Growth Differ.* 58, 31–42. doi: 10.1111/dgd.12249

Yang, L. T., Nichols, J. T., Yao, C., Manilay, J. O., Robey, E. A., and Weinmaster, G. (2005). Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. *Mol. Biol. Cell* 16, 927–942. doi: 10.1091/mbc.E04-07-0614

Zhou, S., Fujimuro, M., Hsieh, J. J., Chen, L., Miyamoto, A., Weinmaster, G., et al. (2000). SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function. *Mol. Cell. Biol.* 20, 2400–2410. doi: 10.1128/MCB.20.7.2400-2410.2000

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

HYPOTHESIS AND THEORY
published: 24 March 2017
doi: 10.3389/fcell.2017.00021

Signaling and Transcription Factors during Inner Ear Development: The Generation of Hair Cells and Otic Neurons

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Integration between cell signals and bHLH transcription factors plays a prominent role during the development of hair cells of the inner ear. Hair cells are the sensory receptors of the inner ear, responsible for the mechano-transduction of sound waves into electrical signals. They derive from multipotent progenitors that reside in the otic placode. Progenitor commitment is the result of cell signaling from the surrounding tissues that result in the restricted expression of SoxB1 transcription factors, Sox2 and Sox3. In turn, they induce the expression of Neurog1 and Atoh1, two bHLH factors that specify neuronal and hair cell fates, respectively. Neuronal and hair cell development, however, do not occur simultaneously. Hair cell development is prevented during neurogenesis and prosensory stages, resulting in the delay of hair cell development with respect to neuron production. Negative interactions between Neurog1 and Atoh1, and of Atoh1 with other bHLH factors driven by Notch signaling, like Hey1 and Hes5, account for this delay. In summary, the regulation of Atoh1 and hair cell development relies on interactions between cell signaling and bHLH transcription factors that dictate cell fate and timing decisions during development. Interestingly, these mechanisms operate as well during hair cell regeneration after damage and during stem cell directed differentiation, making developmental studies instrumental for improving therapies for hearing impairment.

Keywords: atoh1, Neurog1, Hes and Hey factors, Notch signaling pathway, cell fate specification, hair cell regeneration

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *17 December 2016*
Accepted: *02 March 2017*
Published: *24 March 2017*

Citation:
Gálvez H, Abelló G and Giraldez F (2017) Signaling and Transcription Factors during Inner Ear Development: The Generation of Hair Cells and Otic Neurons. Front. Cell Dev. Biol. 5:21. doi: 10.3389/fcell.2017.00021

THE INDUCTION OF NEURAL COMPETENCE IN THE OTIC PLACODE

The ear is one major sensory organ of the vertebrate head that is responsible for the senses of hearing, balance and acceleration. The vertebrate inner ear derives from the otic placode, a thickening of the head ectoderm. The formation of the inner ear requires a series of cell fate decisions and morphogenetic events with a precise temporal and spatial pattern (Fritzsch et al., 2006; Groves and Fekete, 2012). Mature sensory organs of the vestibular and auditory regions of the inner ear are formed by three cells types: hair cells (HC), supporting cells (SC), and neurons, which in amniotes derive from a common neurosensory pool of cells.

One crucial step during inner ear development is the specification of neurosensory progenitors and the diversification of the different cell types. This is probably the first developmental decision in the otic epithelium and it reflects the segregation of two functionally independent domains,

one with neurosensory competence and another devoid of it (Abello and Alsina, 2007). The neurosensory domain gives rise to otic sensory neurons, sensory hair cells and supporting cells in chick and mouse (Satoh and Fekete, 2005; Raft et al., 2007). The expression of Sox3 and Sox2, Fgf10, and also that of members of the Notch pathway like Delta1, Hes5, and Lunatic Fringe is restricted to the neurosensory domain (Abelló et al., 2007). SoxB1 genes have a proneural function (See **Box 1**) and drive the expression of Neurog1 and Atoh1 (Jeon et al., 2011; Neves et al., 2012). The complementary non-neural domain shows two major patterning genes, Lmx1b and Iroquois1, and two members of the Notch pathway, Serrate1 and Hes1 (Abelló et al., 2007, 2010).

FGF and BMP signaling differentially regulate the expression of Sox3 and Lmx1, and their respective restriction to the anterior and posterior domains (Abelló et al., 2007; Schneider-Maunoury and Pujades, 2007). The regionalization of the otic placode into neurosensory and non-sensory territories requires also the functional integrity of the Notch pathway for its stabilization (Abelló et al., 2007). The non-sensory region of the otic placode receives signals that confer posterior identity (Bok et al., 2011). Retinoic acid (RA), which is known to posteriorize the embryonic body axis, is also required to specify the posterior character of the otic placode. Expression of RA synthesizing and degrading enzymes coincides with the AP boundary of the otic placode, and experiments in chicken and zebrafish have disclosed a developmental window during which the otocyst receives and is sensitive to RA posteriorizing signals (Bok et al., 2011; Radosevic et al., 2011).

Two main cell fate decisions are made sequentially during ear development. First, neurosensory progenitors produce either neuronal (neuroblast) or sensory precursors. Secondly, once neurons have delaminated, the progenitors that remain in the epithelia develop into either hair cells or supporting cells. The differentiation of neurons and hair cells is driven by the expression of, respectively, Neurog1 and Atoh1, two basic Helix-Loop-Helix (bHLH) proteins. Notch signaling plays a critical role in these two sequential decisions because it is instrumental in forcing precursors to adopt alternative fates by the mechanism of lateral inhibition (**Figure 1**).

Evidence in different species suggests that neurosensory progenitors are multipotent. Lineage analyses by viral tracing in chicken embryos demonstrated that bipotential neurosensory progenitor cells are present in the otic placode (Satoh and Fekete, 2005) and dye-labeling of otic placode progenitors showed that neurons and hair cells derive from the neurosensory domain of the otic vesicle (Bell et al., 2008). Furthermore, genetic fate mapping in mouse and chick indicates that vestibular sensory hair cells derive from Sox2 expressing progenitors residing in the neurosensory domain of the otic placode (Raft et al., 2007; Neves et al., 2012). In zebrafish, there are three progenitor pools, one specific to neurons, another specific to hair cells and a third one that can give either neurons or hair cells until later stages (Sapède et al., 2012), but all come from a population that expresses Atoh1b, suggesting that they also may share a common progenitor (Millimaki et al., 2007).

SOX2 AND NEURAL COMPETENCE

Sox genes are transcription factors that belong to the High Moblity Group (HMG) box domain proteins (Kamachi and Kondoh, 2013). One subfamily of Sox genes is the SoxB group, which is split in turn into two sub-groups: SoxB1 that includes Sox1, Sox2, and Sox3, being only Sox2 and Sox3 expressed in the vertebrate inner ear (Neves et al., 2007; Abelló et al., 2010), and SoxB2 that comprises Sox14 and Sox21, which are transcriptional repressors (Kamachi and Kondoh, 2013) and from which only Sox21 is expressed during ear development (Freeman and Daudet, 2012).

Sox2 is critical for the specification of neurons and hair cells in the neurosensory domain of the otic placode (Kiernan et al., 2005; Neves et al., 2012). Sox2 is able to activate both Neurog1 and Atoh1, but it is downregulated in differentiated neurons and hair cells. Sox2 expression remains high in supporting cells, suggesting that this cell type retains progenitor properties (Neves et al., 2007; Evsen et al., 2013; Kamachi and Kondoh, 2013). The expression of Sox2 in the inner ear is driven by signals from the surrounding tissues. Like in other regions of the nervous system, FGF signaling is determinant for setting the onset of SoxB1 factors (Alsina et al., 2004; Sweet et al., 2011; Ono et al., 2014). In the inner ear, first Sox3 and then Sox2 expression depends on FGF signaling emanating from the underlying mesoderm, the hindbrain and probably from the otic placode (Schneider-Maunoury and Pujades, 2007; Groves and Fekete, 2012). Sox3,

BOX 1 | bHLH in vertebrates: has Atoh1 lost its proneural function?

What is a proneural gene? A proneural gene must fulfill three main characteristics (Hassan and Bellen, 2000): First, its expression precedes and coincides with the selection of neuronal precursor cells. Secondly, its function is both necessary and sufficient for the specification of a given neuronal lineage in a cell autonomous fashion. Finally, its loss of function results in the deletion (and its missexpression ectopic development) of a given lineage. Proneural genes were first identified in Drosophila peripheral nervous system development. The Achaete–Scute complex (AS-C) genes were identified as proneural genes encoding bHLH factors. Later on, atonal (Atoh1 in mammals) was identified by PCR (Jarman et al., 1993). Atonal in Drosophila is the master gene for the formation of chordotonal organs, which are mechano-receptors of insect muscles. Atonal gene selects the progenitors that give rise to the mature organs. Atonal loss of function abolishes chordotonal organs and its missexpression favors their ectopic formation (Jarman et al., 1993). Are Atoh1 and Neurog1, the vertebrate homologs of atonal, also proneural genes? Atoh1 and Neurog1 overexpression drives, respectively, ectopic hair cell and neuron formation (Izumikawa et al., 2005; Evsen et al., 2013), and their loss of function results in the lack of hair cells or neurons (Ma et al., 1998; Bermingham, 1999). However, their function is far more restricted and, like in other regions of the Nervous System, they do not provide a broad neural competence, but a far more restricted lineage selection (for example, HCs and SCs in the case of Atoh1 and the inner ear). The broad neural competence is rather dependent on SoxB1 (Azuara et al., 2006; Puligilla and Kelley, 2017). This shows a proneural identity crisis in vertebrate development and the taking over by SoxB1 proteins (Hassan and Bellen, 2000).

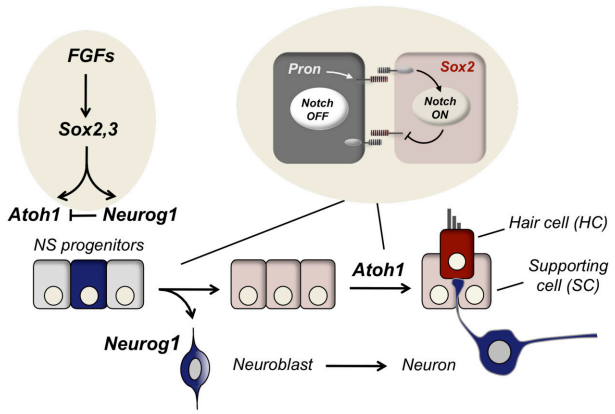

FIGURE 1 | Specification of neurons and hair cells during inner ear development. The three cell types that constitute the functional unit of the sensory patches of the inner ear are: neurons (blue), supporting cells (SC, pink) and hair cells (HC, red). The three cell types derive from the neurosensory epithelium that is specified by Sox2 and Sox3 after FGF signaling. Upon neurosensory induction, HC differentiation is delayed with respect to neurons. Neurog1 is expressed in neuronal precursors and after neurogenesis Atoh1 is expressed in the prospective hair cells. Notch signaling contributes to cell determination in two rounds of lateral inhibition, first to single out neuronal precursors (neurogenesis) and, secondly, to decide between hair cells and supporting cells (sensorigenesis).

which is expressed in the neurosensory domain in chick is not detected in the mouse, where Sox9 is co-expressed along with Sox2 in the prosensory region (Mak et al., 2009).

THE REGULATION OF NEUROG1 AND ATOH1

The Regulation of Neurog1

Neurog1 (Neurogenin1) is a basic helix-loop-helix (bHLH) transcription factor that behaves as master regulator for neuronal differentiation in different vertebrates (Henrique et al., 1997; Ma et al., 1998; Alsina et al., 2004; Evsen et al., 2013). Neurog1 is an Atonal-related protein (ARP; Hassan and Bellen, 2000). On average, it shares with Atoh1 53% amino acid identity in the bHLH domain, and differs from Atoh1 in four basic domain residues (Sommer et al., 1996). Three neurogenins have been described in mammals. Neurog1 and Neurog2 function as neuroblast selector genes in mouse (Ma et al., 1998), but in the chicken and mouse inner ear, only Neurog1 is expressed during ear development (Ma et al., 1996; Evsen et al., 2013).

Sox2 is necessary for Neurog1 up-regulation in the otic epithelium (Jeon et al., 2011; Neves et al., 2012). In mice, Neurog1 is also activated by Six1 and Eya1 that synergize with Sox2 (Zheng et al., 2003; Ahmed et al., 2012b). The neurosensory domain has high Notch activity, and Jeon and colleagues showed that the enhancer of Neurog1 is activated by high levels of NICD (Notch Intracellular Domain), while Atoh1 enhancer is not (Jeon et al., 2011). This may favor that Neurog1 expression precedes Atoh1 in the otic vesicle (Neves et al., 2011). However, later in development Notch signaling represses Neurog1 expression in the cells that remain in the epithelium.

Neurog1 expression is controlled by different cis-elements located 5′ and 3′ to the Neurog1 coding sequence. These enhancers drive the expression of Neurog1 in midbrain, hindbrain, trigeminal ganglia, and ventral neural tube. For Neurog1 expression in the dorsal neural tube only a 5′ enhancer has been identified (Nakada et al., 2004). Another enhancer region drives Neurog1 activity to the VIII cochlea-vestibular ganglion (Murray et al., 2000). The configuration of these enhancers is similar to the cis-elements identified for Neurog2 (Simmons et al., 2001), suggesting that there is a tight regulation of the two Neurogenins. Nakada et al. (2004) speculated that possibly the conservation between Neurog1 and Neurog2 arises from gene duplication. The modular organization of Neurogenins cis-regulatory regions contrasts with the single enhancer regulation described for Atoh1 (Helms et al., 2000 and see below).

The Regulation of Atoh1

Atoh1 expression is regulated by a downstream enhancer, which depends on its interaction with Atoh1. In other words, Atoh1 expression relies on its auto-regulation. This implies that crucial events in the developmental regulation of Atoh1 are the chromatin arrangements that allow the interaction of Atoh1 with its own enhancer, and also the activity of potential repressors that break this loop (See **BOX 2**). Work by Jane Johnson's lab discovered a region in the Atoh1 genome landscape that recapitulates Atoh1 expression during mouse and chicken development (Helms et al., 2000). Transgene expression in mouse identified a region that directed the expression to the neural tube, external granular layer (EGL) of the cerebellum from rhombic lip, and the developing hair cells of the cochlea and semicircular canals. The region contains a 1.7 Kb fragment located 3.4 Kb 3′ of the Atoh1 coding region that recapitulates the expression of Atoh1. This region is called the 3′Atoh1-enhancer (see **Figure 2A**).

Two regions within the 3′Atoh1-enhancer (3′Atoh1-enh) show a high homology between humans and mouse, and they were named Enhancer A and Enhancer B (EnhA and EnhB). The length of A and B is highly conserved in species like chicken, mouse, and human (Ebert, 2003), although the distance in between them varies among species. Interestingly, Helms et al. (2000) observed that transgenic embryos for Atoh1-enh/lacZ transgenic mice had no detectable β-gal activity in the Math1 null background, and this was shown to be also the case for the 3′Atoh1-enhancer-GFP reporter in the inner ear (Raft et al., 2007). This suggested that the activity of the 3′Atoh1-enh is dependent on Atoh1 expression and that autoregulation is one major mechanism for setting Atoh1 transcriptional activity (**Figure 2B**).

The 3′Atoh1-enh contains several E-boxes, which are six-nucelotide DNA sequences that bind bHLH proteins, like Atoh1, Neurog1, and Hes/Hey factors (CANNTG; (Massari and Murre, 2000)). The Enhancer A contains a degenerated E-box and the Enhancer B three E-boxes. E-boxes in Enhancer B are a class A, a

BOX 2 | The priming of Atoh1.

Priming of Atoh1 in the developing ear may be also promoted by other mechanisms rather than Atoh1 autoactivation. Changes in histone modifications can change the transcriptional hierarchy that controls cell differentiation (Azuara et al., 2006). The dynamic changes in the histone modifications H3K4me3/H3K27me3, H3K9ac, and H3K9me3 indicate that there is a progression from poised to active, and finally to repressive marks in the Atoh1 locus. This sequence correlates with the onset of Atoh1 expression and its subsequent silencing during the perinatal period (Stojanova et al., 2015). The inhibition of acetylation blocks Atoh1 mRNA expression in nascent hair cells, as well as the ongoing hair cell differentiation during embryonic organ of Corti development (Stojanova et al., 2015). Contrarily, histone deacetylase inhibition favors the expression of hair cell markers in mouse utricle progenitor cells (Hu and Wang, 2014). Cochlear explants treated with histone deacetylase inhibitor increase the levels of Atoh1 mRNA in early post-natal mice (Stojanova et al., 2015). This suggests that Atoh1 is poised during developmental stages and thereby ready to be activated. However, after birth the Atoh1 locus becomes methylated and cannot be transcribed when hair cells are damaged. The epigenetic status of Atoh1 locus during organ of Corti development shows a bivalent mark of the Atoh1 locus by H3K27me3 and H3K4me3, prior to the upregulation of Atoh1 (Stojanova et al., 2015). This is consistent with the idea that Sox2 poises/primes the Atoh1 locus until Atoh1 itself is able to bind to the E-box A and trigger Atoh1 expression.

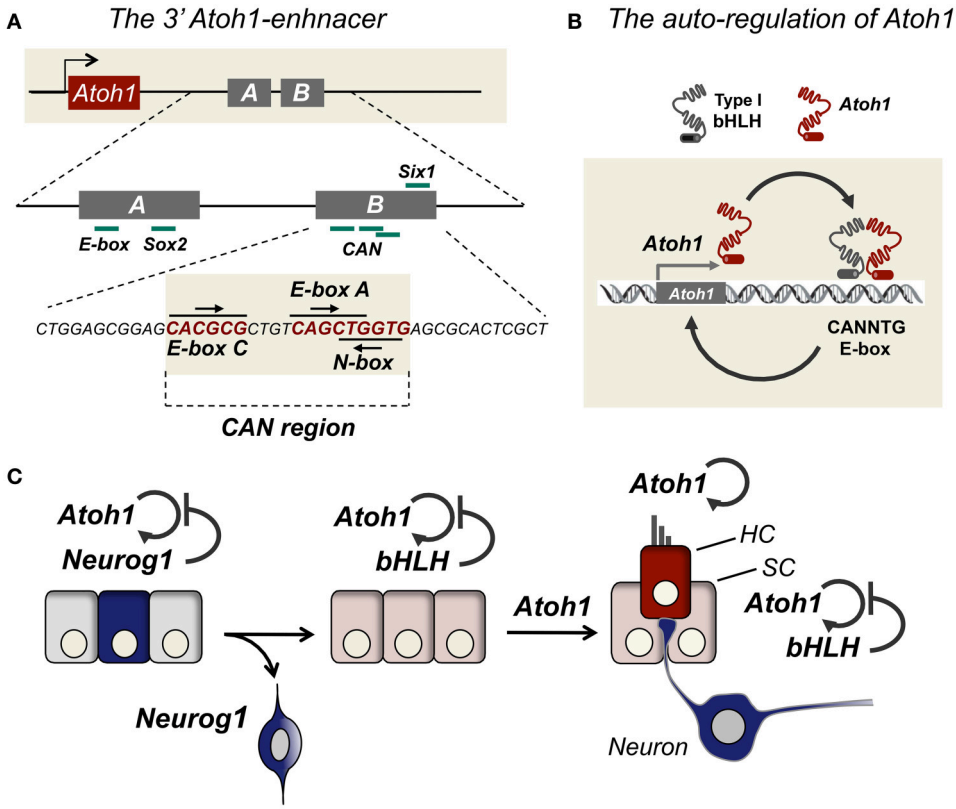

FIGURE 2 | The regulation of Atoh1 by signal integration in the 3′Atoh1 enhancer. (A) The 3′Atoh1 enhancer is located 3.5 Kb downstream Atoh1 coding region and it consists of two enhancer named, Enhancer A and Enhancer B. Different transcription factors bind to this region like Sox2 in Enhancer A, and Six1 in Enhancer B. The three E-boxes in Enhancer B are putative sites for Atoh1 repression. **(B)** Atoh1 binds to the class A E-box located in the Enhancer B and is able to activate its own transcription. **(C)** During neurogenesis Atoh1 expression is silenced by Neurog1 and its expression is further delayed by the counteraction.

class C and a reversed N-box. All three boxes are very close, and class A and N-box overlap (**Figure 2A**, the CAN region for C-, A- and N-boxes). As mentioned above, Helms et al. (2000) already identified the class A E-box located in Enhancer B as crucial for Atoh1 autoactivation. Besides the CAN region, the 3′Atoh1-enh region contains putative binding sites for a menagerie of transcriptional activators and repressors. Some of them like Sox2, Six1/Eya1, and β-catenin bind directly to the enhancer as shown by biochemical assay (Akazawa et al., 1995; Ebert, 2003; Mutoh et al., 2006; Briggs et al., 2008; D'Angelo et al., 2010; Shi et al., 2010).

Sox2 is sufficient to activate Atoh1 and to induce ectopic hair cell formation in the chick otocyst (Neves et al., 2012) and it is rapidly downregulated as hair cells differentiate. This downregulation is required for further maturation because sustained expression of Sox2 in Atoh1 expressing cells blocks the induction of hair cell markers such as Myosin-VIIa (Dabdoub et al., 2008). Six1 and its transcriptional co-activator Eya1 are expressed in the prosensory domain of the cochlea, and they bind directly to the 3′Atoh1-enh (Ahmed et al., 2012a). These two factors are sufficient to induce Atoh1 expression in competent regions and their activation is potentiated by Sox2 (Ahmed et al.,

2012a). As described before, Neurog1 is also upregulated by Six/Eya with Sox2 (Ahmed et al., 2012b).

In summary, the crucial elements in setting neuronal and sensory competence are: (1) FGF signaling that induces the expression of Sox2 in the neurosensory domain of the otic placode, (2) Sox2 that drives the activation of neuronal and sensory master genes Neurog1 and Atoh1 and neurosensory competence, and (3) the onset of Neurog1 and Atoh1 and the determination of neurons and hair cells, respectively.

NEURONS VS. HAIR CELLS: HOW DOES NEUROG1 COUNTERACT ATOH1?

Although Sox2 is able to activate both Neurog1 and Atoh1, Atoh1 expression remains undetected until HC differentiation (**Figure 2C**). Neves et al. (2012) hypothesized that this delay in Atoh1 expression may be explained by an incoherent feed-forward loop (I-FFL) triggered by Sox2, where Sox2 activates both Atoh1 and Atoh1 repressors (Neves et al., 2012). Recent experiments using a conditional gain of function system in mouse support the model by showing that Sox2 is required for prosensory specification, but it must be downregulated to allow Atoh1 expression (Puligilla and Kelley, 2017). Major candidates to mediate the repression of Atoh1 include a variety of bHLH factors that are expressed throughout ear development such Neurog1 or Notch targets.

There is a mutual antagonism between Neurog1 and Atoh1 functions. Neurog1 null mice show a loss of sensory neurons, smaller sensory patches, and premature development of hair cells (Matei et al., 2005). Moreover reduced Neurog1 causes ectopic Atoh1 expression and that excess of Atoh1 suppresses Neurog1 (Raft et al., 2007). During development, Neurog1 overrides Atoh1 expression. Therefore, the functional antagonism between Neurog1 and Atoh1 is resolved in favor of Neurog1, the result being that neurons develop prior to hair cells. The molecular mechanism of this dominance of Neurog1 over Atoh1 is still unknown, but it seems crucial for understanding the timing of cell fate during ear development. Neurog1 is a transcriptional activator, suggesting that the counteractive interaction with Atoh1 is likely complex. In principle, Neurog1 may repress Atoh1 by the following mechanisms: (1) by preventing Atoh1 transcription, (2) by preventing Atoh1 mRNA translation, or (3) by post-translational mechanisms that result in modified Atoh1 protein levels or activity.

Transcriptional Repression of Atoh1

Neurog1 and Atoh1 are two bHLH type II proteins (Massari and Murre, 2000). They are known to dimerize with type I bHLH like E47 and bind to E-box sequences resulting in activation of transcription (Jarman et al., 1993; Koyano-Nakagawa et al., 1998; Bertrand et al., 2002). One simple possibility for Neurog1 acting as a repressor of Atoh1 is that Neurog1 acts as a partial agonist for Atoh1. Neurog1 would compete for the class A E-box located in the 3′Atoh1-enh, resulting in a weak activation but impeding the stronger autoactivation by Atoh1. Atoh1 and Neurog1 may also compete for the same E-protein partners, like E47, the result being that Atoh1 is unable to bind DNA.

Neurog1 may repress Atoh1 transcription in an indirect manner, by activating transcriptional repressors of Atoh1. Among the targets of Neurog1, NeuroD is one major effector of Neurog1 in the ear, being essential for neuroblasts delamination (Ma et al., 1996; Huang et al., 2000; Kim et al., 2001) and for shutting down Sox2 expression in the neurons (Evsen et al., 2013). Conditional NeuroD deficient mice show that NeuroD suppresses Atoh1 expression in auditory-vestibular neurons as indicated by the ectopic expression of Atoh1 after NeuroD deletion (Jahan et al., 2010). However, during early stages of neurosensory development, Neurog1 is expressed homogeneously in the neurosensory epithelium, including hair cell precursors (Raft et al., 2007), and only those cells that express high levels of Neurog1 trigger lateral inhibition and delaminate from the epithelium. Therefore, it is likely that alternative mechanisms may prevent Atoh1 without necessarily driving neuronal differentiation (Sun et al., 2001; Fritzsch et al., 2006).

Post-Transcriptional Regulation of Atoh1: mRNA Processing and Stability

The half-life of many mRNAs can fluctuate during development and mRNA stability depends on RNA-binding proteins that bind mRNAs (Day and Tuite, 1998; Knuckles et al., 2012). Also micro-RNAs (miRNAs) are known regulators of mRNA stability or translation efficiency and modify protein expression levels. Some miRNAs like the miR-183 family (miR-96, miR-182, and miR-183) are expressed at high levels in young hair cells and ganglion neurons (Weston et al., 2006; Li et al., 2010) and the manipulation of miR-183 levels modify the number of hair cells (Li et al., 2010, see Groves et al., 2013 for a review).

Regulation of translation and protein synthesis depends on initiation factors (eIFs), some of which are phosphoproteins susceptible of regulation (Day and Tuite, 1998), but little is known about their behavior during embryonic development. Those are potential candidates to regulate the reduction of Atoh1 induced by Neurog1; however, we have no information on whether they are modified by Neurog1.

Post-Translational Regulation: Atoh1 Activity and Degradation

Degradation of bHLH proteins has been extensively documented in different model systems and it is accounted by phosphorylations in their C-terminus domain (Forget et al., 2014; Hardwick and Philpott, 2015; Quan et al., 2016). Atoh1 post-transcriptional downregulation has been reported during cerebellar granule neuron differentiation, where BMP2 and BMP4 inhibit proliferation and induce differentiation through proteosome mediated degradation of Atoh1 (Zhao et al., 2008). BMPs induce the expression of Id1 and Id2 that upon dimerization with Atoh1 target the complex for degradation. In cerebellar granule neuron progenitors, Shh prevents Atoh1 degradation by preventing the recruitment of Atoh1 by Huwe3, an E3 ligase (Forget et al., 2014). Atoh1 is degraded by the

proteosome pathway when dimerizing with Huwe1 in HEK cells, and the conditional deletion of Huwe1 generates supernumerary HCs in the mouse cochlea (Cheng et al., 2016).

Atoh1 contains in the C-terminus a potential PEST sequence (Jarman et al., 1993). This is a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T). This sequence is associated with proteins that have a short intracellular half-life and it is hypothesized that the PEST sequence acts as signal for protein degradation. Atoh1 protein stability is very short and it is extinguished in 2 h after protein synthesis blockade (Forget et al., 2014; Cheng et al., 2016). Aminoacid residues located at the C-terminus region of Atoh1 protein that are susceptible to phosphorylation are conserved among different species (Mulvaney and Dabdoub, 2012). Like Atoh1, other bHLH proteins as Neurog2 and NeuroD4 are also less stable upon phosphorylation (Hindley et al., 2012; Hardwick and Philpott, 2015).

Cyclin-dependent kinases (Cdks) drive cell cycle progression and are known to target Serine Proline (SP) and Threonine Proline (TP) sites (Errico et al., 2010). bHLH proteins like Atoh1 and Neurog1 contain several putative ST and TP in their C and N-terminal regions. Phosphorylation in these SP/TP residues may be crucial for regulating activity and is linked to the cell cycle. For example, in Xenopus embryos and P19 cells, progenitor cells that divide rapidly show Neurog2 phosphorylation and degradation, whereas when cell cycle is lengthened, Neurog2 accumulates and activates down-stream targets (Ali et al., 2011). Cell cycle exit in the cochlea is dependent on the expression of the cyclin inhibitor p27kip (White et al., 2006), and it is possible that Atoh1 is degraded in dividing prosensory progenitors until p27kip expression and cell cycle withdrawal. One plausible mechanism is that Atoh1 protein is degraded in the presence of Neurog1. This type of regulation has been recently described for Atoh1 protein when targeted by the E-3 ubiquitin ligase Huwe1 (Cheng et al., 2016). Finally, Neurog1 may also interfere with Atoh1 translation (see **Figure 3**).

NOTCH SIGNALING AND THE SINGLING OUT OF HAIR CELLS

Notch signaling is an evolutionarily conserved juxtacrine signaling pathway used by metazoans. It controls a broad spectrum of developmental processes in organisms ranging from sea urchins to humans (Artavanis-Tsakonas et al., 1999; Neves et al., 2013). Lateral inhibition is one major operation mode of the pathway by which a ligand-producing cell signals its neighbors to reduce ligand expression (see (Neves et al., 2013) for review on the different modes of operation of Notch during ear development). Lateral inhibition mediates binary cell fate decisions by ensuring that the cells adopt one of two alternative fates. In the inner ear Notch mediates the determination of two major cell types, neurons and hair cells. Driven by Sox2, progenitors residing in the neurosensory domain express Neurog1, some of them with enough strength so to unfold the neuronal program and become neuroblasts. Nascent neuroblasts express the ligand Delta-like1 (Dll1), which activates Notch1 in the neighboring cells and suppress Neurog1 expression. Neuroblasts delaminate, and the cells that remain in the neurosensory epithelium are fated to become sensory precursors (the prosensory patches). Later in development, some cells from the prosensory patches start expressing Atoh1, which

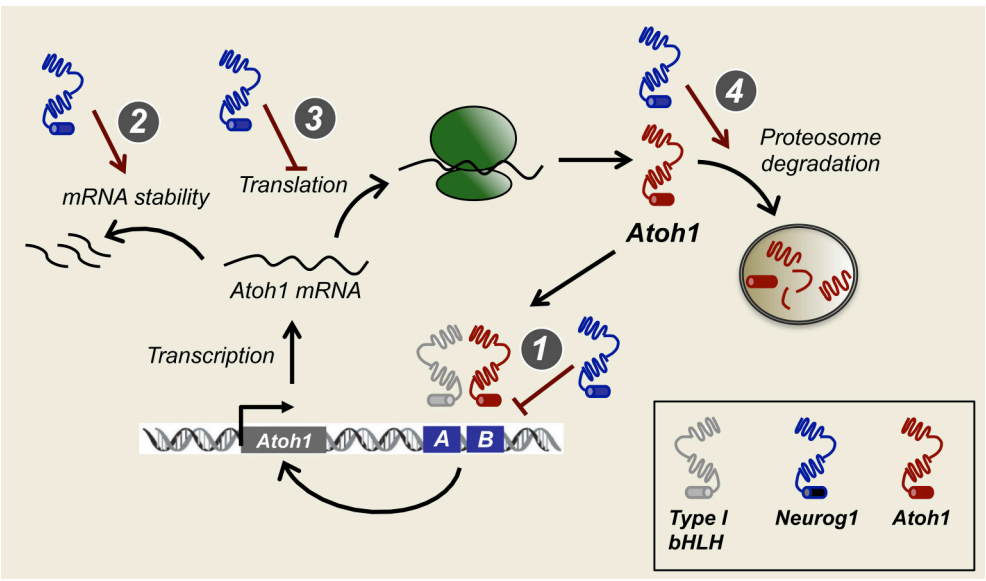

FIGURE 3 | How does Neurog1 force neurogenesis before sensorigenesis? Possible models for Neurog1 repression of Atoh1. Neurog1 prevents Atoh1 activation by binding to the CAN region of the 3′Atoh1 enhancer or by sequestering type I bHLH factors necessary for Atoh1 binding (1). Neurog1 reduces Atoh1 protein levels by mRNA degradation (2), inhibition of protein synthesis (3), or reducing protein stability by promoting Atoh1 protein to proteosome degradation (4), of other bHLH in the prosensory epithelia.

initiates a second round of lateral inhibition by which some precursors activate the ligands Delta1 (in mammals also Jag2) that drive lateral inhibition. The result is that those cells that express Atoh1 become hair cells and prevent the neighbors to do so, generating the typical mosaic of alternate cell types (Adam et al., 1998; Eddison et al., 2000; Neves et al., 2013).

Both during neurogenesis and hair cell generation, the action of Notch ligands results in the expression of the typical Notch targets like Hes and Hey factors (Petrovic et al., 2014, 2015). The most studied Notch canonical effectors are Hairy and Enhancer of Split (Hes) and Hairy and enhancer of split related (Hey). Hes and Hey genes belong to the type VI bHLH group. Seven Hes members have been identified in vertebrates (Hes1–7), while the Hey subfamily of genes encodes three members in mammals (Hey1, Hey2, and HeyL; Iso et al., 2001, 2003).

The core structure of Hes and Hey proteins contains a basic and Helix-loop-Helix domain and an Orange domain at the C-terminus region. The Orange domain serves as a region for protein-protein interactions and for partner selection (Iso et al., 2001). Hey proteins differ from the Hes subgroup by two striking features: first a glycine present in the basic domain of Hey proteins instead of a conserved proline in Hes proteins, which confers DNA-binding specificity (Leimeister et al., 1999). Secondly, the C-terminal WRPW motif that is characteristic of Hes proteins and allow Groucho co-repressor recruitment, is replaced with YRPW or YXXW (HeyL; Fisher et al., 1996). The C-terminal WRPW of Hes motif acts as polyubiquitination signal, making Hes proteins short-living (Hirata et al., 2002; Iso et al., 2003).

Hes factors bind with high affinity to E-box class C or N-box. Hey1, due to the presence of a glycine residue in the basic domain has preference to class C or class B E-boxes (Iso et al., 2003). The repressive function can be either active or passive. Active repression involves DNA binding, whilst in passive repression Hey/Hes proteins sequester bHLH type I family and impair their heterodimerization with class II bHLH (Iso et al., 2003).

During development, several Hes and Hey genes are expressed in the inner ear. Hes5 is the major Notch target expressed during lateral inhibition. It is detected in the precursors that are not selected as neurons or hair cells. Its expression correlates well with that of Dll1 in nascent neurons and hair cells (Petrovic et al., 2014). Hey1 is also expressed in the prosensory epithelium, concomitantly with Jagged1, and co-expressed with Hes5 during hair cell formation (Petrovic et al., 2014). Although Hey1 and Hes5 are direct Notch downstream targets, they differ in the level of Notch required for their activation.

Knockout mice of different Hey and Hes factors exhibit supernumerary hair cells in the cochlea, suggesting a repressor

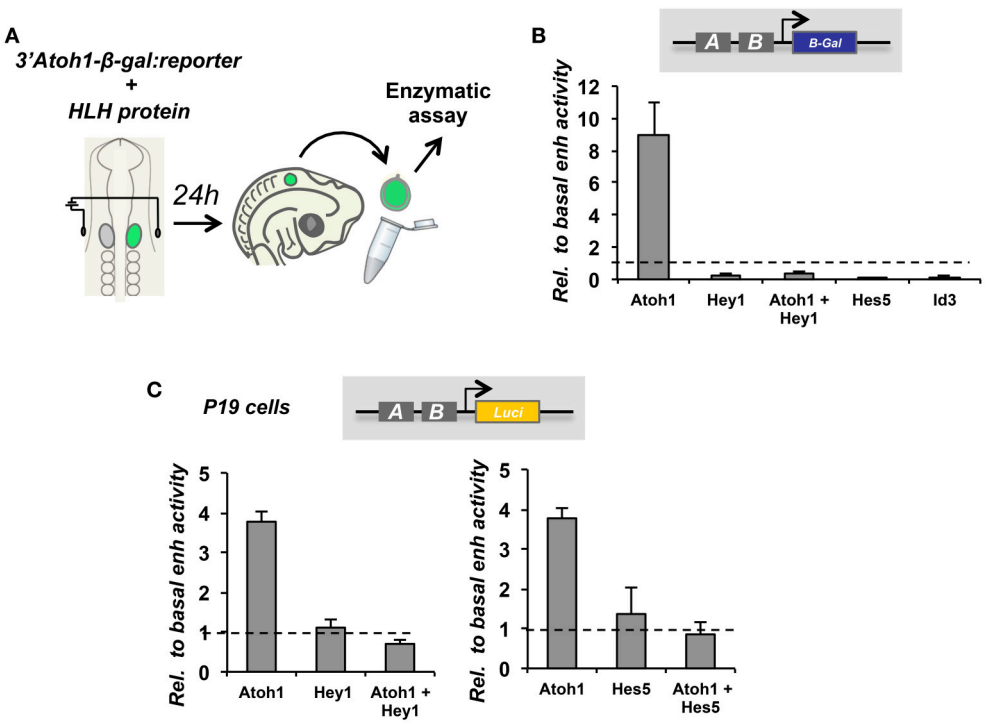

FIGURE 4 | 3′Atoh1 enhancer regulation by the Notch targets, Hey1 and Hes5. (A) Schematic representation of in ovo chicken electroporation of the 3′Atoh1 enhancer reporter in combination with bHLH factors. **(B)** Otic vesicles were isolated and developed in ovo for 24 h after in ovo electroporation (E2+1). Reporter β-gal activity measured in the conditions indicated ($n = 3$–4). Levels of electroporation were normalized by luciferase activity. Atoh1 activated its own enhancer and Hey1 prevented Atoh1 autoactivation. All three bHLH factors repressed 3′Atoh1 enhancer basal activity. **(C)** Hey1 (left) and Hes5 (right) also prevented Atoh1 autoactivation in P19 cells. Values of luciferase correspond to enhancer activity relative to the basal activity of the 3′Atoh1 enhancer in the conditions indicated in abscissa ($n = 3$). Values are normalized by renilla activity. Data displayed as Mean + S.E.M.

function during hair cell development. The combined loss of function of Hes5, Hey1, and Hes1 results in supernumerary hair cells (Tateya et al., 2011) and Atoh1 is upregulated after interference of Hey1/Hes5 expression with siRNAs (Du et al., 2013) or treatment with the Notch inhibitor DAPT (Ren et al., 2016). Notch inhibition of damaged sensory epithelia favors HC regeneration (Lin et al., 2011; Mizutari et al., 2013), suggesting that these factors may also regulate the ability to regenerate HCs (see below).

THE REPRESSION OF ATOH1 BY HES AND HEY: IS IT ALL IN THE 3′ATOH1 ENHANCER?

Hey1 and Hes5 repress Atoh1 and silence the 3′Atoh1-enh (**Figures 4A,B**). In addition, both factors are able to block Atoh1 autoactivation (**Figure 4C**), suggesting that the repression of Atoh1 by Hes5 and Hey1 prevails upon its own activation. Accordingly, and parallel to 3′Atoh1-enh repression, Hey1 overexpression is sufficient to prevent HC generation in chick sensory epithelia (**Figure 5**). Taken together, these observations suggest that during development, Notch targets Hey1 and Hes5 act on the 3′Atoh1-enh and repress Atoh1 expression in prosensory precursors and supporting cells.

Hey1 is also able to prevent both, the basal activity and the autoactivation of EnhB of the 3′Atoh1-enh in chicken otic vesicles and P19 cells (**Figures 6A,B**). Hes5 can also prevent Atoh1 autoactivation (**Figure 6B**). Moreover, the importance of the CAN region of EnhB is illustrated by multimer reporter analysis showing that Hey1 requires the E-boxes flanking the class A E-box to act as a repressor (**Figure 6C**). The CAN multimer mimics the 3′Atoh1-enh repression promoted by Hey1, indicating that the minimal region of the enhancer to explain the repression is the CAN region.

Hey1 needs to bind DNA in order to repress Atoh1, since the mutation of Hey1 DNA binding domain abolishes repression (**Figure 6D**). However, the identification of the region bound by Hey1 and Hes5 has been difficult and still remains elusive. On the one hand, mutations of either the class C E-box or the N-box of the CAN region are unable to prevent Atoh1 repression by Hey1 and Hes5 (**Figure 7**). This is in agreement with the results of ChIP-seq analysis performed on HEK 293, which shows that Hey1 does not bind to the 3′Atoh1-enh (Heisig et al., 2012). But it is also somehow surprising and suggests that there are alternative binding sites and/or mechanisms of repression for Hes and Hey factors. For example, it is possible that Hey1 blocks the transcription of Atoh1 by interfering with the class A E-box. This possibility is difficult to explore since the mutation of E-box A silences the 3′Atoh1-enh.

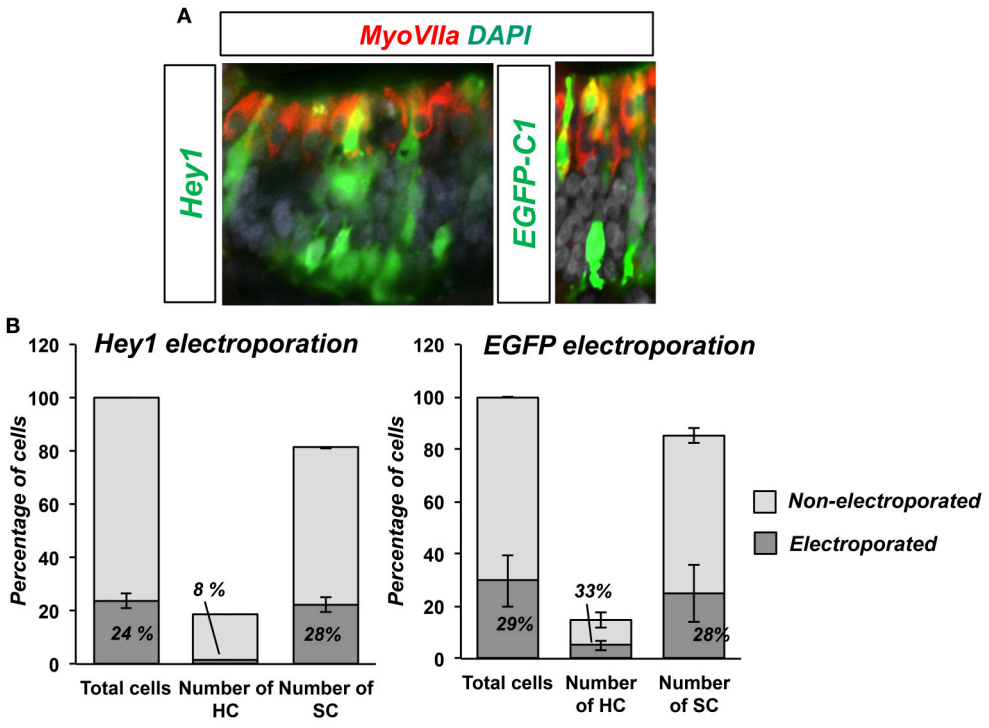

FIGURE 5 | Hey1 prevents HC formation in ovo. (A) E3.5 chicken embryos were electroporated with Hey1 (left image) or EGFP-C1 (right image) and then sectioned after 3 days of incubation (E3.5+3). Electroporated cells in the macula sacularis were found mainly in the SC layer, and very few developed as HCs. Control electroporation with EGFP-C1 (E3.5+3) is shown on the right. **(B)** Hey1 electroporation biased electroporated cells toward supporting cell fate. The fraction of HCs that were electroporated (8%) was smaller than that of SCs (28%), similar to the efficiency of the electroporation (24%). Bars represent the number of cells counted in two consecutive frames of electroporated macula sacularis, from three independent embryos ($n = 3$). Electroporation with EGFP-C1 did not show any bias for either HCs or SC.

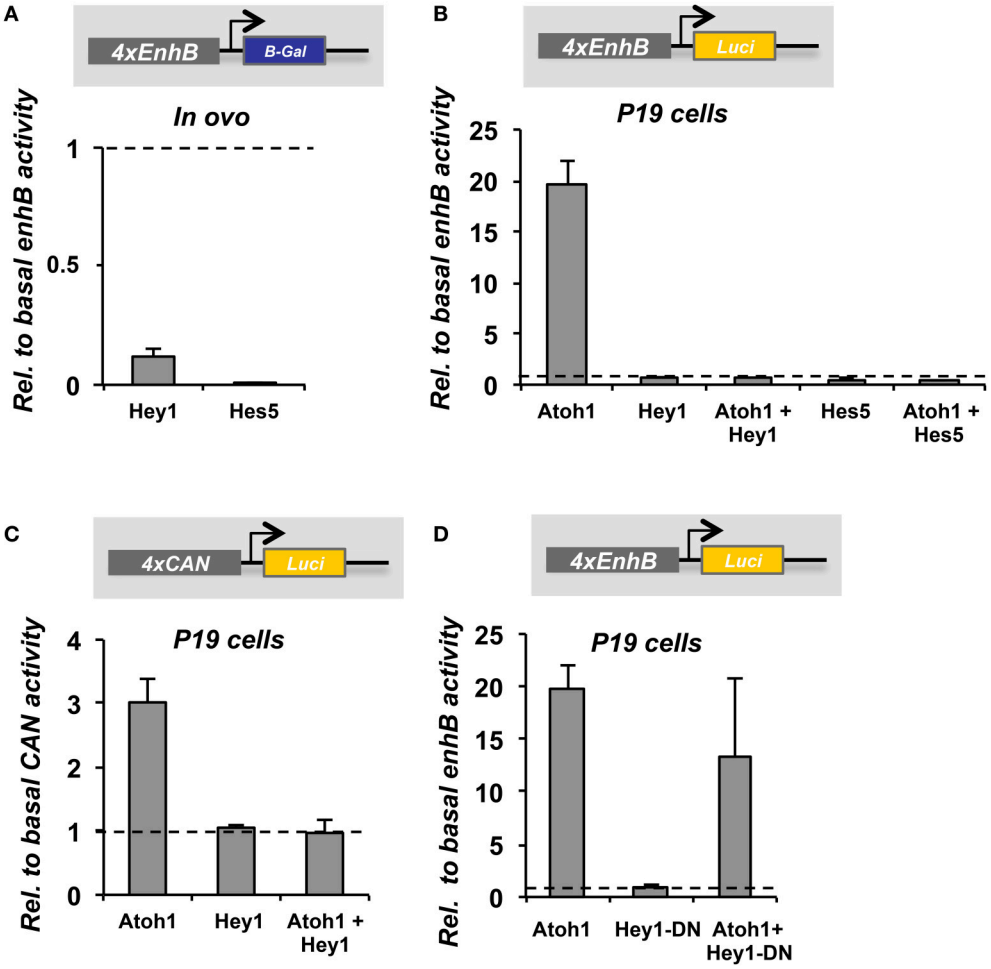

FIGURE 6 | The regulation of the 3′Atoh1 enhancer by Hey1 and Hes5 is recapitulated by the CAN region. (A) Quantification of EnhB activity in the presence of Hey1 or Hes5 in E2+1 otic vesicles. Hey1 and Hes5 factors were able to prevent the basal activity of 4 × EnhB ($n = 3$). **(B)** In P19, Atoh1 was able to activate 4 × EnhB and the autoactivation was suppressed by either Hey1 or Hes5 ($n = 3$). **(C)** The CAN multimer was activated by Atoh1, but Hey1 was not able to repress the basal reporter activity. However, it prevented Atoh1 autoactivation ($n = 3$). **(D)** Hey1 requires its DNA binding domain to repress the CAN region. Quantification of 4 × EnhB activity with Atoh1 and Hey1-DN (Hey1 dominant negative) in P19 cells. Data displayed as Mean + S.E.M.

A recent study has shown that Hes5 and Hey2 are able to prevent Atoh1 expression by binding to the promoter region of Atoh1, and that the repression of Atoh1 in supporting cells depends on this interaction, rather than on the 3′Atoh1 enhancer, which would operate mainly for Atoh1 activation (Abdolazimi et al., 2016). Gene repression and binding by Hey1 is also dependent on the chromatin signature of the promoter regions. Heisig et al. (2012) found that Hey1 bound sequences overlapped with the presence of polymerase II and the active chromatin mark H3K4m3, characteristic of active and poised promoters. H3K4m3 chromatin marks are found in Atoh1 promoter and enhancer prior to Atoh1 upregulation (Stojanova et al., 2015). Therefore, the regulation of Atoh1 is likely to be dependent on multiple sites, and not only mediated by the 3′Atoh1 enhancer. The relationship between the Enhancer and promoter regions may be crucial to fully solve the complex regulation by Hey1.

FROM DEVELOPMENT TO REGENERATION

Hearing loss is a major problem affecting more than 360 million people in the industrialized world (WHO). It affects speech and language and leads to severe deficits in communication, and a strong negative impact in the quality of life. Hearing impairment is mainly caused by the failure of hair cells and/or otic neurons (sensorineural hearing loss), hair cell damage being the most frequent triggering factor. Hair cell damage arises from genetic defects, aging, noise, traumatic lesions, infections, or therapeutic substances. The main problem of hair cell damage is that, unlike other animal species, mammals are not able to regenerate hair cells of the auditory epithelia and there is no treatment for hearing deficiencies in humans.

In contrast to mammals, non-mammalian vertebrates like chicken, zebrafish, or lizards, are able to repair and heal

A

Reporter	Sequence
Wt 3'Atoh1-enh	AG**CACGCG**CTGTC**AGCTGGTG**AG
Class C E-box mut	AG**ATT**GCGCTGTCAGCTGGTGAG
Class C-N box mut	AG**ATT**GCGCTGTCAGCTG**AAT**AG

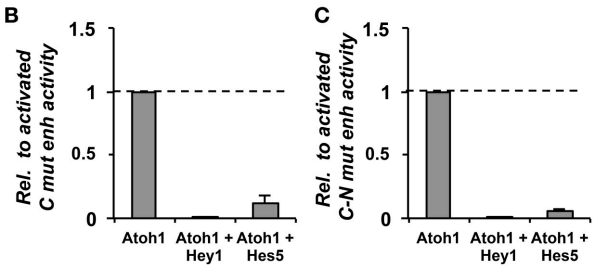

FIGURE 7 | Hey1 and Hes5 predicted binding sites were not enough to prevent the repression of the 3′Atoh1 enhancer. (A) Table showing the mutations generated in the E-boxes of the CAN region (or located in enhancer B). The three E-boxes are depicted in black and bold, in red the mutated nucleotides. **(B)** Hey1 and Hes5 were able to block Atoh1 autoactivation even after mutation of the class C E-box ($n = 3$). **(C)** Similarly, the simultaneous mutation of the E-box C and N-box had no effect on the repression of the 3′Atoh1 enhancer ($n = 2$, Hey1, and $n = 3$, Hes5). Data displayed as Mean + S.E.M.

damaged sensory epithelia. In the chick, damaged hair cells trigger supporting cells to replace lost hair cells by two different mechanisms: (1) mitotic regeneration, where SC divides asymmetrically and one daughter cell remains as SC and another as HC, and (2) transdifferentiation of SC into HC. In transdifferentiation, HCs are generated at the expense of SCs, which become exhausted and hence, the epithelium is disorganized. The consequence is that, although HCs are recovered, hearing function is not (Stone and Cotanche, 2007). In birds, hair cell regeneration starts with direct transdifferentiation of SCs into HCs, followed by mitotic regeneration and the correct replacement of the sensory epithelium and auditory function (Roberson et al., 2004).

Although mammals have some capacity to regenerate hair cells in the vestibular organs and the early post-natal cochlea, the adult auditory organ is completely devoid of this capacity. The question arises as to what are the differences between birds and mammals that explain their different regenerative capacity. Are there signals that regulate SC quiescence and activation after HC loss in chicken? Are they similar to mammalian early post-natal regeneration? Why mammals lose the capacity of regeneration after birth?

Studies in the chick have shown that hair cell regeneration reuses mechanisms that operate during embryonic development. Several molecular pathways known to regulate embryonic hair cell progenitors are reactivated in mature chicken epithelia after HC loss. Upon HC damage, Atoh1 becomes reactivated in transdifferentiating and mitotically active SCs (Cafaro et al., 2007). Atoh1 reactivation is essential to form new hair cells, like it is to form hair cells during development (Bermingham, 1999). Notch signaling is down-regulated upon damage in the basilar papilla suggesting that in the mature organ it maintains a repressive state that prevents Atoh1 expression. In agreement, different laboratories have shown that treatment with Notch inhibitors favors Atoh1 reactivation and HC regeneration in the chick basilar papilla and also in the post-natal mammalian cochlea under certain conditions (Cafaro et al., 2007; Mizutari et al., 2013). The ability of SC to respond to Notch blockade dramatically declines after birth, and is lost by post-natal day 6 (Maass et al., 2015).

Human stem cells constitute a reasonable alternative to replace damaged hair cells. Major problems of this approach are the difficulty to deliver treated cells to the damaged areas and their limited ability to integrate in the epithelium. Several groups have developed protocols to differentiate hair cells by mimicking the hair cell development in the embryo. Although this has proved successful, the efficiency of the procedures in hair cell regeneration is still very low (Chen et al., 2012; Ronaghi et al., 2014). In contrast to the low efficiency in replacing hair cells, stem cell therapy has proven surprisingly effective at restoring auditory neurons. The first reports of otic guidance with monolayer cultured human ESCs (hESCs) revealed a propensity to differentiate along an otic neurogenic lineage rather than HC lineage (Chen et al., 2012). Different attempts to generate HCs in culture commonly face the problem that most cells go to the neuronal cell fate, making it very difficult to enrich them in HCs (Chen et al., 2012; Ronaghi et al., 2014). This problem is directly related to the question addressed in the present work. During early stages of development, Neurog1 prevails over Atoh1, thereby forcing neurogenesis and delaying sensorigenesis. This suggests that the default fate is to become a neuron and that sensory competence is silenced. If this is so, the consequence is that production relies mainly on relieving the repression of hair cell competence rather than on the expression of activators. The cellular context of conditionally derived stem cells may be similar to that in the embryo and interference with Neurog1 may open a way to improve the efficiency of HC production.

In summary, understanding the developmental mechanisms involving interactions among cell-to-cell signals and transcription factors is crucial for designing strategies for hearing repair. Developmental studies have shown that the connection between FGF signaling and neurosensory commitment relies on the induction of SoxB1 factors, which set the expression of Neurog1 and Atoh1. The further interaction between Neurog1 and Atoh1 is crucial for neuronal and hair cell specification and for setting the timing for cell diversification. Notch operates at several stages during ear development, but one of them is linked to proneural gene expression and the irreversible commitment to neuronal and hair cell fate of only a fraction of the competent progenitors. Its function is crucial for maintaining dormant the potential of prosensory cells to develop as hair cells, and to keep the regenerative potential of supporting cells. Further work is required to better understand the details of the molecular mechanisms of Atoh1 and Neurog1 regulation, and the development and regeneration of neurons and hair cells.

ETHICS STATEMENT

The experiments reported in this paper were carried out on E2-E3 chick embryos according to national regulations following a protocol approved by the Ethics Committee of the PRBB.

AUTHOR CONTRIBUTIONS

HG, GA, and FG: Planning, executing, and writing.

FUNDING

La Marató and BFU2015-67400-P (MINECO/FEDER, UE).

ACKNOWLEDGMENTS

We are grateful to Joana Neves, Jelena Petrovic, Thomas Schimmang, Marta Ibañes, Juan Camilo Luna, Diogo Castro, and Domingos Henrique for their contribution. We like to thank Marta Linares and Sara Calatayud for technical assistance.

REFERENCES

Abdolazimi, Y., Stojanova, Z., and Segil, N. (2016). Selection of cell fate in the organ of Corti involves the integration of Hes/Hey signaling at the Atoh1 promoter. *Development* 143, 841–850. doi: 10.1242/dev.129320

Abello, G., and Alsina, B. (2007). Establishment of a proneural field in the inner ear. *Int. J. Dev. Biol.* 51, 483–493. doi: 10.1387/ijdb.072343ga

Abelló, G., Khatri, S., Giráldez, F., and Alsina, B. (2007). Early regionalization of the otic placode and its regulation by the Notch signaling pathway. *Mech. Dev.* 124, 631–645. doi: 10.1016/j.mod.2007.04.002

Abelló, G., Khatri, S., Radosevic, M., Scotting, P. J., Giráldez, F., and Alsina, B. (2010). Independent regulation of Sox3 and Lmx1b by FGF and BMP signaling influences the neurogenic and non-neurogenic domains in the chick otic placode. *Dev. Biol.* 339, 166–178. doi: 10.1016/j.ydbio.2009.12.027

Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D., et al. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development. *Development* 125, 4645–4654.

Ahmed, M., Wong, E. Y., Sun, J., Xu, J., Wang, F., and Xu, P. X. (2012a). Eya1-Six1 interaction is sufficient to induce hair cell fate in the cochlea by activating atoh1 expression in cooperation with Sox2. *Dev. Cell* 22, 377–390. doi: 10.1016/j.devcel.2011.12.006

Ahmed, M., Xu, J., and Xu, P. X. (2012b). EYA1 and SIX1 drive the neuronal developmental program in cooperation with the SWI/SNF chromatin-remodeling complex and SOX2 in the mammalian inner ear. *Development* 139, 1965–1977. doi: 10.1242/dev.071670

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S., and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. *J. Biol. Chem.* 270, 8730–8738. doi: 10.1074/jbc.270.15.8730

Ali, F., Hindley, C., McDowell, G., Deibler, R., Jones, A., Kirschner, M., et al. (2011). Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. *Development* 138, 4267–4277. doi: 10.1242/dev.067900

Alsina, B., Abelló, G., Ulloa, E., Henrique, D., Pujades, C., and Giraldez, F. (2004). FGF signaling is required for determination of otic neuroblasts in the chick embryo. *Dev. Biol.* 267, 119–134. doi: 10.1016/j.ydbio.2003.11.012

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch Signaling: cell fate control and signal integration in development. *Science* 284, 770–776. doi: 10.1126/science.284.5415.770

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jørgensen, H. F., John, R. M., et al. (2006). Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* 8, 532–538. doi: 10.1038/ncb1403

Bell, D., Streit, A., Gorospe, I., Varela-Nieto, I., Alsina, B., and Giraldez, F. (2008). Spatial and temporal segregation of auditory and vestibular neurons in the otic placode. *Dev. Biol.* 322, 109–120. doi: 10.1016/j.ydbio.2008.07.011

Bermingham, N. A. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* 284, 1837–1841. doi: 10.1126/science.284.5421.1837

Bertrand, N., Castro, D. S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* 3, 517–530. doi: 10.1038/nrn874

Bok, J., Raft, S., Kong, K. A., Koo, S. K., Dräger, U. C., and Wu, D. K. (2011). Transient retinoic acid signaling confers anterior-posterior polarity to the inner ear. *Proc. Natl. Acad. Sci. U.S.A.* 108, 161–166. doi: 10.1073/pnas.1010547108

Briggs, K. J., Corcoran-schwartz, I. M., Zhang, W., Harcke, T., Devereux, W. L., Baylin, S. B., et al. (2008). Tumor suppressors in medulloblastoma. *Genes Dev.* 22, 770–785. doi: 10.1101/gad.1640908

Cafaro, J., Lee, G. S., and Stone, J. S. (2007). Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. *Dev. Dyn.* 236, 156–170. doi: 10.1002/dvdy.21023

Chen, W., Jongkamonwiwat, N., Abbas, L., Eshtan, S. J., Johnson, S. L., Kuhn, S., et al. (2012). ES-cell-derived otic progenitors. *Nature* 490, 278–282. doi: 10.1038/nature11415

Cheng, Y. F., Tong, M., and Edge, A. S. (2016). Destabilization of atoh1 by e3 ubiquitin ligase huwe1 and casein kinase 1 is essential for normal sensory hair cell development. *J. Biol. Chem.* 291, 21096–21109. doi: 10.1074/jbc.m116.722124

D'Angelo, A., Bluteau, O., Garcia-Gonzalez, M. A., Gresh, L., Doyen, A., Garbay, S., et al. (2010). Hepatocyte nuclear factor 1alpha and beta control terminal differentiation and cell fate commitment in the gut epithelium. *Development* 137, 1573–1582.

Dabdoub, A., Puligilla, C., Jones, J. M., Fritzsch, B., Cheah, K. S., Pevny, L. H., et al. (2008). Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc. Natl. Acad. Sci. U.S.A.* 105, 18396–18401. doi: 10.1073/pnas.0808175105

Day, D. A., and Tuite, M. F. (1998). Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview. *J. Endocrinol.* 157, 361–371. doi: 10.1677/joe.0.1570361

Du, X., Li, W., Gao, X., West, M. B., Saltzman, W. M., Cheng, C. J., et al. (2013). Regeneration of mammalian cochlear and vestibular hair cells through Hes1/Hes5 modulation with siRNA. *Hear. Res.* 304, 91–110. doi: 10.1016/j.heares.2013.06.011

Ebert, P. J. (2003). Zic1 represses Math1 expression via interactions with the Math1 enhancer and modulation of Math1 autoregulation. *Development* 130, 1949–1959. doi: 10.1242/dev.00419

Eddison, M., Le Roux, I., and Lewis, J. (2000). Notch signaling in the development of the inner ear: lessons from Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11692–11699. doi: 10.1073/pnas.97.22.11692

Errico, A., Deshmukh, K., Tanaka, Y., Pozniakovsky, A., and Hunt, T. (2010). Identification of substrates for cyclin dependent kinases. *Adv. Enzyme Regul.* 50, 375–399. doi: 10.1016/j.advenzreg.2009.12.001

Evsen, L., Sugahara, S., Uchikawa, M., Kondoh, H., and Wu, D. K. (2013). Progression of neurogenesis in the inner ear requires inhibition of Sox2 transcription by neurogenin1 and neurod1. *J. Neurosci.* 33, 3879–3890. doi: 10.1523/JNEUROSCI.4030-12.2013

Fisher, A. L., Ohsako, S., and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Mol. Cell. Biol.* 16, 2670–2677. doi: 10.1128/MCB.16.6.2670

Forget, A., Bihannic, L., Cigna, S. M., Lefevre, C., Remke, M., Barnat, M., et al. (2014). Shh signaling protects atoh1 from degradation mediated by the e3ubiquitin ligase huwe1 in neural precursors. *Dev. Cell* 29, 649–661. doi: 10.1016/j.devcel.2014.05.014

Freeman, S. D., and Daudet, N. (2012). Artificial induction of Sox21 regulates sensory cell formation in the embryonic chicken inner ear. *PLoS ONE* 7:e46387. doi: 10.1371/journal.pone.0046387

Fritzsch, B., Beisel, K. W., and Hansen, L. A. (2006). The molecular basis of neurosensory cell formation in ear development: a blueprint for hair cell and sensory neuron regeneration? *Bioessays* 28, 1181–1193. doi: 10.1002/bies.20502

Groves, A. K., and Fekete, D. M. (2012). Shaping sound in space: the regulation of inner ear patterning. *Development* 139, 826–826. doi: 10.1242/dev.078257

Groves, A. K., Zhang, K. D., and Fekete, D. M. (2013). The genetics of hair cell development and regeneration. *Annu. Rev. Neurosci.* 36, 361–381. doi: 10.1146/annurev-neuro-062012-170309

Hardwick, L. J., and Philpott, A. (2015). Multi-site phosphorylation regulates NeuroD4 activity during primary neurogenesis: a conserved mechanism amongst proneural proteins. *Neural Dev.* 10:15. doi: 10.1186/s13064-015-0044-8

Hassan, B. A., and Bellen, H. J. (2000). Doing the MATH: is the mouse a good model for fly development? *Genes Dev.* 14, 1852–1865. doi: 10.1101/gad.14.15.1852

Heisig, J., Weber, D., Englberger, E., Winkler, A., Kneitz, S., Sung, W. K., et al. (2012). Target gene analysis by microarrays and chromatin immunoprecipitation identifies HEY proteins as highly redundant bHLH repressors. *PLoS Genet.* 8:e1002728. doi: 10.1371/journal.pgen.1002728

Helms, A. W., Abney, A. L., Ben-Arie, N., Zoghbi, H. Y., and Johnson, J. E. (2000). Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* 127, 1185–1196.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquié, O., Ish-Horowicz, D., et al. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr. Biol.* 7, 661–670. doi: 10.1016/S0960-9822(06)00293-4

Hindley, C., Ali, F., McDowell, G., Cheng, K., Jones, A., Guillemot, F., et al. (2012). Post-translational modification of Ngn2 differentially affects transcription of distinct targets to regulate the balance between progenitor maintenance and differentiation. *Development* 139, 1718–1723. doi: 10.1242/dev.077552

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., et al. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* 298, 840–843. doi: 10.1126/science.1074560

Hu, Z., and Wang, J. (2014). Histone deacetylase inhibitor induces the expression of select epithelial genes in mouse utricle sensory epithelia-derived progenitor cells. *Cell. Reprogram.* 16, 266–275. doi: 10.1089/cell.2013.0086

Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M., and Tsai, M. J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. *Mol. Cell. Biol.* 20, 3292–3307. doi: 10.1128/MCB.20.9.3292-3307.2000

Iso, T., Kedes, L., and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell. Physiol.* 194, 237–255. doi: 10.1002/jcp.10208

Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H. Y., Chung, G., et al. (2001). HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol. Cell. Biol.* 21, 6080–6089. doi: 10.1128/MCB.21.17.6080-6089.2001

Izumikawa, M., Minoda, R., Kawamoto, K., Abrashkin, K. A., Swiderski, D. L., Dolan, D. F., et al. (2005). Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. *Nat. Med.* 11, 271–276. doi: 10.1038/nm1193

Jahan, I., Pan, N., Kersigo, J., and Fritzsch, B. (2010). Neurod1 suppresses hair cell differentiation in ear ganglia and regulates hair cell subtype development in the cochlea. *PLoS ONE* 5:e11661. doi: 10.1371/journal.pone.0011661

Jarman, A. P., Grau, Y., Jan, L. Y., and Jan, Y. N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell* 73, 1307–1321. doi: 10.1016/0092-8674(93)90358-W

Jeon, S. J., Fujioka, M., Kim, S. C., and Edge, A. S. (2011). Notch signaling alters sensory or neuronal cell fate specification of inner ear stem cells. *J. Neurosci.* 31, 8351–8358. doi: 10.1523/JNEUROSCI.6366-10.2011

Kamachi, Y., and Kondoh, H. (2013). Sox proteins: regulators of cell fate specification and differentiation. *Development* 140, 4129–4144. doi: 10.1242/dev.091793

Kiernan, A. E., Pelling, A. L., Leung, K. K., Tang, A. S., Bell, D. M., Tease, C., et al. (2005). Sox2 is required for sensory organ development in the mammalian inner ear. *Nature* 434, 1031–1035. doi: 10.1038/nature03487

Kim, W. Y., Fritzsch, B., Serls, A., Bakel, L. A., Huang, E. J., Reichardt, L. F., et al. (2001). NeuroD-null mice are deaf due to a severe loss of the inner ear sensory neurons during development. *Development* 128, 417–426.

Knuckles, P., Vogt, M. A., Lugert, S., Milo, M., Chong, M. M., Hautbergue, G. M., et al. (2012). Drosha regulates neurogenesis by controlling neurogenin 2 expression independent of microRNAs. *Nat. Neurosci.* 15, 962–969. doi: 10.1038/nn.3139

Koyano-Nakagawa, N., Wettstein, D., and Kintner, C. (1998). Activation of Xenopus genes required for lateral inhibition and neuronal differentiation during primary neurogenesis. *Mol. Cell. Neurosci.* 14, 327–339. doi: 10.1006/mcne.1999.0783

Leimeister, C., Externbrink, A., Klamt, B., and Gessler, M. (1999). Hey genes: a novel subfamily of hairy- and Enhancer of split related genes specifically expressed during mouse embryogenesis. *Mech. Dev.* 85, 173–177. doi: 10.1016/S0925-4773(99)00080-5

Li, H., Kloosterman, W., and Fekete, D. M. (2010). MicroRNA-183 family members regulate sensorineural fates in the inner ear. *J. Neurosci.* 30, 3254–3263. doi: 10.1523/JNEUROSCI.4948-09.2010

Lin, V., Golub, J. S., Nguyen, T. B., Hume, C. R., Oesterle, E. C., and Stone, J. S. (2011). Inhibition of Notch activity promotes nonmitotic regeneration of hair cells in the adult mouse utricles. *J. Neurosci.* 31, 15329–15339. doi: 10.1523/JNEUROSCI.2057-11.2011

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L., and Anderson, D. J. (1998). Neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* 20, 469–482. doi: 10.1016/S0896-6273(00)80988-5

Ma, Q., Kintner, C., and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* 87, 43–52. doi: 10.1016/S0092-8674(00)81321-5

Maass, J. C., Gu, R., Basch, M. L., Waldhaus, J., Lopez, E. M., Xia, A., et al. (2015). Changes in the regulation of the Notch signaling pathway are temporally correlated with regenerative failure in the mouse cochlea. *Front. Cell. Neurosci.* 9:110. doi: 10.3389/fncel.2015.00110

Mak, A. C., Szeto, I. Y., Fritzsch, B., and Cheah, K. S. (2009). Differential and overlapping expression pattern of SOX2 and SOX9 in inner ear development. *Gene Expr. Patterns* 9, 444–453. doi: 10.1016/j.gep.2009.04.003

Massari, M. E., and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* 20, 429–440. doi: 10.1128/MCB.20.2.429-440.2000

Matei, V., Pauley, S., Kaing, S., Rowitch, D., Beisel, K. W., Morris, K., et al. (2005). Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. *Dev. Dyn.* 234, 633–650. doi: 10.1002/dvdy.20551

Millimaki, B. B., Sweet, E. M., Dhason, M. S., and Riley, B. B. (2007). Zebrafish atoh1 genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. *Development* 134, 295–305. doi: 10.1242/dev.02734

Mizutari, K., Fujioka, M., Hosoya, M., Bramhall, N., Okano, H. J., Okano, H., et al. (2013). Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after Acoustic Trauma. *Neuron* 77, 58–69. doi: 10.1016/j.neuron.2012.10.032

Mulvaney, J., and Dabdoub, A. (2012). Atoh1, an essential transcription factor in neurogenesis and intestinal and inner ear development: function, regulation, and context dependency. *J. Assoc. Res. Otolaryngol.* 13, 281–293. doi: 10.1007/s10162-012-0317-4

Murray, R. C., Tapscott, S. J., Petersen, J. W., Calof, A. L., and McCormick, M. B. (2000). A fragment of the Neurogenin1 gene confers regulated expression of a reporter gene *in vitro* and *in vivo*. *Dev. Dyn.* 218, 189–194. doi: 10.1002/(SICI)1097-0177(200005)218:1<189::AID-DVDY16>3.0.CO%-4

Mutoh, H., Sakamoto, H., Hayakawa, H., Arao, Y., Satoh, K., Nokubi, M., et al. (2006). The intestine-specific homeobox gene Cdx2 induces expression of the basic helix-loop-helix transcription factor Math1. *Differentiation* 74, 313–321. doi: 10.1111/j.1432-0436.2006.00074.x

Nakada, Y., Parab, P., Simmons, A., Omer-abdalla, A., and Johnson, J. E. (2004). Separable enhancer sequences regulate the expression of the neural bHLH transcription factor neurogenin 1. *Dev. Biol.* 271, 479–487. doi: 10.1016/j.ydbio.2004.04.021

Neves, J., Abelló, G., Petrovic, J., and Giraldez, F. (2013). Patterning and cell fate in the inner ear: a case for Notch in the chicken embryo. *Dev. Growth Differ.* 55, 96–112. doi: 10.1111/dgd.12016

Neves, J., Kamaid, A., Alsina, B., and Giraldez, F. (2007). Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick. *J. Comp. Neurol.* 503, 487–500. doi: 10.1002/cne.21299

Neves, J., Parada, C., Chamizo, M., and Giráldez, F. (2011). Jagged 1 regulates the restriction of Sox2 expression in the developing chicken inner ear: a mechanism for sensory organ specification. *Development* 138, 735–744. doi: 10.1242/dev.060657

Neves, J., Uchikawa, M., Bigas, A., and Giraldez, F. (2012). The prosensory function of Sox2 in the chicken inner ear relies on the direct regulation of Atoh1. *PLoS ONE* 7:e30871. doi: 10.1371/journal.pone.0030871

Ono, K., Kita, T., Sato, S., O'Neill, P., Mak, S. S., Paschaki, M. Ito, M., et al. (2014). FGFR1-Frs2/3 signalling maintains sensory progenitors during inner ear hair cell formation. *PLoS Genet.* 10:e1004118. doi: 10.1371/journal.pgen.1004118

Petrovic, J., Formosa-Jordan, P., Luna-Escalante, J. C., Abelló, G., Ibañes, M., Neves, J., et al. (2014). Ligand-dependent Notch signaling strength orchestrates lateral induction and lateral inhibition in the developing inner ear. *Development* 141, 2313–2324. doi: 10.1242/dev.108100

Petrovic, J., Gálvez, H., Neves, J., Abelló, G., and Giraldez, F. (2015). Differential regulation of Hes/Hey genes during inner ear development. *Dev. Neurobiol.* 75, 703–720. doi: 10.1002/dneu.22243

Puligilla, C., and Kelley, M. W. (2017). Dual role for Sox2 in specification of sensory competence and regulation of Atoh1 function. *Dev. Neurobiol.* 77, 3–13. doi: 10.1002/dneu.22401

Quan, X. J., Yuan, L., Tiberi, L., Claeys, A., De Geest, N., Yan, J., et al. (2016). Post-translational control of the temporal dynamics of transcription factor activity regulates neurogenesis. *Cell* 164, 460–475. doi: 10.1016/j.cell.2015.12.048

Radosevic, M., Robert-Moreno, A., Coolen, M., Bally-Cuif, L., and Alsina, B. (2011). Her9 represses neurogenic fate downstream of Tbx1 and retinoic acid signaling in the inner ear. *Development* 138, 397–408. doi: 10.1242/dev.056093

Raft, S., Koundakjian, E. J., Quinones, H., Jayasena, C. S., Goodrich, L. V., Johnson, J. E., et al. (2007). Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells during inner ear development. *Development* 134, 4405–4415. doi: 10.1242/dev.009118

Ren, H., Guo, W., Liu, W., Gao, W., Xie, D., Yin, T., et al. (2016). DAPT mediates atoh1 expression to induce hair cell-like cells. *Am. J. Transl. Res.* 8, 634–643.

Roberson, D. W., Alosi, J. A., and Cotanche, D. A. (2004). Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. *J. Neurosci. Res.* 78, 461–471. doi: 10.1002/jnr.20271

Ronaghi, M., Nasr, M., Ealy, M., Durruthy-Durruthy, R., Waldhaus, J., Diaz, G. H., et al. (2014). Inner ear hair cell-like cells from human embryonic stem cells. *Stem Cells Dev.* 23, 1275–1284. doi: 10.1089/scd.2014.0033

Sapède, D., Dyballa, S., and Pujades, C. (2012). Cell lineage analysis reveals three different progenitor pools for neurosensory elements in the otic vesicle. *J. Neurosci.* 32, 16424–16434. doi: 10.1523/JNEUROSCI.3686-12.2012

Satoh, T., and Fekete, D. M. (2005). Clonal analysis of the relationships between mechanosensory cells and the neurons that innervate them in the chicken ear. *Development* 132, 1687–1697. doi: 10.1242/dev.01730

Schneider-Maunoury, S., and Pujades, C. (2007). Hindbrain signals in otic regionalization: walk on the wild side. *Int. J. Dev. Biol.* 51, 495–506. doi: 10.1387/ijdb.072345ss

Shi, F., Cheng, Y. F., Wang, X. L., and Edge, A. S. (2010). Beta-catenin up-regulates Atoh1 expression in neural progenitor cells by interaction with an Atoh1 3' enhancer. *J. Biol. Chem.* 285, 392–400. doi: 10.1074/jbc.M109.059055

Simmons, A. D., Horton, S., Abney, A. L., and Johnson, J. E. (2001). *Neurogenin2* expression in ventral and dorsal spinal neural tube progenitor cells is regulated by distinct enhancers. *Dev. Biol.* 229, 327–339. doi: 10.1006/dbio.2000.9984

Sommer, L., Ma, Q., and Anderson, D. J. (1996). *Neurogenins*, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* 8, 221–241. doi: 10.1006/mcne.1996.0060

Stojanova, Z. P., Kwan, T., and Segil, N. (2015). Epigenetic regulation of Atoh1 guides hair cell development in the mammalian cochlea. *Development* 142, 3529–3536. doi: 10.1242/dev.126763

Stone, J. S., and Cotanche, D. A. (2007). Hair cell regeneration in the avian auditory epithelium. *Int. J. Dev. Biol.* 51, 633–647. doi: 10.1387/ijdb.072408js

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M. Z., Zubiaga, A., Hua, X., et al. (2001). Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 104, 365–376. doi: 10.1016/S0092-8674(01)00224-0

Sweet, E. M., Vemaraju, S., and Riley, B. B. (2011). Sox2 and Fgf interact with Atoh1 to promote sensory competence throughout the zebrafish inner ear. *Dev. Biol.* 358, 113–121. doi: 10.1016/j.ydbio.2011.07.019

Tateya, T., Imayoshi, I., Tateya, I., Ito, J., and Kageyama, R. (2011). Cooperative functions of Hes/Hey genes in auditory hair cell and supporting cell development. *Dev. Biol.* 352, 329–340. doi: 10.1016/j.ydbio.2011.01.038

Weston, M. D., Pierce, M. L., Rocha-Sanchez, S., Beisel, K. W., and Soukup, G. A. (2006). MicroRNA gene expression in the mouse inner ear. *Brain Res.* 1111, 95–104. doi: 10.1016/j.brainres.2006.07.006

White, P. M., Doetzlhofer, A., Lee, Y. S., Groves, A. K., and Segil, N. (2006). Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature* 441, 984–987. doi: 10.1038/nature04849

Zhao, H., Ayrault, O., Zindy, F., Kim, J. H., and Roussel, M. F. (2008). Post-transcriptional down-regulation of Atoh1/Math1 by bone morphogenic proteins suppresses medulloblastoma development. *Genes Dev.* 22, 722–727. doi: 10.1101/gad.1636408

Zheng, W., Huang, L., Wei, Z. B., Silvius, D., Tang, B., and Xu, P. X. (2003). The role of Six1 in mammalian auditory system development. *Development* 130, 3989–4000. doi: 10.1242/dev.00628

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 30 November 2016
doi: 10.3389/fcell.2016.00138

WNT/β-Catenin Signaling in Vertebrate Eye Development

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The vertebrate eye is a highly specialized sensory organ, which is derived from the anterior neural plate, head surface ectoderm, and neural crest-derived mesenchyme. The single central eye field, generated from the anterior neural plate, divides to give rise to the optic vesicle, which evaginates toward the head surface ectoderm. Subsequently, the surface ectoderm, in conjunction with the optic vesicle invaginates to form the lens vesicle and double-layered optic cup, respectively. This complex process is controlled by transcription factors and several intracellular and extracellular signaling pathways including WNT/β-catenin signaling. This signaling pathway plays an essential role in multiple developmental processes and has a profound effect on cell proliferation and cell fate determination. During eye development, the activity of WNT/β-catenin signaling is tightly controlled. Faulty regulation of WNT/β-catenin signaling results in multiple ocular malformations due to defects in the process of cell fate determination and differentiation. This mini-review summarizes recent findings on the role of WNT/β-catenin signaling in eye development. Whilst this mini-review focuses on loss-of-function and gain-of-function mutants of WNT/β-catenin signaling components, it also highlights some important aspects of β-catenin-independent WNT signaling in the eye development at later stages.

Keywords: retina, WNT, β-catenin, development, differentiation

OPEN ACCESS

Edited by:
Andrea Erika Münsterberg, University of East Anglia, UK

Reviewed by:
Paola Bovolenta, Spanish National Research Council, Spain
Gunnar Schulte, Karolinska Institutet, Sweden
Andrea Streit, King's College London, UK

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *24 August 2016*
Accepted: *09 November 2016*
Published: *30 November 2016*

Citation:
Fujimura N (2016) WNT/β-Catenin Signaling in Vertebrate Eye Development
Front. Cell Dev. Biol. 4:138.
doi: 10.3389/fcell.2016.00138

OVERVIEW OF EYE DEVELOPMENT IN MICE

During gastrulation, the eye field, a group of the retinal precursor cells, is specified within the anterior neural plate. At this stage, these cells are anteriorly and laterally surrounded by the telencephalic progenitor cells. Subsequently, the eye field is divided into two lateral parts, which extend toward the surface ectoderm and give rise to the optic vesicle (**Figure 1A**; Inoue et al., 2000; Cavodeassi and Houart, 2012; Heavner and Pevny, 2012). The head surface ectoderm thickens to give rise to the lens placode while the optic vesicle subdivides into three parts, namely the presumptive retinal pigment epithelium (RPE), the presumptive neural retina, and the presumptive optic stalk (**Figure 1B**). The optic vesicle subsequently invaginates together with the lens placode to form the double-layered optic cup (**Figure 1C**). The inner part of the optic cup gives rise to the neural retina, meanwhile the outer layer forms the RPE. The ciliary margin (peripheral part of the optic cup) develops to generate the iris and the ciliary body. The lens placodes progresses to form a hollow lens vesicle. Cells in the posterior region differentiate as primary lens fiber cells and elongate to fill the cavity, while the cells in the anterior region become proliferative lens epithelial cells (**Figure 1D**; Fuhrmann, 2008; Cvekl and Ashery-Padan, 2014; Fuhrmann et al., 2014). The retinal vessels arise from the optic nerve head shortly after birth and extend radically to the retinal periphery in the superficial retina. The vasculature then sprouts ventrally to form the deep vascular layer (Gariano and Gardner, 2005).

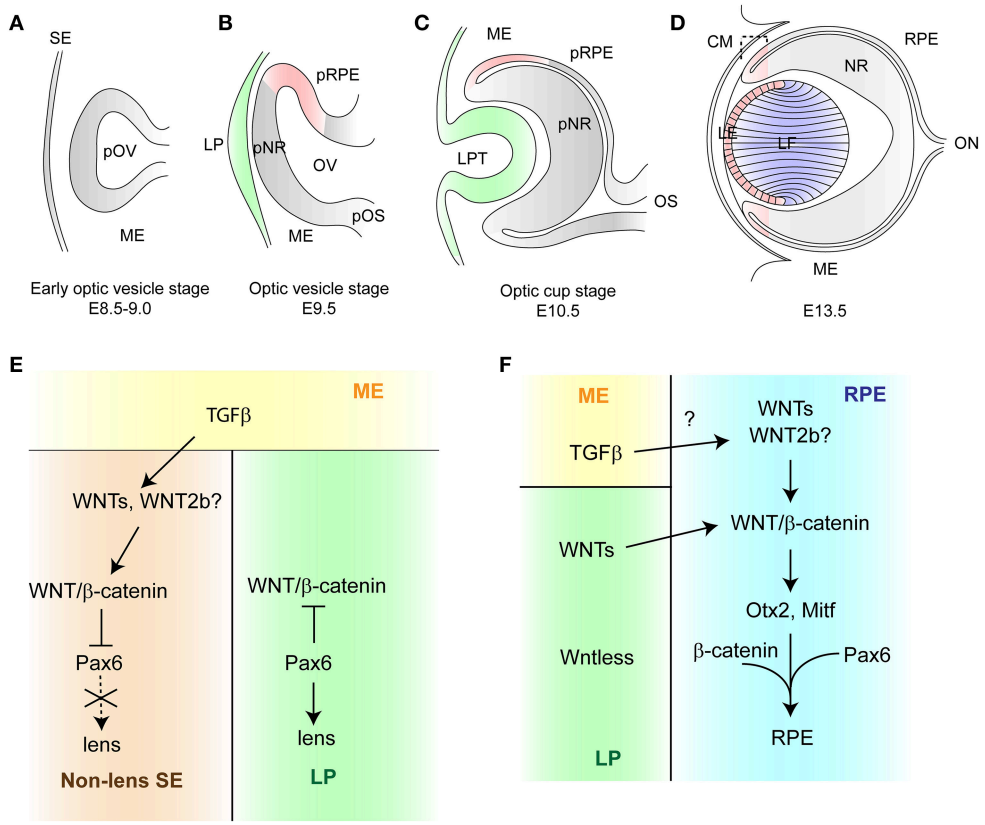

FIGURE 1 | Schematic diagram of vertebrate eye development (A). The early optic vesicle stage (E8.5–9.0). The presumptive optic vesicle envaginates toward the head surface ectoderm through the mesenchyme. **(B)** The optic vesicle stage (E9.5). As the optic vesicle comes into contact with the head surface ectoderm, it becomes partitioned into three domains: a dorsal, a distal and a proximal domain, which give rise to the retinal pigment epithelium, the neural retina and the optic stalk, respectively. The head surface ectoderm thickens to form the lens placode. **(C)** The optic cup stage (E10.5). The optic vesicle invaginates in coordination with the lens placode to form the optic cup and the lens pit. **(D)** The closure of the lens vesicle (E13.5). The cells located at the posterior lens vesicle elongate anteriorly to fill the cavity and differentiate as primary lens fiber cells. The cells in the anterior part of lens vesicle give rise to lens epithelial cells which migrate posteriorly to the equator and differentiate as secondary lens fiber cells. Pink color represents the region where the activity of WNT/β-catenin signaling is active, green shows the source of WNTs, blue indicates the region where WNT/PCP signaling is active. **(E, F)** Schematic representation of WNT/β-catenin signaling in the early lens development and in the RPE development, respectively. E. The periocular mesenchyme secretes TGFβ, which signals to the non-lens surface ectoderm. *WNT2b* is induced by TGFβ and activates WNT/β-catenin signaling in order to suppress the lens fate by repressing expression of *Pax6*. In the lens placode, WNT/β-catenin is inhibited by *Pax6* which initiates lens development. **(F)** The surface ectoderm secretes WNTs which activate WNT/β-catenin signaling in the RPE. This signaling induces expression of *Otx2* and *Mitf* which in cooperation with *Pax6* control the RPE developments.

WNT SIGNALING

WNTs can couple to various receptors and trigger different downstream signaling cascades including the non-canonical WNT/planar cell polarity (PCP), WNT/Ca^{2+}, and the canonical WNT/β-catenin signaling pathway, the focus of this review. WNT/β-catenin signaling is initiated by binding of the WNTs to the Frizzled/LRP5/6 receptor complex, which leads to the accumulation of β-catenin and nuclear translocation. In the nucleus, β-catenin interacts with the TCF/LEF family of transcription factors and regulates their target genes. In the absence of WNTs, β-catenin is phosphorylated by a "destruction complex" composed of multiple proteins, including AXIN2 and GSK3β, and targeted for degradation (Loh et al., 2016). In addition to its critical role as a transcriptional co-activator, β-catenin acts as a central component of the adherens junction by forming a link between cadherins and the actin cytoskeleton (Heuberger and Birchmeier, 2010). WNT/PCP signaling does not use β-catenin, but activates the Rho family GTPases and JNK pathway, which results in changes in cytoskeleton and cell polarity (Loh et al., 2016). WNT signaling is modulated by a number of WNT-sequestering proteins, such as DKKs and SFRPs, which prevent ligand-receptor interactions (Cruciat and Niehrs, 2013).

Abbreviations: pOV, presumptive optic vesicle; OV, optic vesicle; SE, head surface ectoderm; ME, extraocular mesenchyme; pRPE, presumptive retinal pigment epithelium; pNR, presumptive neural retina; pOS, presumptive optic stalk; LP, lens placode; RPE, retinal pigment epithelium; LPT, lens pit; OS, optic stalk; CM, ciliary margin; LE, lens epithelium; ON, optic nerve.

THE LENS

WNT signaling plays essential roles in eye organogenesis (Fuhrmann, 2008). During lens development, WNT/β-catenin signaling is active in the periocular surface ectoderm and lens epithelium (Stump et al., 2003; Smith et al., 2005; Kreslova et al., 2007; Machon et al., 2010; Carpenter et al., 2015). Conditional deletion of β-*catenin* in the presumptive lens placode and surrounding head surface ectoderm results in abnormal lens morphogenesis due to cell-cell adhesion defects. Conversely, the lens induction in the β-*catenin* loss-of-function mutant is not affected as expression of lens-specific markers is maintained (Smith et al., 2005). Consistently, a null mutation in *Lrp6*, which is expressed throughout the eye at the optic vesicle stage, does not have a profound effect on the lens induction (Stump et al., 2003; Smith et al., 2005). Interestingly, ectopic lentoid bodies are formed in the periocular surface ectoderm, where WNT/β-catenin signaling is inactivated in β-*catenin*-deficient mutants. Although the adherens junction is disrupted, ectopic lentoid bodies are not observed in the *E-cadherin*/*N-cadherin* or *Scribs* conditional knockout mice generated using the same Cre line (Pontoriero et al., 2009; Yamben et al., 2013). Thus, formation of ectopic lentoid bodies is mediated by the inactivation of WNT/β-catenin signaling rather than by cell-cell adhesion defects. In addition, ectopic activation of WNT/β-catenin signaling by expression of constitutively active β-catenin leads to inhibition of the lens formation (Smith et al., 2005; Machon et al., 2010). Taken together, WNT/β-catenin signaling is not required for the lens fate determination, however it inhibits the lens formation and appears to suppress the lens fate in the periocular ectoderm. The precise regulation of WNT/β-catenin signaling is required to ensure the correct patterning of the ocular tissue.

WNT/β-catenin signaling is regulated by TGFβ signaling and *Pax6* in the surface ectoderm at the optic vesicle stage (**Figure 1E**). The migrating neural crest cells inhibit the lens specification, while their ablation results in ectopic lens formation (Bailey et al., 2006). In chick embryos, the neural crest cells secrete multiple TGFβs which activate WNT/β-catenin signaling by inducing *WNT2b* in the adjacent non-lens ectoderm. The lens fate in presumptive lens ectoderm explants can be suppressed by the neural crest, constitutively active β-catenin, as well as TGFβ. Interestingly, the expression of lens markers is restored when these explants are cultured with TGFβ and WNT-sequestering protein FZD8-CRD, a truncated and soluble form of the WNT receptor. This indicates that lens suppression by the neural crest-derived TGFβ is dependent on WNT/β-catenin signaling (Grocott et al., 2011). *WNT2b* null mice display no ocular defects and multiple WNTs are expressed in the surface ectoderm, therefore additional WNTs are required for the process in mice (Tsukiyama and Yamaguchi, 2012; Carpenter et al., 2015).

Pax6 is expressed in the presumptive lens placode and *Pax6* null mutation results in failure of the lens formation (Hill et al., 1991; Grindley et al., 1995). It has been shown that *Pax6* regulates the expression of *Sfrp2*, and *Dkk1*. In *Pax6*-deficient presumptive lens placode, *Sfrp2* is down-regulated and WNT/β-catenin signaling is ectopically activated (Machon et al., 2010). However, it is unlikely that *Sfrp2* acts as a downstream effector as lens induction is not affected in the $Sfrp1^{-/-}$; $Sfrp2^{-/-}$ mice (Sugiyama et al., 2013). On the other hand, the role of *Dkk1* in the lens induction remains elusive as *Dkk1* null embryos lack the anterior head structure including the eyes (Mukhopadhyay et al., 2001). Interestingly, PAX6 ChIP sequencing using human neuroectodermal cells has shown that PAX6 binds to a variety of genes, which regulate WNT signaling (Bhinge et al., 2014). Further studies are necessary to understand how *Pax6* counteracts WNT/β-catenin signaling.

At later stages of development, WNT/β-catenin signaling is required for the formation and maintenance of the lens epithelium (Stump et al., 2003; Cain et al., 2008; Martinez et al., 2009). Interestingly, WNT/β-catenin signaling is reduced in the lens epithelium of the $Sfrp1^{-/-}$; $Sfrp2^{-/-}$ embryos (Sugiyama et al., 2013). SFRP1/2 are primarily characterized as WNT-sequestering proteins, however they can activate WNT/β-catenin signaling by facilitating the diffusion of WNTs or suppressing WNT/PCP pathway which can antagonize WNT/β-catenin signaling (Satoh et al., 2008; Mii and Taira, 2009). Additionally, *Sfrp1/2* can also inhibit BMP and Notch signaling, which are required for lens development, thus mis-regulation of these signaling pathways might also be responsible for the defects in the *Sfrp1/2*-deficient lens (Misra and Matise, 2010; Esteve et al., 2011a).

Although WNT/β-catenin signaling is not required for the lens fiber development, there are indications that the alignment and orientation of lens fiber cells are dependent on the WNT/PCP signaling pathways (Chen et al., 2008; Sugiyama et al., 2010, 2011). In the lens overexpressing *Sfrp2,* the fiber orientation is severely disrupted and expression of components of the WNT/PCP pathway is down-regulated (Chen et al., 2008; Sugiyama et al., 2010). WNT5, which activates the PCP pathway is secreted from the lens epithelium and WNT5 promotes the directed behavior of lens fiber cells in the lens explants (Dawes et al., 2014).

THE RPE

Signals from neighboring tissues are crucial for the accurate specification of the neural retina and the RPE within the optic vesicle. The dorsal optic vesicle receives signals from the extraocular mesenchyme and the head surface ectoderm to differentiate into the RPE (Fuhrmann et al., 2000; Martínez-Morales et al., 2004; Bharti et al., 2006; Steinfeld et al., 2013; Carpenter et al., 2015). During retinal development, WNT/β-catenin signaling is active in the dorsal optic vesicle which gives rise to presumptive RPE at the optic vesicle stage and is subsequently restricted to the peripheral RPE (Liu et al., 2006; Fujimura et al., 2009; Westenskow et al., 2009; Hägglund et al., 2013). The RPE transdifferentiates into the neural retina in the β-*catenin*-deficient RPE at the optic cup stage, as evidenced by loss of the RPE markers *Mitf* and *Otx2* and by the ectopic expression of neural retinal markers, such as *Chx10* and *Rax* (Fujimura et al., 2009; Westenskow et al., 2009; Hägglund et al., 2013). The β-catenin-deficient RPE preserves intact adherens junctions at the optic cup stage, although

cell-cell adhesion is disrupted at later stages (Fujimura et al., 2009; Westenskow et al., 2009). Interestingly, γ-catenin, a paralog of β-catenin, can substitute β-catenin in cell adhesion complexes in various developmental contexts (Huelsken et al., 2000; Posthaus et al., 2002; Zhou et al., 2007). The lack of β-catenin in the adherens junctions might be compensated by γ-catenin as evidenced by the presence of γ-catenin in the β-catenin-deficient RPE at the optic cup stage. Thus, the transdifferentiation is probably caused by loss of WNT/β-catenin signaling (Fujimura et al., 2009). A similar phenomenon is observed in the optic cup derived from the mouse embryonic stem cell aggregates *in vitro* (Eiraku et al., 2011). Treatment with a WNT secretion inhibitor reduces the number of the RPE cells, while WNT3a promotes the RPE differentiation and suppresses the neural retina generation (Eiraku et al., 2011). Interestingly, ectopic activation of WNT/β-catenin signaling in the entire RPE also results in disruption of the RPE patterning. The peripheral RPE remains normal, while the central part, in which WNT/β-catenin signaling is ectopically active, loses expression of the RPE markers. In contrast to β-*catenin*-deficient mutants, the RPE is not transdifferentiated to the neural retina (Fujimura et al., 2009). Thus, the activity of WNT/β-catenin signaling is spatially and temporally regulated during the RPE development.

WNT/β-catenin signaling regulates RPE development in cooperation with *Mitf*, *Otx2*, and *Pax6* (**Figure 1F**). Expression of *Mitf* and *Otx2* is directly regulated by WNT/β-catenin signaling (Fujimura et al., 2009; Westenskow et al., 2009). Furthermore, ectopic expression of both *Otx2* and β-*catenin* in the presumptive chick neural retina promotes the RPE fate while the ectopic expression of *Otx2* or β-*catenin* alone is not sufficient. Therefore, β-*catenin,* together with *Otx2,* induces a change in cell fate from retinal progenitor cells to the presumptive RPE (Westenskow et al., 2010). Furthermore, β-catenin directly interacts with MITF and promotes *Mitf*-mediated transcription (Schepsky et al., 2006). A recent study has shown that PAX6 acts in synergy with β-catenin and MITF to activate the promoters of melanogenic genes *Tyr* and *Trp-1* (Fujimura et al., 2015).

Although the identity of the specific WNTs involved in RPE development remains elusive, a recent study has shown that WNTs from the surface ectoderm are necessary for this process (Carpenter et al., 2015). During early eye development, the WNT transporter *Wntless* is expressed in the presumptive lens placode, the periocular surface ectoderm, the periocular mesenchyme at the optic vesicle stage, and it is also detected in the peripheral retina and the RPE at later stages (Carpenter et al., 2015). Conditional deletion of *Wntless* in the presumptive lens leads to inactivation of WNT/β-catenin signaling in the peripheral retina and periocular mesenchyme (Carpenter et al., 2015). Moreover, the number of RPE cells is reduced in *Wntless*-deficient mice (Carpenter et al., 2015). Despite the presence of multiple WNTs and *Wntless* in the periocular mesenchyme, conditional inactivation of *Wntless* in the periocular mesenchyme and RPE does not affect the eye development or the activity of WNT/β-catenin signaling (Carpenter et al., 2015). It remains elusive how WNTs disperse from the periocular mesenchyme to the WNT-responsive tissue in the optic cup. There are, however, indications that heparan sulfate proteoglycans (HSPG) are involved in the distribution of WNTs within the eye. HSPGs are located on the cell surface and in the extracellular matrix and have been implicated in a number of signaling pathways including WNT (Sarrazin et al., 2011). In the context of WNT signaling transduction, HSPGs play an essential role in organizing the extracellular distribution of WNTs and they maintain the activity of WNTs by preventing their aggregation in the extracellular environment (Fuerer et al., 2010; Matsuo and Kimura-Yoshida, 2014). Interestingly, conditional deletion of *Ext1,* a key HSPG synthetic enzyme, in the periocular mesenchyme leads to severe ocular malformations including the defects in the peripheral RPE development (Iwao et al., 2010). It has not been shown whether WNT/β-catenin signaling is affected in the peripheral optic cup of the *Ext1*-deficient mice, however *Ext1* is required for the activation of the WNT11/β-catenin pathway in *Xenopus* embryos (Tao et al., 2005). Thus, HSPG in the periocular mesenchyme might mediate the distribution of WNTs from the surface ectoderm.

THE CILIARY MARGIN

WNT/β-catenin signaling is active in the developing ciliary margin or peripheral retina, but it is inactive in the central retina (Liu et al., 2003, 2007; Cho and Cepko, 2006). Several WNT signaling members, such as *WNT2b*, *Frizzled-4* (FZD_4), and *Lef1* are expressed in the ciliary margin (Trimarchi et al., 2009). Overexpression of a constitutively active form of β-catenin leads to the expansion of the ciliary margin at the expense of the central retina (Cho and Cepko, 2006; Liu et al., 2007; Trimarchi et al., 2009). In addition, *Axin2* null embryos display multiple ocular phenotypes including expansion of the ciliary margin (Alldredge and Fuhrmann, 2016).

Several studies indicate that WNT/β-catenin signaling activity in the peripheral retina is controlled by *Sfrp1/2*, *Foxg1*, and *Sox2* (Matsushima et al., 2011; Esteve et al., 2011b; Fotaki et al., 2013). As mentioned above, it has been suggested that WNT-sequestering proteins SFRP1/2 can activate WNT/β-catenin signaling (Bovolenta et al., 2008). In the $Sfrp1^{-/-}; Sfrp2^{-/-}$ embryos, this signaling is inactive in the peripheral retina, which displays neural retinal characteristics (Esteve et al., 2011b). Conversely, restriction of WNT/β-catenin signaling to the ciliary margin has been shown to be mediated by *Foxg1* and *Sox2* (Matsushima et al., 2011; Fotaki et al., 2013). In *Foxg1*−or *Sox2*-deficient retina, WNT/β-catenin signaling are up-regulated in the peripheral retina and the ciliary margin expands at the expense of the neural retina (Matsushima et al., 2011; Fotaki et al., 2013). *foxg1* suppresses WNT/β-catenin signaling by directly repressing the transcription of WNTs in the forebrain of zebrafish (Matsushima et al., 2011). SOX2 interferes with WNT/β-catenin signaling by binding β-catenin in the osteoblast lineage (Seo et al., 2011). Taken together, it is likely that multiple mechanisms control the activity of WNT/β-catenin signaling in the ciliary margin.

THE DORSO-VENTRAL PATTERNING IN THE OPTIC CUP

In addition to the correct patterning of the lens and the RPE development, WNT/β-catenin signaling is required for the maintenance of the dorsal retinal identity (Veien et al., 2008; Zhou et al., 2008; Hägglund et al., 2013). Conditional inactivation of β-*catenin* in the early optic cup results in the down-regulation of dorsal retinal markers, such as *Bmp4* and expansion of the ventral retinal markers, such as *Vax2* (Hägglund et al., 2013). Similarly, loss of *Lrp6* causes dorso-ventral patterning defects in the neural retina (Zhou et al., 2008). Consistently, the expression of dorsal retinal markers are attenuated in a transgenic fish which overexpresses *dkk1* or dominant-repressor form of *tcf3.* This phenotype is rescued by LiCl, which promotes the accumulation of cytoplasmic β-catenin by inhibiting GSK3β (Veien et al., 2008). Thus, the role of WNT/β-catenin signaling in the dorso-ventral patterning within the retina seems to be evolutionarily conserved.

THE RETINAL VASCULAR SYSTEM

WNT/β-catenin signaling plays an essential role in the retinal vascular development. In genetic disorders, such as Norrie disease and Familial Exudative Vitreoretinopathy, retinal hypovascularization is caused by loss-of-function mutations in the *Norrin disease protein (Norrin)*, *FZD*$_4$, or *LRP5* genes. Norrin contains separate binding sites for FZD$_4$ and for LRP5 (Ke et al., 2013). Activation of FZD$_4$/β-catenin signaling by Norrin requires the presence of either LRP5 or LRP6 (Ye et al., 2009). Although *Lrp5* can compensate for the loss of *Lrp6* (and vice versa) in the postnatal brain vasculature, *Lrp5* plays a major role and *Lrp6* plays a minor role in the retinal vascularization (Zhou et al., 2014; Huang et al., 2016). Norrin secreted from Müller glial cells binds to FZD$_4$ in the endothelial cells and regulates retinal vascular development (Xu et al., 2004; Junge et al., 2009; Ye et al., 2009; Wang et al., 2012). The retinal vascular defects caused by ablation of *Norrin* are rescued by stabilizing β-catenin, while ectopic expression of dominant negative *Tcf4* in the endothelial cells mimics the phenotype. This indicates that Norrin/FZD$_4$ signaling acts via β-catenin signaling (Zhou et al., 2014). In addition, WNT/β-catenin signaling in the retinal vascular system is regulated by the EST transcription factor *Erg,* which plays a critical role in vascular development and angiogenesis (Birdsey et al., 2015). *Erg* controls WNT/β-catenin signaling by promoting β-catenin stability and regulating transcription of *FZD*$_4$ (Birdsey et al., 2015).

β-catenin-independent WNT signaling pathway is also required for the retinal vascular system development (Stefater et al., 2011; Korn et al., 2014; Franco et al., 2016). The endothelial cells express preferentially non-canonical WNTs, such as *WNT5a* and *WNT11*. Conditional deletion of *Wntless* or *WNT5a* in the endothelial cells leads to significant decrease in vascular density due to excessive vessel regression (Korn et al., 2014; Franco et al., 2016).

CONCLUSION

The activity of WNT/β-catenin signaling is tightly regulated during eye development and mis-regulation of the signaling results in multiple ocular malformations due to defects in the process of cell fate determination and differentiation. Studies of conditional knockout mice of various members of the WNT/β-catenin signaling pathway indicate that WNT/β-catenin signaling is essential for eye development by controlling the correct patterning of the ocular tissue, promoting the differentiation of the retinal pigment epithelium, controlling the morphogenesis of the optic cup, and maintaining the dorsal retinal identity. Further research is necessary to clarify the mechanisms through which WNT/β-catenin signaling integrates into the genetic regulatory networks controlling the eye development in the vertebrate.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

FUNDING

This work was supported by the Ministry of Education, Youth and Sports of CR within the LQ1604 National Sustainability Program II (Project BIOCEV-FAR).

ACKNOWLEDGMENTS

I apologize to the investigators whose original work was not cited due to space constraints. I thank Drs. Z. Kozmik for advice, continuous support, and mentoring and N. Chambers and C. Pantzartzi for critical reading of the manuscript.

REFERENCES

Alldredge, A., and Fuhrmann, S. (2016). Loss of Axin2 causes ocular defects during mouse eye development. *Invest. Ophthalmol. Vis. Sci.* 57, 5253–5262. doi: 10.1167/iovs.15-18599

Bailey, A. P., Bhattacharyya, S., Bronner-Fraser, M., and Streit, A. (2006). Lens specification is the ground state of all sensory placodes, from which FGF promotes olfactory identity. *Dev. Cell* 11, 505–517. doi: 10.1016/j.devcel.2006.08.009

Bharti, K., Nguyen, M. T., Skuntz, S., Bertuzzi, S., and Arnheiter, H. (2006). The other pigment cell: specification and development of the pigmented epithelium of the vertebrate eye. *Pigment Cell Res.* 19, 380–394. doi: 10.1111/j.1600-0749.2006.00318.x

Bhinge, A., Poschmann, J., Namboori, S. C., Tian, X., Jia Hui Loh, S., Traczyk, A., et al. (2014). MiR-135b is a direct PAX6 target and specifies human neuroectoderm by inhibiting TGF-beta/BMP signaling. *EMBO J.* 33, 1271–1283. doi: 10.1002/embj.201387215

Birdsey, G. M., Shah, A. V., Dufton, N., Reynolds, L. E., Osuna Almagro, L., Yang, Y., et al. (2015). The endothelial transcription factor ERG promotes vascular stability and growth through Wnt/β-catenin signaling. *Dev. Cell* 32, 82–96. doi: 10.1016/j.devcel.2014.11.016

Bovolenta, P., Esteve, P., Ruiz, J. M., Cisneros, E., and Lopez-Rios, J. (2008). Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J. Cell Sci.* 121(Pt 6), 737–746. doi: 10.1242/jcs.026096

Cain, S., Martinez, G., Kokkinos, M. I., Turner, K., Richardson, R. J., Abud, H. E., et al. (2008). Differential requirement for beta-catenin in epithelial and fiber cells during lens development. *Dev. Biol.* 321, 420–433. doi: 10.1016/j.ydbio.2008.07.002

Carpenter, A. C., Smith, A. N., Wagner, H., Cohen-Tayar, Y., Rao, S., Wallace, V., et al. (2015). Wnt ligands from the embryonic surface ectoderm regulate 'bimetallic strip' optic cup morphogenesis in mouse. *Development* 142, 972–982. doi: 10.1242/dev.120022

Cavodeassi, F., and Houart, C. (2012). Brain regionalization: of signaling centers and boundaries. *Dev. Neurobiol.* 72, 218–233. doi: 10.1002/dneu.20938

Chen, Y., Stump, R. J., Lovicu, F. J., Shimono, A., and McAvoy, J. W. (2008). Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. *Dev. Biol.* 324, 161–176. doi: 10.1016/j.ydbio.2008.09.002

Cho, S. H., and Cepko, C. L. (2006). Wnt2b/β-catenin-mediated canonical Wnt signaling determines the peripheral fates of the chick eye. *Development* 133, 3167–3177. doi: 10.1242/dev.02474

Cruciat, C. M., and Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harb. Perspect. Biol.* 5:a015081. doi: 10.1101/cshperspect.a015081

Cvekl, A., and Ashery-Padan, R. (2014). The cellular and molecular mechanisms of vertebrate lens development. *Development* 141, 4432–4447. doi: 10.1242/dev.107953

Dawes, L. J., Sugiyama, Y., Lovicu, F. J., Harris, C. G., Shelley, E. J., and McAvoy, J. W. (2014). Interactions between lens epithelial and fiber cells reveal an intrinsic self-assembly mechanism. *Dev. Biol.* 385, 291–303. doi: 10.1016/j.ydbio.2013.10.030

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., et al. (2011). Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature* 472, 51–56. doi: 10.1038/nature09941

Esteve, P., Sandonis, A., Cardozo, M., Malapeira, J., Ibañez, C., Crespo, I., et al. (2011a). SFRPs act as negative modulators of ADAM10 to regulate retinal neurogenesis. *Nat. Neurosci.* 14, 562–569. doi: 10.1038/nn.2794

Esteve, P., Sandonis, A., Ibanez, C., Shimono, A., Guerrero, I., and Bovolenta, P. (2011b). Secreted frizzled-related proteins are required for Wnt/β-catenin signalling activation in the vertebrate optic cup. *Development* 138, 4179–4184. doi: 10.1242/dev.065839

Fotaki, V., Smith, R., Pratt, T., and Price, D. J. (2013). Foxg1 is required to limit the formation of ciliary margin tissue and Wnt/β-catenin signalling in the developing nasal retina of the mouse. *Dev. Biol.* 380, 299–313. doi: 10.1016/j.ydbio.2013.04.017

Franco, C. A., Jones, M. L., Bernabeu, M. O., Vion, A. C., Barbacena, P., Fan, J., et al. (2016). Non-canonical Wnt signalling modulates the endothelial shear stress flow sensor in vascular remodelling. *Elife* 5:e07727. doi: 10.7554/eLife.07727

Fuerer, C., Habib, S. J., and Nusse, R. (2010). A study on the interactions between heparan sulfate proteoglycans and Wnt proteins. *Dev. Dyn.* 239, 184–190. doi: 10.1002/dvdy.22067

Fuhrmann, S. (2008). Wnt signaling in eye organogenesis. *Organogenesis* 4, 60–67. doi: 10.4161/org.4.2.5850

Fuhrmann, S., Levine, E. M., and Reh, T. A. (2000). Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. *Development* 127, 4599–4609.

Fuhrmann, S., Zou, C., and Levine, E. M. (2014). Retinal pigment epithelium development, plasticity, and tissue homeostasis. *Exp. Eye Res.* 123, 141–150. doi: 10.1016/j.exer.2013.09.003

Fujimura, N., Klimova, L., Antosova, B., Smolikova, J., Machon, O., and Kozmik, Z. (2015). Genetic interaction between Pax6 and β-catenin in the developing retinal pigment epithelium. *Dev. Genes Evol.* 225, 121–128. doi: 10.1007/s00427-015-0493-4

Fujimura, N., Taketo, M. M., Mori, M., Korinek, V., and Kozmik, Z. (2009). Spatial and temporal regulation of Wnt/β-catenin signaling is essential for development of the retinal pigment epithelium. *Dev. Biol.* 334, 31–45. doi: 10.1016/j.ydbio.2009.07.002

Gariano, R. F., and Gardner, T. W. (2005). Retinal angiogenesis in development and disease. *Nature* 438, 960–966. doi: 10.1038/nature04482

Grindley, J. C., Davidson, D. R., and Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development* 121, 1433–1442.

Grocott, T., Johnson, S., Bailey, A. P., and Streit, A. (2011). Neural crest cells organize the eye via TGF-β and canonical Wnt signalling. *Nat. Commun.* 2, 265. doi: 10.1038/ncomms1269

Hägglund, A. C., Berghard, A., and Carlsson, L. (2013). Canonical Wnt/β-catenin signalling is essential for optic cup formation. *PLoS ONE* 8:e81158. doi: 10.1371/journal.pone.0081158

Heavner, W., and Pevny, L. (2012). Eye development and retinogenesis. *Cold Spring Harb. Perspect. Biol.* 4:a008391. doi: 10.1101/cshperspect.a008391

Heuberger, J., and Birchmeier, W. (2010). Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. *Cold Spring Harb. Perspect. Biol.* 2:a002915. doi: 10.1101/cshperspect.a002915

Hill, R. E., Favor, J., Hogan, B. L., Ton, C. C., Saunders, G. F., Hanson, I. M., et al. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* 354, 522–525. doi: 10.1038/354522a0

Huang, W., Li, Q., Amiry-Moghaddam, M., Hokama, M., Sardi, S. H., Nagao, M., et al. (2016). Critical endothelial regulation by LRP5 during retinal vascular development. *PLoS ONE* 11:e0152833. doi: 10.1371/journal.pone.0152833

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., and Birchmeier, W. (2000). Requirement for beta-catenin in anterior-posterior axis formation in mice. *J. Cell Biol.* 148, 567–578. doi: 10.1083/jcb.148.3.567

Inoue, T., Nakamura, S., and Osumi, N. (2000). Fate mapping of the mouse prosencephalic neural plate. *Dev. Biol.* 219, 373–383. doi: 10.1006/dbio.2000.9616

Iwao, K., Inatani, M., Ogata-Iwao, M., Yamaguchi, Y., Okinami, S., and Tanihara, H. (2010). Heparan sulfate deficiency in periocular mesenchyme causes microphthalmia and ciliary body dysgenesis. *Exp. Eye Res.* 90, 81–88. doi: 10.1016/j.exer.2009.09.017

Junge, H. J., Yang, S., Burton, J. B., Paes, K., Shu, X., French, D. M., et al. (2009). TSPAN12 regulates retinal vascular development by promoting Norrin- but not Wnt-induced FZD4/beta-catenin signaling. *Cell* 139, 299–311. doi: 10.1016/j.cell.2009.07.048

Ke, J., Harikumar, K. G., Erice, C., Chen, C., Gu, X., Wang, L., et al. (2013). Structure and function of Norrin in assembly and activation of a Frizzled 4-Lrp5/6 complex. *Genes Dev.* 27, 2305–2319. doi: 10.1101/gad.228544.113

Korn, C., Scholz, B., Hu, J., Srivastava, K., Wojtarowicz, J., Arnsperger, T., et al. (2014). Endothelial cell-derived non-canonical Wnt ligands control vascular pruning in angiogenesis. *Development* 141, 1757–1766. doi: 10.1242/dev.104422

Kreslova, J., Machon, O., Ruzickova, J., Lachova, J., Wawrousek, E. F., Kemler, R., et al. (2007). Abnormal lens morphogenesis and ectopic lens formation in the absence of beta-catenin function. *Genesis* 45, 157–168. doi: 10.1002/dvg.20277

Liu, H., Mohamed, O., Dufort, D., and Wallace, V. A. (2003). Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. *Dev. Dyn.* 227, 323–334. doi: 10.1002/dvdy.10315

Liu, H., Thurig, S., Mohamed, O., Dufort, D., and Wallace, V. A. (2006). Mapping canonical Wnt signaling in the developing and adult retina. *Invest. Ophthalmol. Vis. Sci.* 47, 5088–5097. doi: 10.1167/iovs.06-0403

Liu, H., Xu, S., Wang, Y., Mazerolle, C., Thurig, S., Coles, B. L., et al. (2007). Ciliary margin transdifferentiation from neural retina is controlled by canonical Wnt signaling. *Dev. Biol.* 308, 54–67. doi: 10.1016/j.ydbio.2007.04.052

Loh, K. M., van Amerongen, R., and Nusse, R. (2016). Generating cellular diversity and spatial form: Wnt signaling and the evolution of multicellular animals. *Dev. Cell* 38, 643–655. doi: 10.1016/j.devcel.2016.08.011

Machon, O., Kreslova, J., Ruzickova, J., Vacik, T., Klimova, L., Fujimura, N., et al. (2010). Lens morphogenesis is dependent on Pax6-mediated inhibition of the canonical Wnt/β-catenin signaling in the lens surface ectoderm. *Genesis* 48, 86–95. doi: 10.1002/dvg.20583

Martinez, G., Wijesinghe, M., Turner, K., Abud, H. E., Taketo, M. M., Noda, T., et al. (2009). Conditional mutations of β-catenin and APC reveal roles for canonical Wnt signaling in lens differentiation. *Invest. Ophthalmol. Vis. Sci.* 50, 4794–4806. doi: 10.1167/iovs.09-3567

Martínez-Morales, J. R., Rodrigo, I., and Bovolenta, P. (2004). Eye development: a view from the retina pigmented epithelium. *Bioessays* 26, 766–777. doi: 10.1002/bies.20064

Matsuo, I., and Kimura-Yoshida, C. (2014). Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 369:20130545. doi: 10.1098/rstb.2013.0545

Matsushima, D., Heavner, W., and Pevny, L. H. (2011). Combinatorial regulation of optic cup progenitor cell fate by SOX2 and PAX6. *Development* 138, 443–454. doi: 10.1242/dev.055178

Mii, Y., and Taira, M. (2009). Secreted Frizzled-related proteins enhance the diffusion of Wnt ligands and expand their signalling range. *Development* 136, 4083–4088. doi: 10.1242/dev.032524

Misra, K., and Matise, M. P. (2010). A critical role for sFRP proteins in maintaining caudal neural tube closure in mice via inhibition of BMP signaling. *Dev. Biol.* 337, 74–83. doi: 10.1016/j.ydbio.2009.10.015

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., et al. (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev. Cell* 1, 423–434. doi: 10.1016/S1534-5807(01)00041-7

Pontoriero, G. F., Smith, A. N., Miller, L. A., Radice, G. L., West-Mays, J. A., and Lang, R. A. (2009). Co-operative roles for E-cadherin and N-cadherin during lens vesicle separation and lens epithelial cell survival. *Dev. Biol.* 326, 403–417. doi: 10.1016/j.ydbio.2008.10.011

Posthaus, H., Williamson, L., Baumann, D., Kemler, R., Caldelari, R., Suter, M. M., et al. (2002). β-Catenin is not required for proliferation and differentiation of epidermal mouse keratinocytes. *J. Cell Sci.* 115(Pt 23), 4587–4595. doi: 10.1242/jcs.00141

Sarrazin, S., Lamanna, W. C., and Esko, J. D. (2011). Heparan sulfate proteoglycans. *Cold Spring Harb. Perspect. Biol.* 3:a004952. doi: 10.1101/cshperspect.a004952

Satoh, W., Matsuyama, M., Takemura, H., Aizawa, S., and Shimono, A. (2008). Sfrp1, Sfrp2, and Sfrp5 regulate the Wnt/beta-catenin and the planar cell polarity pathways during early trunk formation in mouse. *Genesis* 46, 92–103. doi: 10.1002/dvg.20369

Schepsky, A., Bruser, K., Gunnarsson, G. J., Goodall, J., Hallsson, J. H., Goding, C. R., et al. (2006). The microphthalmia-associated transcription factor Mitf interacts with β-catenin to determine target gene expression. *Mol. Cell. Biol.* 26, 8914–8927. doi: 10.1128/MCB.02299-05

Seo, E., Basu-Roy, U., Zavadil, J., Basilico, C., and Mansukhani, A. (2011). Distinct functions of Sox2 control self-renewal and differentiation in the osteoblast lineage. *Mol. Cell. Biol.* 31, 4593–4608. doi: 10.1128/MCB.05798-11

Smith, A. N., Miller, L. A., Song, N., Taketo, M. M., and Lang, R. A. (2005). The duality of beta-catenin function: a requirement in lens morphogenesis and signaling suppression of lens fate in periocular ectoderm. *Dev. Biol.* 285, 477–489. doi: 10.1016/j.ydbio.2005.07.019

Stefater, J. A. III, Lewkowich, I., Rao, S., Mariggi, G., Carpenter, A. C., Burr, A. R., et al. (2011). Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in myeloid cells. *Nature* 474, 511–515. doi: 10.1038/nature10085

Steinfeld, J., Steinfeld, I., Coronato, N., Hampel, M. L., Layer, P. G., Araki, M., et al. (2013). RPE specification in the chick is mediated by surface ectoderm-derived BMP and Wnt signalling. *Development* 140, 4959–4969. doi: 10.1242/dev.096990

Stump, R. J., Ang, S., Chen, Y., von Bahr, T., Lovicu, F. J., Pinson, K., et al. (2003). A role for Wnt/beta-catenin signaling in lens epithelial differentiation. *Dev. Biol.* 259, 48–61. doi: 10.1016/S0012-1606(03)00179-9

Sugiyama, Y., Lovicu, F. J., and McAvoy, J. W. (2011). Planar cell polarity in the mammalian eye lens. *Organogenesis* 7, 191–201. doi: 10.4161/org.7.3.18421

Sugiyama, Y., Shelley, E. J., Wen, L., Stump, R. J., Shimono, A., Lovicu, F. J., et al. (2013). Sfrp1 and Sfrp2 are not involved in Wnt/β-catenin signal silencing during lens induction but are required for maintenance of Wnt/beta-catenin signaling in lens epithelial cells. *Dev. Biol.* 384, 181–193. doi: 10.1016/j.ydbio.2013.10.008

Sugiyama, Y., Stump, R. J., Nguyen, A., Wen, L., Chen, Y., Wang, Y., et al. (2010). Secreted frizzled-related protein disrupts PCP in eye lens fiber cells that have polarised primary cilia. *Dev. Biol.* 338, 193–201. doi: 10.1016/j.ydbio.2009.11.033

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., et al. (2005). Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell* 120, 857–871. doi: 10.1016/j.cell.2005.01.013

Trimarchi, J. M., Cho, S. H., and Cepko, C. L. (2009). Identification of genes expressed preferentially in the developing peripheral margin of the optic cup. *Dev. Dyn.* 238, 2327–2329. doi: 10.1002/dvdy.21973

Tsukiyama, T., and Yamaguchi, T. P. (2012). Mice lacking Wnt2b are viable and display a postnatal olfactory bulb phenotype. *Neurosci. Lett.* 512, 48–52. doi: 10.1016/j.neulet.2012.01.062

Veien, E. S., Rosenthal, J. S., Kruse-Bend, R. C., Chien, C. B., and Dorsky, R. I. (2008). Canonical Wnt signaling is required for the maintenance of dorsal retinal identity. *Development* 135, 4101–4111. doi: 10.1242/dev.027367

Wang, Y., Rattner, A., Zhou, Y., Williams, J., Smallwood, P. M., and Nathans, J. (2012). Norrin/Frizzled4 signaling in retinal vascular development and blood brain barrier plasticity. *Cell* 151, 1332–1344. doi: 10.1016/j.cell.2012.10.042

Westenskow, P. D., McKean, J. B., Kubo, F., Nakagawa, S., and Fuhrmann, S. (2010). Ectopic Mitf in the embryonic chick retina by co-transfection of beta-catenin and Otx2. *Invest. Ophthalmol. Vis. Sci.* 51, 5328–5335. doi: 10.1167/iovs.09-5015

Westenskow, P., Piccolo, S., and Fuhrmann, S. (2009). Beta-catenin controls differentiation of the retinal pigment epithelium in the mouse optic cup by regulating Mitf and Otx2 expression. *Development* 136, 2505–2510. doi: 10.1242/dev.032136

Xu, Q., Wang, Y., Dabdoub, A., Smallwood, P. M., Williams, J., Woods, C., et al. (2004). Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. *Cell* 116, 883–895. doi: 10.1016/S0092-8674(04)00216-8

Yamben, I. F., Rachel, R. A., Shatadal, S., Copeland, N. G., Jenkins, N. A., Warming, S., et al. (2013). Scrib is required for epithelial cell identity and prevents epithelial to mesenchymal transition in the mouse. *Dev. Biol.* 384, 41–52. doi: 10.1016/j.ydbio.2013.09.027

Ye, X., Wang, Y., Cahill, H., Yu, M., Badea, T. C., Smallwood, P. M., et al. (2009). Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization. *Cell* 139, 285–298. doi: 10.1016/j.cell.2009.07.047

Zhou, C. J., Molotkov, A., Song, L., Li, Y., Pleasure, D. E., Pleasure, S. J., et al. (2008). Ocular coloboma and dorsoventral neuroretinal patterning defects in Lrp6 mutant eyes. *Dev. Dyn.* 237, 3681–3689. doi: 10.1002/dvdy.21770

Zhou, J., Qu, J., Yi, X. P., Graber, K., Huber, L., Wang, X., et al. (2007). Upregulation of gamma-catenin compensates for the loss of beta-catenin in adult cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* 292, H270–H276. doi: 10.1152/ajpheart.00576.2006

Zhou, Y., Wang, Y., Tischfield, M., Williams, J., Smallwood, P. M., Rattner, A., et al. (2014). Canonical WNT signaling components in vascular development and barrier formation. *J. Clin. Invest.* 124, 3825–3846. doi: 10.1172/JCI76431

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 05 April 2017
doi: 10.3389/fcell.2017.00031

PTK7 Faces the Wnt in Development and Disease

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PTK7 (protein tyrosine kinase 7) is an evolutionarily conserved transmembrane receptor regulating various processes in embryonic development and tissue homeostasis. On a cellular level PTK7 affects the establishment of cell polarity, the regulation of cell movement and migration as well as cell invasion. The PTK7 receptor has been shown to interact with ligands, co-receptors, and intracellular transducers of Wnt signaling pathways, pointing to a function in the fine-tuning of the Wnt signaling network. Here we will review recent findings implicating PTK7 at the crossroads of Wnt signaling pathways in development and disease.

Keywords: PTK7, Wnt signaling, planar cell polarity, cancer, neural tube defect, scoliosis

OPEN ACCESS

Edited by:
Sigmar Stricker,
Free University of Berlin, Germany

Reviewed by:
Madelon Maurice,
Utrecht University, Netherlands
Frank Thévenod,
Witten/Herdecke University, Germany

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *23 December 2016*
Accepted: *21 March 2017*
Published: *05 April 2017*

Citation:
Berger H, Wodarz A and Borchers A (2017) PTK7 Faces the Wnt in Development and Disease. Front. Cell Dev. Biol. 5:31. doi: 10.3389/fcell.2017.00031

INTRODUCTION

PTK7 (protein tyrosine kinase 7) is an evolutionarily conserved transmembrane receptor with a broad range of functions in tissue development and homeostasis. Originally identified as a gene upregulated in colon carcinoma cells and accordingly named colon carcinoma kinase 4 (CCK-4) (Mossie et al., 1995) it was later shown to affect various aspects of cell-cell communication and movement. PTK7 controls tissue morphogenesis and patterning by affecting cell polarity, migration as well as tissue regeneration and wound healing (Lu et al., 2004; Shnitsar and Borchers, 2008; Caddy et al., 2010; Lee et al., 2011; Lander and Petersen, 2016). Additionally its function in adult tissue homeostasis is demonstrated by the fact that misregulation of PTK7 expression correlates with development of cancer and its progression to metastasis in various cellular contexts (reviewed in Dunn and Tolwinski, 2016). Furthermore, mutations in PTK7 have been implicated in scoliosis and human neural tube closure defects, demonstrating its clinical relevance (Hayes et al., 2014; Wang et al., 2015; Grimes et al., 2016). Since the first publication on PTK7/CCK-4 (Mossie et al., 1995) more than 20 years ago over 120 publications have followed. Although its signaling function is still far from being understood, recent findings provide compelling evidence that PTK7 is a regulator of Wnt signaling pathways. In this review we will summarize recent findings and take a look at PTK7's function in distinct Wnt signaling pathways.

Secreted glycoproteins of the Wnt family are key regulators of development and disease. Wnt ligands regulate a wide range of processes including primary embryonic axis specification, organogenesis and stem cell proliferation. Further, deregulated Wnt signaling has been implicated in various diseases like colon and breast cancer, melanoma, and neurodegenerative disorders (MacDonald et al., 2009; Clevers and Nusse, 2012; Anastas and Moon, 2013; Inestrosa and Varela-Nallar, 2014). Wnt ligands activate distinct downstream signaling pathways, and historically the first described, ß-catenin-dependent, signaling cascade is referred to as the "canonical" Wnt signaling pathway, while later discovered, ß-catenin-independent pathways were termed "non-canonical." Canonical Wnt signaling (Logan and Nusse, 2004; MacDonald et al., 2009)

is activated by binding of the Wnt ligand to a receptor complex consisting of the seven-pass transmembrane Frizzled (Fz) receptor and the low-density lipoprotein receptor-related protein 6 (LRP6) (MacDonald and He, 2012). Wnt binding to the Fz/LRP6 receptor complex leads to inactivation of glycogen synthase kinase 3ß (GSK3ß) regulating various intracellular substrates. One of these is the transcriptional co-activator ß-catenin, which is phosphorylated and thereby targeted for proteasomal degradation. Thus, in the presence of Wnt ligands, ß-catenin is stabilized, enters the nucleus and regulates in combination with transcription factors of the Lef (lymphoid enhancer-binding factor) and Tcf (T cell factor) family the transcription of target genes. In contrast to canonical Wnt signaling, non-canonical Wnt signaling pathways encompass a complex network of signal transducers that do not activate ß-catenin, but use alternative modes of downstream signaling (reviewed in Niehrs, 2012). Here, we will focus on the planar cell polarity (PCP) pathway, as PTK7 has been implicated in its regulation.

The PCP pathway (Goodrich and Strutt, 2011; Yang and Mlodzik, 2015) determines the orientation of cells in the plane of an epithelium and is one of the best-characterized non-canonical Wnt signaling pathways. PCP was first described in *Drosophila*, where genetic screens discovered its function in the polarization of adult cuticular structures. According to mutant phenotypes showing wing hair polarity defects the genes *Frizzled* (Fz) and *Disheveled* (Dsh) were identified. Other core PCP proteins include the four-pass transmembrane protein Van Gogh (Vang, Strabismus), the atypical cadherin Flamingo (Fmi, Celsr) and intracellular components like Prickle (Pk) and Diego (Dgo). These proteins confer intra- and intercellular signaling, thereby aligning PCP in neighboring cells. Complementary studies in vertebrates revealed that these core PCP proteins are also required for the polarization of vertebrate tissues, like the orientation of hair follicles in the epidermis or the sensory hair cells in the inner ear (Montcouquiol et al., 2006; Simons and Mlodzik, 2008; Wallingford, 2012). Furthermore, these proteins are also involved in the polarized localization of cilia, microtubule-based protrusions that are found on the surface of most vertebrate cells and required for fluid movement during development and homeostasis (Wallingford, 2010; Wallingford and Mitchell, 2011). In addition to the polarization of tissues, loss of function studies using the mouse, zebrafish and *Xenopus* model systems demonstrated that PCP signaling also affects morphogenetic cell movements shaping the embryonic body. One of these is convergent extension, a cell movement whereby cells intercalate in a way that a tissue converges in one direction and extends in the perpendicular direction (Wallingford et al., 2002; Wallingford, 2012). Convergent extension is required to drive gastrulation and neural tube closure. Consequently, misregulation of PCP signaling leads to severe gastrulation and neurulation defects in mouse, zebrafish and *Xenopus* embryos. Since the discovery of vertebrate PCP phenotypes, these have also contributed to the identification of novel vertebrate regulators of PCP without previous knowledge of a *Drosophila* phenotype. One of these genes, which was identified by its mouse neural tube closure and inner ear hair polarity defect, is PTK7.

PTK7 AFFECTS Wnt SIGNALING PATHWAYS

Vertebrate PTK7 is according to the current criteria a bona fide PCP regulator. Using a mouse gene trap-screen for transmembrane proteins with a function in neural development, PTK7 mutants were identified showing a combination of severe neural tube closure and inner ear polarity defects (Lu et al., 2004). Based on this mutant phenotype, which is typical for known regulators of PCP (Hamblet et al., 2002; Curtin et al., 2003; Montcouquiol et al., 2003), as well as its genetic interaction with Vangl2, PTK7 was added to the list of vertebrate PCP regulators. Further functional studies using mouse, zebrafish and *Xenopus* confirmed a function for PTK7 in processes that are regulated by PCP signaling, including convergent extension movements during gastrulation, neurulation and Wolffian duct elongation, as well as neural crest migration and wound healing (**Table 1**). Surprisingly, although PTK7 appears to be a core regulator of vertebrate PCP, classical PCP phenotypes have so far not been reported for the *Drosophila* orthologs of PTK7, *off-track* (*otk*), and *off-track2* (*otk2*). These two genes, which are the result of a tandem gene duplication, function redundantly in the tubular morphogenesis of the male ejaculatory duct, leading to male sterility in the *otk, otk2* double mutant (Linnemannstons et al., 2014). Intriguingly, mesoderm-specific knock-out of PTK7 in the mouse resulted in tubular morphogenesis defects in the Wolffian duct, again leading to male sterility (Xu et al., 2016). In both cases, tubular morphogenesis defects upon loss of Otk/Otk2 or PTK7 may be caused by the failure to properly execute convergent extension movements. Thus, although the *Drosophila* mutants do not display the classical PCP defects, PTK7/Otk may play an evolutionarily conserved role in the regulation of cell movements.

The molecular mechanism by which PTK7 affects PCP signaling is currently unclear. However, as PTK7 interacts with Wnt ligands and known Wnt receptors (**Table 2**) it likely affects PCP by functioning as a Wnt receptor. This is also supported by the structure of PTK7, which is highly reminiscent of receptor tyrosine kinases. PTK7 consists of seven extracellular immunoglobulin domains, a transmembrane domain, and an evolutionarily conserved tyrosine kinase homology domain. The kinase homology domain of PTK7 lacks catalytic activity (Miller and Steele, 2000; Kroiher et al., 2001), but serves as an interaction site for intracellular signaling molecules like ß-catenin, Dsh, and Src (Shnitsar and Borchers, 2008; Puppo et al., 2011; Andreeva et al., 2014). PTK7 interacts with distinct Wnt receptors including Fz7, LRP6, and Ror2 (Peradziryi et al., 2011; Bin-Nun et al., 2014; Linnemannstons et al., 2014; Martinez et al., 2015; Podleschny et al., 2015), indicating that PTK7 affects canonical and non-canonical Wnt signaling pathways. This is also reflected by its evolutionarily conserved interaction with different Wnt ligands that are supposed to signal via both canonical and non-canonical pathways (Peradziryi et al., 2011; Linnemannstons et al., 2014; Martinez et al., 2015). While its requirement for PCP signaling is firmly established, the function of PTK7 in canonical Wnt signaling remains controversial. PTK7 has been reported to activate canonical Wnt signaling in the context of Spemann's organizer formation (Puppo et al., 2011) and the specification

TABLE 1 | PCP phenotypes upon PTK7 loss of function in vertebrates.

PCP phenotype	Process	Mutant	References
Craniorachischisis	Neural tube closure	Mouse, hypomorphic mutant (*Ptk7*^{XST87})	Lu et al., 2004
		Mouse, *chuzhoi* mutant (insertion of MT1-MMP splice site)	Paudyal et al., 2010
Convergent extension defect	Neural tube closure	Mouse, hypomorphic mutant (*Ptk7*^{XST87})	Lu et al., 2004; Williams et al., 2014
		Xenopus, Morpholino knockdown	Lu et al., 2004; Wehner et al., 2011
		Zebrafish, maternal-zygotic mutant (*ptk7*^{hsc9})	Hayes et al., 2013
	Gastrulation	Mouse, hypomorphic mutant (*Ptk7*^{XST87})	Yen et al., 2009
		Zebrafish, maternal-zygotic mutant (*ptk7*^{hsc9})	Hayes et al., 2013
	Wolffian duct morphogenesis	Mouse, hypomorphic mutant (*Ptk7*^{XST87})	Xu et al., 2016
Impaired stereociliary bundle orientation	Development of the organ of corti	Mouse, hypomorphic mutant (*Ptk7*^{XST87})	Lu et al., 2004; Lee et al., 2012; Andreeva et al., 2014
		Mouse, *chuzhoi* mutant (insertion of MT1-MMP splice site)	Paudyal et al., 2010
Impaired neural crest migration	Neural crest migration	*Xenopus* Morpholino knockdown	Shnitsar and Borchers, 2008; Podleschny et al., 2015
Defective wound repair	Epidermal wound repair	Mouse, hypomorphic mutant (*Ptk7*^{XST87})	Caddy et al., 2010
Defect in cilia development	Development of ependymal cell cilia	Zebrafish, zygotic mutant (*ptk7*^{hsc9})	Grimes et al., 2016

TABLE 2 | PTK7 interaction partners with a known function in Wnt signaling.

Interaction partner		Interaction domain	Biological context	References
Wnt ligand	Wnt3a, Wnt8	Extracellular domain	*Xenopus* double axis assay	Peradziryi et al., 2011
	Wnt4	Unknown	*Xenopus* double axis assay	Peradziryi et al., 2011
	Wnt5a	Extracellular domain (Ig4-7)	*Xenopus* morphogenesis	Martinez et al., 2015
	Wnt2	Unknown	*Drosophila* male fertility	Linnemannstons et al., 2014
Wnt receptor	Fz1	Unknown	*Drosophila* male fertility	Linnemannstons et al., 2014
	Fz2	Unknown	*Drosophila* male fertility	Linnemannstons et al., 2014
	Fz7	Extracellular domain	*Xenopus* luciferase reporter assay	Peradziryi et al., 2011
	Ror2	Extracellular domain Ig1-7	*Xenopus* morphogenesis and neural crest migration	Martinez et al., 2015; Podleschny et al., 2015
	LRP6	Transmembrane domain	*Xenopus* posterior neural development	Bin-Nun et al., 2014
Intracellular Wnt components	Dsh	Kinase homology domain (via Rack1/ PKCδ1)	*Xenopus* neural crest migration and neural tube closure	Shnitsar and Borchers, 2008; Wehner et al., 2011
	β-catenin	Kinase homology domain	*Xenopus* Spemann Organizer formation	Puppo et al., 2011

of posterior neural tissue (Bin-Nun et al., 2014) in *Xenopus* embryos. However, PTK7 inhibits canonical Wnt signaling in *Xenopus* double axis and luciferase reporter assays (Peradziryi et al., 2011). This was confirmed by *ptk7* mutant zebrafish, which showed an upregulation of ß-catenin target gene expression, suggesting that PTK7 functions in attenuating canonical Wnt signaling (Hayes et al., 2013). Conflicting results were also obtained analyzing the interaction of PTK7 with Wnt ligands using immunoprecipitation of overexpressed/tagged constructs. While we found interaction of PTK7 with canonical Wnt3a and Wnt8 but not non-canonical Wnt5a (Peradziryi et al., 2011) in *Xenopus* lysates, Martinez et al. observed an interaction with non-canonical Wnt5a, but not canonical Wnt1 (Martinez et al., 2015) in HEK293T cells. Some of these contradictions may be explained by receptor context. Using secreted proteins we showed that the extracellular domain of PTK7 requires the extracellular Fz7 domain for interaction with recombinant Wnt3a (Peradziryi et al., 2011). Conversely, Wnt5a binding may require the Ror2 co-receptor. Although Martinez et al. confirmed interaction of PTK7 and Wnt5a in cells that were

depleted of Ror2 using a specific siRNA, there may still be sufficient endogenous Ror2 present to mediate binding. Thus, studies analyzing direct interaction of PTK7 and Wnt ligands are currently lacking. Furthermore, experiments testing Wnt binding of combinatorial PTK7 co-receptor complexes are required. As PTK7 is a versatile receptor interacting not only with Wnt co-receptors but also with plexin and VEGF receptors (reviewed in Peradziryi et al., 2012), the latter interactions may also contribute to tissue-specific differences. Thus, future research will have to elucidate how receptor context affects PTK7 signaling and its functions in distinct Wnt signaling pathways.

PTK7 AND DISEASE

As PTK7 has a crucial function in the regulation of Wnt signaling pathways known to be essential for embryonic development and homeostasis, mutations in the human PTK7 gene are likely of clinical relevance. PTK7 was identified as a gene upregulated in colon carcinoma cells and appears to be misregulated in a variety of cancers (Dunn and Tolwinski, 2016). Furthermore, PTK7 mutations have recently been implicated in the etiology of neural tube defects and scoliosis (Hayes et al., 2014; Wang et al., 2015). Here, we will briefly describe these respective disorders and look at the human PTK7 gene variants identified in this context as well as their functional implications.

The connection between PTK7 and cancer has so far mostly been deduced on the basis of up- or downregulation of PTK7 in a variety of cancer types. PTK7 levels were reported to be increased in esophageal (Shin et al., 2013), gastric (Lin et al., 2012), colorectal (Lhoumeau et al., 2015), breast (Gartner et al., 2014), intrahepatic bile duct (Jin et al., 2014), prostate (Zhang et al., 2014), and lung carcinoma (Chen et al., 2014), as well as liposarcoma (Gobble et al., 2011). In other cancer types PTK7 was shown to be downregulated, including lung squamous cell carcinoma (Kim et al., 2014), ovarian carcinoma (Wang et al., 2014) and metastatic melanoma (Easty et al., 1997). While the mechanistic contribution of PTK7 to the respective tumor phenotypes is unclear at present, the upregulation of PTK7 in many tumor types makes it an attractive tumor marker and therapeutic target. Indeed, the first PTK7 specific reagents with potential clinical applications have now been published, including a PTK7-specific fluorescently labeled aptamer for *in vivo* detection of tumor tissue (Calzada et al., 2017). Very interestingly, PTK7 has recently been established as a marker for normal colon stem cells (Jung et al., 2015) and as a marker for tumor initiating cells in triple-negative breast cancer, ovarian cancer and non-small cell lung cancer (Damelin et al., 2017). The authors of the latter study also developed a PTK7-targeted antibody-drug conjugate and showed that its application reduces tumor initiating cells and induces sustained tumor regressions, paving the way for a PTK7-directed anti-tumor therapy (Damelin et al., 2017).

Neural tube defects are among the most common human birth defects affecting 1 per 1000 live births and are caused by environmental as well as genetic factors (Wilde et al., 2014). PCP genes are likely among the genetic factors contributing to the etiology of human neural tube closure defects as loss of function mutants of PTK7, Vangl, Celsr, Fz, Dvl, and Scribble result in the most severe neural tube closure defects called craniorachischisis (Gerrelli and Copp, 1997; Kibar et al., 2001; Hamblet et al., 2002; Curtin et al., 2003; Murdoch et al., 2003; Lu et al., 2004; Wang et al., 2006), whereby the neural tube fails to close from the midbrain-hindbrain boundary to the base of the spine. Indeed, rare mutations with a predicted damaging role were identified for a number of PCP genes including Vangl1/2, Celsr1, Fzd6, Dvl2, Prickle, and Scribble (Kibar et al., 2007; De Marco et al., 2014). Furthermore, the analysis of a cohort of 473 patients with various forms of neural tube defects identified six rare PTK7 sequence variants (Wang et al., 2015). Interestingly, five of these mutations are located in the extracellular domain of PTK7, which serves as interaction site for Wnt ligands as well as Fz7 and Ror2 receptors (**Table 2**; Peradziryi et al., 2011; Martinez et al., 2015; Podleschny et al., 2015). Whether these interactions are affected in the potentially pathogenic sequence variants is currently unclear and functional validation assays testing their efficiency to rescue for example *Xenopus* or zebrafish loss of function phenotypes are still missing. Nevertheless, the extracellular domain was shown to be important for promoting PCP and inhibiting canonical Wnt signaling. In fact, deletion of the extracellular domain abolished PTK7's ability to inhibit canonical Wnt signaling in *Xenopus* reporter assays (Peradziryi et al., 2011). Conversely, a membrane-tethered PTK7 extracellular fragment was sufficient to rescue excess Wnt/ß-catenin signaling and PCP morphogenesis defects in maternal-zygotic *ptk7* mutant zebrafish (Hayes et al., 2013). Thus, these data point to PTK7 as a risk factor for neural tube closure defects and stress the functional importance of its extracellular domain.

In addition to neural tube defects, PTK7 has also been implicated in the pathogenesis of scoliosis, a complex genetic disorder characterized by a three-dimensional spinal curvature. Congenital scoliosis (CS) is apparent at birth and involves abnormal vertebrae development, while idiopathic scoliosis is diagnosed during adolescence and does not show vertebral malformations. *Ptk7* mutant zebrafish were recently discovered as a model for congenital and idiopathic scoliosis. Maternal-zygotic *ptk7* (*MZptk7*) mutant zebrafish exhibit vertebral abnormalities at larval stages, phenotypically resembling congenital scoliosis. Further, zygotic *ptk7* (*Zptk7*) mutants show late onset spinal curvatures consistent with the idiopathic form of scoliosis (Hayes et al., 2014). Analysis of maternal-zygotic mutants showed that PTK7 positively regulates PCP-dependent morphogenesis, while it attenuates ß-catenin-dependent canonical Wnt signaling (Hayes et al., 2013). Thus, segmentation and somite patterning are disturbed, likely causing the observed vertebral abnormalities. In contrast, zygotic *ptk7* mutants did not show defects in segmentation and somite patterning, but developed late spinal curvatures resembling idiopathic scoliosis (Hayes et al., 2014; Grimes et al., 2016). They showed defects in ependymal cell cilia development leading to irregularities in the cerebrospinal fluid (CSF) flow. Moreover, the brain ventricles revealed a severe hydrocephalus, a condition associated with loss of cilia function. Consistently, the number of motile cilia was reduced and if cilia were present they lacked the correct

polarization. Transgenic reintroduction of wild-type PTK7 in motile ciliated cell lineages rescued all phenotypes, proving a specific function of PTK7 in motile ciliated cells. The authors hypothesized that impaired cerebrospinal fluid flow due to abnormal cilia function is most likely the cause of scoliosis in *ptk7*-deficient zebrafish (Grimes et al., 2016). The connection of PTK7 to scoliosis was further evidenced by the isolation of a novel PTK7 mutation from a single patient suffering from idiopathic scoliosis. This mutation, hPTK7^{P545A}, exhibits a proline to alanine substitution in the sixth extracellular immunoglobulin domain thereby affecting PCP and canonical Wnt signaling function (Hayes et al., 2014). In fact, in contrast to wild-type human PTK7, the hPTK7^{P545A} failed to rescue PCP-dependent axial extension defects as well as nervous system patterning defects caused by Wnt8 overexpression (Hayes et al., 2014). Further, the mutant protein accumulated at the plasma membrane, indicating altered protein stability and/or trafficking of this mutant compared to the wild-type protein. As PTK7 forms co-receptor complexes with Fz7 and LRP6 (Peradziryi et al., 2011; Bin-Nun et al., 2014; Linnemannstons et al., 2014), which were shown to be subject to Wnt-dependent receptor complex trafficking (Yamamoto et al., 2006; Kim et al., 2008; Ohkawara et al., 2011), this is likely also the case for PTK7-containing receptor complexes. Thus, it is tempting to speculate that the proline residue in position 545—which is conserved in mammals—is required for interaction with Wnt ligands or co-receptors, respectively. Interestingly, this conserved P545 residue is also mutated in one of the six sequence variants identified in patients with neural tube closure defects. In a patient affected with myelomeningocele and interestingly also hydrocephalus, which is indicative of a cilia-defect, the non-polar proline residue was changed to a positively charged arginine (Wang et al., 2015). These data indicate that this conserved residue is important for protein function and mutations are likely pathogenic. Future studies are required to elucidate the molecular pathomechanism.

CONCLUSIONS

During the last two decades our understanding of the function of PTK7 has significantly advanced. Diverse biological processes that are regulated by PTK7 have been identified and its role in the establishment of polarity and coordinated cell movements has been acknowledged. Recent publications shed light on a literary "complex" function of PTK7 in Wnt signaling. While its role in non-canonical PCP signaling has been confirmed in different animal model systems and biological contexts, its function with respect to canonical Wnt signaling remains controversial. Possibly, these contradictory findings can be explained by the cell type-specific formation and subcellular localization of distinct co-receptor complexes. Further characterization of the formation and dynamics of these ligand-receptor complexes may help us to understand how PTK7 affects development as well as disease-related processes.

AUTHOR CONTRIBUTIONS

AB conceptualized and wrote the main part of the paper. AW wrote the *Drosophila* and the cancer section and edited the manuscript; HB wrote the section on scoliosis and designed the two tables.

REFERENCES

Anastas, J. N., and Moon, R. T. (2013). WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* 13, 11–26. doi: 10.1038/nrc3419

Andreeva, A., Lee, J., Lohia, M., Wu, X., Macara, I. G., and Lu, X. (2014). PTK7-Src signaling at epithelial cell contacts mediates spatial organization of actomyosin and planar cell polarity. *Dev. Cell* 29, 20–33. doi: 10.1016/j.devcel.2014.02.008

Bin-Nun, N., Lichtig, H., Malyarova, A., Levy, M., Elias, S., and Frank, D. (2014). PTK7 modulates Wnt signaling activity via LRP6. *Development* 141, 410–421. doi: 10.1242/dev.095984

Caddy, J., Wilanowski, T., Darido, C., Dworkin, S., Ting, S. B., Zhao, Q., et al. (2010). Epidermal wound repair is regulated by the planar cell polarity signaling pathway. *Dev. Cell* 19, 138–147. doi: 10.1016/j.devcel.2010.06.008

Calzada, V., Moreno, M., Newton, J., Gonzalez, J., Fernandez, M., Gambini, J. P., et al. (2017). Development of new PTK7-targeting aptamer-fluorescent and -radiolabelled probes for evaluation as molecular imaging agents: lymphoma and melanoma *in vivo* proof of concept. *Bioorg. Med. Chem.* 25, 1163–1171. doi: 10.1016/j.bmc.2016.12.026

Chen, R., Khatri, P., Mazur, P. K., Polin, M., Zheng, Y., Vaka, D., et al. (2014). A meta-analysis of lung cancer gene expression identifies PTK7 as a survival gene in lung adenocarcinoma. *Cancer Res.* 74, 2892–2902. doi: 10.1158/0008-5472.CAN-13-2775

Clevers, H., and Nusse, R. (2012). Wnt/beta-Catenin signaling and disease. *Cell* 149, 1192–1205. doi: 10.1016/j.cell.2012.05.012

Curtin, J. A., Quint, E., Tsipouri, V., Arkell, R. M., Cattanach, B., Copp, A. J., et al. (2003). Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. *Curr. Biol.* 13, 1129–1133. doi: 10.1016/S0960-9822(03)00374-9

Damelin, M., Bankovich, A., Bernstein, J., Lucas, J., Chen, L., Williams, S., et al. (2017). A PTK7-targeted antibody-drug conjugate reduces tumor-initiating cells and induces sustained tumor regressions. *Sci. Trans. Med.* 9:eaag2611. doi: 10.1126/scitranslmed.aag2611

De Marco, P., Merello, E., Piatelli, G., Cama, A., Kibar, Z., and Capra, V. (2014). Planar cell polarity gene mutations contribute to the etiology of human neural tube defects in our population. *Birth Defects Res. A Clin. Mol. Teratol.* 100, 633–641. doi: 10.1002/bdra.23255

Dunn, N. R., and Tolwinski, N. S. (2016). Ptk7 and Mcc, unfancied components in non-canonical wnt signaling and cancer. *Cancers* 8:E68. doi: 10.3390/cancers8070068

Easty, D. J., Mitchell, P. J., Patel, K., Florenes, V. A., Spritz, R. A., and Bennett, D. C. (1997). Loss of expression of receptor tyrosine kinase family genes PTK7 and SEK in metastatic melanoma. *Int. J. Cancer* 71, 1061–1065. doi: 10.1002/(SICI)1097-0215(19970611)71:6<1061::AID-IJC24>3.0.CO;2-F

Gartner, S., Gunesch, A., Knyazeva, T., Wolf, P., Hogel, B., Eiermann, W., et al. (2014). PTK 7 is a transforming gene and prognostic marker for breast cancer and nodal metastasis involvement. *PLoS ONE* 9:e84472. doi: 10.1371/journal.pone.0084472

Gerrelli, D., and Copp, A. J. (1997). Failure of neural tube closure in the loop-tail (Lp) mutant mouse: analysis of the embryonic mechanism. *Brain Res. Dev. Brain Res.* 102, 217–224. doi: 10.1016/S0165-3806(97)00099-0

Gobble, R. M., Qin, L. X., Brill, E. R., Angeles, C. V., Ugras, S., O'Connor, R. B., et al. (2011). Expression profiling of liposarcoma yields a multigene predictor of patient outcome and identifies genes that contribute to liposarcomagenesis. *Cancer Res.* 71, 2697–2705. doi: 10.1158/0008-5472.CAN-10-3588

Goodrich, L. V., and Strutt, D. (2011). Principles of planar polarity in animal development. *Development* 138, 1877–1892. doi: 10.1242/dev.054080

Grimes, D. T., Boswell, C. W., Morante, N. F., Henkelman, R. M., Burdine, R. D., and Ciruna, B. (2016). Zebrafish models of idiopathic scoliosis link cerebrospinal fluid flow defects to spine curvature. *Science* 352, 1341–1344. doi: 10.1126/science.aaf6419

Hamblet, N. S., Lijam, N., Ruiz-Lozano, P., Wang, J., Yang, Y., Luo, Z., et al. (2002). Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. *Development* 129, 5827–5838. doi: 10.1242/dev.00164

Hayes, M., Gao, X., Yu, L. X., Paria, N., Henkelman, R. M., Wise, C. A., et al. (2014). ptk7 mutant zebrafish models of congenital and idiopathic scoliosis implicate dysregulated Wnt signalling in disease. *Nat. Commun.* 5:5777. doi: 10.1038/ncomms5777

Hayes, M., Naito, M., Daulat, A., Angers, S., and Ciruna, B. (2013). Ptk7 promotes non-canonical Wnt/PCP-mediated morphogenesis and inhibits Wnt/beta-catenin-dependent cell fate decisions during vertebrate development. *Development* 140, 1807–1818. doi: 10.1242/dev.090183

Inestrosa, N. C., and Varela-Nallar, L. (2014). Wnt signaling in the nervous system and in Alzheimer's disease. *J. Mol. Cell Biol.* 6, 64–74. doi: 10.1093/jmcb/mjt051

Jin, J., Ryu, H. S., Lee, K. B., and Jang, J. J. (2014). High expression of protein tyrosine kinase 7 significantly associates with invasiveness and poor prognosis in intrahepatic cholangiocarcinoma. *PLoS ONE* 9:e90247. doi: 10.1371/journal.pone.0090247

Jung, P., Sommer, C., Barriga, F. M., Buczacki, S. J., Hernando-Momblona, X., Sevillano, M., et al. (2015). Isolation of human colon stem cells using surface expression of PTK7. *Stem Cell Rep.* 5, 979–987. doi: 10.1016/j.stemcr.2015.10.003

Kibar, Z., Torban, E., McDearmid, J. R., Reynolds, A., Berghout, J., Mathieu, M., et al. (2007). Mutations in VANGL1 associated with neural-tube defects. *New Engl. J. Med.* 356, 1432–1437. doi: 10.1056/NEJMoa060651

Kibar, Z., Vogan, K. J., Groulx, N., Justice, M. J., Underhill, D. A., and Gros, P. (2001). Ltap, a mammalian homolog of Drosophila Strabismus/Van Gogh, is altered in the mouse neural tube mutant Loop-tail. *Nat. Gen.* 28, 251–255. doi: 10.1038/90081

Kim, G. H., Her, J. H., and Han, J. K. (2008). Ryk cooperates with Frizzled 7 to promote Wnt11-mediated endocytosis and is essential for *Xenopus laevis* convergent extension movements. *J. Cell Biol.* 182, 1073–1082. doi: 10.1083/jcb.200710188

Kim, J. H., Kwon, J., Lee, H. W., Kang, M. C., Yoon, H. J., Lee, S. T., et al. (2014). Protein tyrosine kinase 7 plays a tumor suppressor role by inhibiting ERK and AKT phosphorylation in lung cancer. *Oncol. Rep.* 31, 2708–2712. doi: 10.3892/or.2014.3164

Kroiher, M., Miller, M. A., and Steele, R. E. (2001). Deceiving appearances: signaling by "dead" and "fractured" receptor protein-tyrosine kinases. *Bioessays* 23, 69–76. doi: 10.1002/1521-1878(200101)23:1<69::AID-BIES1009>3.3.CO;2-B

Lander, R., and Petersen, C. P. (2016). Wnt, Ptk7, and FGFRL expression gradients control trunk positional identity in planarian regeneration. *Elife* 5:e12850. doi: 10.1056/NEJMoa060651

Lee, J., Andreeva, A., Sipe, C. W., Liu, L., Cheng, A., and Lu, X. (2012). PTK7 regulates myosin II activity to orient planar polarity in the mammalian auditory epithelium. *Curr. Biol.* 22, 956–966. doi: 10.1016/j.cub.2012.03.068

Lee, H. K., Chauhan, S. K., Kay, E., and Dana, R. (2011). Flt-1 regulates vascular endothelial cell migration via a protein tyrosine kinase-7-dependent pathway. *Blood* 117, 5762–5771. doi: 10.1182/blood-2010-09-306928

Lhoumeau, A. C., Martinez, S., Boher, J. M., Monges, G., Castellano, R., Goubard, A., et al. (2015). Overexpression of the promigratory and prometastatic PTK7 receptor Is associated with an adverse clinical outcome in colorectal cancer. *PLoS ONE* 10:e0123768. doi: 10.1371/journal.pone.0123768

Lin, Y., Zhang, L. H., Wang, X. H., Xing, X. F., Cheng, X. J., Dong, B., et al. (2012). PTK7 as a novel marker for favorable gastric cancer patient survival. *J. Surg. Oncol.* 106, 880–886. doi: 10.1002/jso.23154

Linnemannstons, K., Ripp, C., Honemann-Capito, M., Brechtel-Curth, K., Hedderich, M., and Wodarz, A. (2014). The PTK7-related transmembrane proteins off-track and off-track 2 are co-receptors for Drosophila Wnt2 required for male fertility. *PLoS Genet.* 10:e1004443. doi: 10.1371/journal.pgen.1004443

Logan, C. Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Ann. Rev. Cell Dev. Biol.* 20, 781–810. doi: 10.1146/annurev.cellbio.20.010403.113126

Lu, X., Borchers, A. G., Jolicoeur, C., Rayburn, H., Baker, J. C., and Tessier-Lavigne, M. (2004). PTK7/CCK-4 is a novel regulator of planar cell polarity in vertebrates. *Nature* 430, 93–98. doi: 10.1038/nature02677

MacDonald, B. T., and He, X. (2012). Frizzled and LRP5/6 receptors for Wnt/beta-catenin signaling. *Cold Spring Harb. Perspect. Biol.* 4:a007880. doi: 10.1101/cshperspect.a007880

MacDonald, B. T., Tamai, K., and He, X. (2009). Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17, 9–26. doi: 10.1016/j.devcel.2009.06.016

Martinez, S., Scerbo, P., Giordano, M., Daulat, A. M., Lhoumeau, A. C., Thome, V., et al. (2015). The PTK7 and ROR2 protein receptors interact in the vertebrate WNT/Planar Cell Polarity (PCP) Pathway. *J. Biol. Chem.* 290, 30562–30572. doi: 10.1074/jbc.M115.697615

Miller, M. A., and Steele, R. E. (2000). Lemon encodes an unusual receptor protein-tyrosine kinase expressed during gametogenesis in Hydra. *Dev. Biol.* 224, 286–298. doi: 10.1006/dbio.2000.9786

Montcouquiol, M., Crenshaw, E. B. III, and Kelley, M. W. (2006). Noncanonical Wnt signaling and neural polarity. *Ann. Rev. Neurosci.* 29, 363–386. doi: 10.1146/annurev.neuro.29.051605.112933

Montcouquiol, M., Rachel, R. A., Lanford, P. J., Copeland, N. G., Jenkins, N. A., and Kelley, M. W. (2003). Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. *Nature* 423, 173–177. doi: 10.1038/nature01618

Mossie, K., Jallal, B., Alves, F., Sures, I., Plowman, G. D., and Ullrich, A. (1995). Colon carcinoma kinase-4 defines a new subclass of the receptor tyrosine kinase family. *Oncogene* 11, 2179–2184.

Murdoch, J. N., Henderson, D. J., Doudney, K., Gaston-Massuet, C., Phillips, H. M., Paternotte, C., et al. (2003). Disruption of scribble (Scrb1) causes severe neural tube defects in the circletail mouse. *Hum. Mol. Genet.* 12, 87–98. doi: 10.1093/hmg/ddg014

Niehrs, C. (2012). The complex world of WNT receptor signalling. *Nat. Rev. Mol. Cell Biol.* 13, 767–779. doi: 10.1038/nrm3470

Ohkawara, B., Glinka, A., and Niehrs, C. (2011). Rspo3 binds syndecan 4 and induces Wnt/PCP signaling via clathrin-mediated endocytosis to promote morphogenesis. *Dev. Cell* 20, 303–314. doi: 10.1016/j.devcel.2011.01.006

Paudyal, A., Damrau, C., Patterson, V. L., Ermakov, A., Formstone, C., Lalanne, Z., et al. (2010). The novel mouse mutant, chuzhoi, has disruption of Ptk7 protein and exhibits defects in neural tube, heart and lung development and abnormal planar cell polarity in the ear. *BMC Dev. Biol.* 286, 20970–20976. doi: 10.1186/1471-213X-10-87

Peradziryi, H., Kaplan, N. A., Podleschny, M., Liu, X., Wehner, P., Borchers, A., et al. (2011). PTK7/Otk interacts with Wnts and inhibits canonical Wnt signalling. *EMBO J.* 30, 3729–3740. doi: 10.1038/emboj.2011.236

Peradziryi, H., Tolwinski, N. S., and Borchers, A. (2012). The many roles of PTK7: a versatile regulator of cell-cell communication. *Arch. Biochem. Biophys.* 524, 71–76. doi: 10.1016/j.abb.2011.12.019

Podleschny, M., Grund, A., Berger, H., Rollwitz, E., and Borchers, A. (2015). A PTK7/Ror2 Co-receptor complex affects xenopus neural crest migration. *PLoS ONE* 10:e0145169. doi: 10.1371/journal.pone.0145169

Puppo, F., Thome, V., Lhoumeau, A. C., Cibois, M., Gangar, A., Lembo, F., et al. (2011). Protein tyrosine kinase 7 has a conserved role in Wnt/beta-catenin canonical signalling. *EMBO Rep.* 12, 43–49. doi: 10.1038/embor.2010.185

Shin, W. S., Kwon, J., Lee, H. W., Kang, M. C., Na, H. W., Lee, S. T., et al. (2013). Oncogenic role of protein tyrosine kinase 7 in esophageal squamous cell carcinoma. *Cancer Sci.* 104, 1120–1126. doi: 10.1111/cas.12194

Shnitsar, I., and Borchers, A. (2008). PTK7 recruits dsh to regulate neural crest migration. *Development* 135, 4015–4024. doi: 10.1242/dev.023556

Simons, M., and Mlodzik, M. (2008). Planar cell polarity signaling: from fly development to human disease. *Ann. Rev. Genet.* 42, 517–540. doi: 10.1146/annurev.genet.42.110807.091432

Wallingford, J. B. (2010). Planar cell polarity signaling, cilia and polarized ciliary beating. *Curr. Opin. Cell Biol.* 22, 597–604. doi: 10.1016/j.ceb.2010.07.011

Wallingford, J. B. (2012). Planar cell polarity and the developmental control of cell behavior in vertebrate embryos. *Ann. Rev. Cell Dev. Biol.* 28, 627–653. doi: 10.1146/annurev-cellbio-092910-154208

Wallingford, J. B., Fraser, S. E., and Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during

embryonic development. *Dev. Cell* 2, 695–706. doi: 10.1016/S1534-5807(02)00197-1

Wallingford, J. B., and Mitchell, B. (2011). Strange as it may seem: the many links between Wnt signaling, planar cell polarity, and cilia. *Genes Dev.* 25, 201–213. doi: 10.1101/gad.2008011

Wang, H., Li, G., Yin, Y., Wang, J., Wei, W., Guo, Q., et al. (2014). PTK7 protein is decreased in epithelial ovarian carcinomas with poor prognosis. *Int. J. Clin. Exp. Pathol.* 7, 7881–7889.

Wang, M., De Marco, P., Merello, E., Drapeau, P., Capra, V., and Kibar, Z. (2015). Role of the planar cell polarity gene Protein tyrosine kinase 7 in neural tube defects in humans. *Birth Defects Res. A Clin. Mol. Teratol.* 103, 1021–1027. doi: 10.1002/bdra.23422

Wang, Y., Guo, N., and Nathans, J. (2006). The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. *J. Neurosci.* 26, 2147–2156. doi: 10.1523/JNEUROSCI.4698-05.2005

Wehner, P., Shnitsar, I., Urlaub, H., and Borchers, A. (2011). RACK1 is a novel interaction partner of PTK7 that is required for neural tube closure. *Development* 138, 1321–1327. doi: 10.1242/dev.056291

Wilde, J. J., Petersen, J. R., and Niswander, L. (2014). Genetic, epigenetic, and environmental contributions to neural tube closure. *Ann. Rev. Genet.* 48, 583–611. doi: 10.1146/annurev-genet-120213-092208

Williams, M., Yen, W., Lu, X., and Sutherland, A. (2014). Distinct apical and basolateral mechanisms drive planar cell polarity-dependent convergent extension of the mouse neural plate. *Dev. Cell* 29, 34–46. doi: 10.1016/j.devcel.2014.02.007

Xu, B., Washington, A. M., Domeniconi, R. F., Ferreira Souza, A. C., Lu, X., Sutherland, A., et al. (2016). Protein tyrosine kinase 7 is essential for tubular morphogenesis of the Wolffian duct. *Dev. Biol.* 412, 219–233. doi: 10.1016/j.ydbio.2016.02.029

Yamamoto, H., Komekado, H., and Kikuchi, A. (2006). Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. *Dev. Cell* 11, 213–223. doi: 10.1016/j.devcel.2006.07.003

Yang, Y., and Mlodzik, M. (2015). Wnt-Frizzled/planar cell polarity signaling: cellular orientation by facing the wind (Wnt). *Ann. Rev. Cell Dev. Biol.* 31, 623–646. doi: 10.1146/annurev-cellbio-100814-125315

Yen, W. W., Williams, M., Periasamy, A., Conaway, M., Burdsal, C., Keller, R., et al. (2009). PTK7 is essential for polarized cell motility and convergent extension during mouse gastrulation. *Development* 136, 2039–2048. doi: 10.1242/dev.030601

Zhang, H., Wang, A., Qi, S., Cheng, S., Yao, B., and Xu, Y. (2014). Protein tyrosine kinase 7 (PTK7) as a predictor of lymph node metastases and a novel prognostic biomarker in patients with prostate cancer. *Int. J. Mol. Sci.* 15, 11665–11677. doi: 10.3390/ijms150711665

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 26 May 2017
doi: 10.3389/fcell.2017.00059

Dishevelled Paralogs in Vertebrate Development: Redundant or Distinct?

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Dishevelled (DVL) proteins are highly conserved in the animal kingdom and are important key players in β-Catenin-dependent and -independent Wnt signaling pathways. Vertebrate genomes typically comprise three DVL genes, DVL1, DVL2, and DVL3. Expression patterns and developmental functions of the three vertebrate DVL proteins however, are only partially redundant in any given species. Moreover, expression and function of DVL isoforms have diverged between different vertebrate species. All DVL proteins share basic functionality in Wnt signal transduction. Additional, paralog-specific interactions and functions combined with context-dependent availability of DVL isoforms may play a central role in defining Wnt signaling specificity and add selectivity toward distinct downstream pathways. In this review, we recapitulate briefly cellular functions of DVL paralogs, their role in vertebrate embryonic development and congenital disease.

Keywords: Dishevelled, Wnt signaling, vertebrate embryonic development, embryonic expression, autosomal dominant robinow syndrome

OPEN ACCESS

Edited by:
Andrea Erika Münsterberg, University of East Anglia, United Kingdom

Reviewed by:
Christine Hartmann, University of Münster, Germany
Victoria Sherwood, University of Dundee, United Kingdom

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *31 January 2017*
Accepted: *12 May 2017*
Published: *26 May 2017*

Citation:
Gentzel M and Schambony A (2017) Dishevelled Paralogs in Vertebrate Development: Redundant or Distinct? Front. Cell Dev. Biol. 5:59. doi: 10.3389/fcell.2017.00059

INTRODUCTION

The Dishevelled (dsh[1]) phenotype has been described the first time in Drosophila close to 60 years ago (Fahmy and Fahmy, 1959) and the diverse molecular functions of Dishevelled (DVL) proteins still stimulate intensive research. To date DVL is considered the central intracellular effector of Wnt signaling pathways, which play key roles in establishing and patterning of the body axes and in the control of proliferation, differentiation, planar cell polarity (PCP), and cell movements throughout the animal kingdom. Although evidence is accumulating that Wnt pathways should be considered as a signaling network, individual pathways have been subdivided into the Wnt/β-Catenin pathway and the β-Catenin–independent pathways including Wnt/PCP, Wnt/Ca^{2+}, and Wnt/STOP signaling, all of which involve DVL (reviewed in Kühl et al., 2000; Kohn and Moon, 2005; Macdonald et al., 2009; Niehrs and Acebron, 2012; van Amerongen, 2012; Collu and Mlodzik, 2015, **Figure 1A**).

DVL and its core functions in β-Catenin-dependent and -independent Wnt pathways are highly conserved. Notably, the genomes of Drosophila and most other invertebrates harbor only a single *DVL* gene. By contrast vertebrate genomes comprise genes for three *DVL* paralogs (*DVL1-3*), which have most likely arisen from two rounds of genome duplication (reviewed in Kasahara, 2007; Dillman et al., 2013). The still high degree of conservation among the three vertebrate DVL paralogs raises the question to which extent they have undergone functional diversification since obviously a single protein is sufficient to mediate DVL functions in invertebrates.

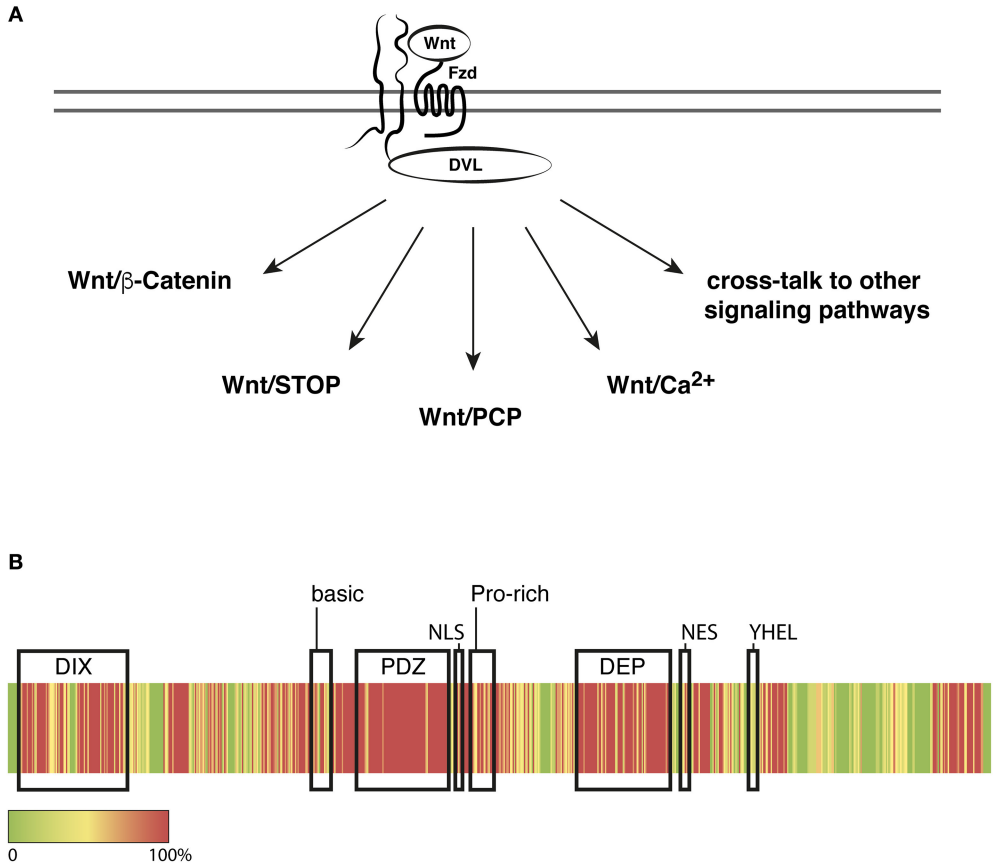

FIGURE 1 | Dishevelled in Wnt/Frizzled signaling. (A) DVL relays Wnt/Frizzled signals to multiple signaling pathways to regulate cellular functions including mitosis, transcription, polarity, and migration. **(B)** Heatmap representation of the conservation of all three DVL proteins in human, rat, mouse, and frog. Sequence alignments were calculated using Clustal Omega with the following input sequences: *Homo sapiens* DVL1: O14640, DVL2: O14641, DVL3: Q92997, *Rattus norvegicus* DVL1: Q9WVB9, DVL2: D3ZB71, DVL3: D4ADV8, *Mus musculus* DVL1: P51141, DVL2: Q60838, DVL3: Q61062, *Xenopus laevis* DVL2: P51142, DVL3: Q6DKE2 (Uniprot Accession numbers), *Xenopus laevis* DVL1: NCBI XP_018081523. Red indicates 100% identity and Green indicates no identity of amino acids at the respective position. Conservation scores were calculated according to Livingstone and Barton (1993). Functional domains or motifs are indicated by correspondingly labeled boxes; for details and references see text.

CELLULAR FUNCTIONS OF DISHEVELLED PARALOGS

All DVL proteins share an N-terminal DIX domain, a PDZ and a DEP domain (Axelrod et al., 1998; Boutros et al., 1998; Li et al., 1999). Additional sequence motifs have been identified that provide interfaces for protein-protein interactions like the basic and proline-rich regions, or functional motifs as the nuclear export sequence (NES), a putative nuclear localization sequence (NLS), the—YHEL—motif that is required for internalization of the activated receptor complex and motifs that mediate the association of DVL with the cytoskeleton or with phospholipids (Capelluto et al., 2002; Penton et al., 2002; Itoh et al., 2005; Yu et al., 2007, **Figure 1B**). The lack of striking differences in the primary structure with no obvious additional or missing functional motifs and domains poses a challenge to predict functional differences and to develop assays that would reveal potential dissimilarities.

DVL interacts with the cytoplasmic interface of Frizzled receptors (Tauriello et al., 2012), regulates receptor internalization and maintenance of Frizzled membrane equilibrium through protein-protein interaction (Yu et al., 2007; Jiang et al., 2015) and serves as scaffold for numerous proteins including multiple protein kinases (reviewed in Gao and Chen, 2010; Mlodzik, 2016). Depending on the composition of the receptor complex and its interaction partners, DVL contributes to β-Catenin stabilization, inhibition of GSK3β or activation of β-Catenin-independent signaling cascades (Gao and Chen, 2010). DVL also enters the nucleus and interacts with TCF/c-Jun/β-Catenin or FOXK1/2 transcription factor complexes (Itoh et al., 2005; Gan et al., 2008; Wang et al., 2015). In addition, DVL proteins play a role in microtubule stabilization (Ciani et al., 2004), centrosome positioning and separation (Sepich et al., 2011; Cervenka et al., 2016) and mediate cross-talk with other signaling pathways such as Notch or NF-κB (Deng et al., 2010; Collu et al., 2012).

Notably, for most of the abovementioned DVL functions no preference for a DVL paralog has been detected, although some studies suggest dose-dependent effects (e.g., Cervenka et al., 2016). A different picture was observed for the role of DVL paralogs in PCP in ciliated cells, which is required to position the basal bodies. DVL1 was required for intact PCP signaling and localized asymmetrically in multiciliated cells in the epidermis of *Xenopus* tadpoles. DVL2 was concentrated in dots in vicinity to the basal bodies that led to a local concentration of active RhoA and was required for basal body positioning. Localization of DVL2 itself was mediated by Inturned and according to current knowledge neither of both proteins plays a role in ciliogenesis in the fly (Park et al., 2008). In the mouse node, DVL2 and DVL3 were apically localized and polarized to the posterior side. Positioning of the basal body and directional flow was disturbed or absent in DVL1/DVL2 or DVL1/DVL3 deficient embryos (Hashimoto et al., 2010). Moreover, polarized localization of DVL, planar polarity of basal bodies and their docking could be separated experimentally although the detailed mechanism remains elusive (Park et al., 2008; Vladar et al., 2009; Hashimoto et al., 2010).

DVL PARALOGS IN VERTEBRATE EMBRYONIC DEVELOPMENT

Embryonic Expression Patterns

In early *Xenopus* embryos, *DVL2* and *DVL3* are present maternally, whereas *DVL1* expression is upregulated after the mid-blastula transition (Tadjuidje et al., 2011). At early gastrula stages, all three *DVL* paralogs are expressed in the prospective mesoderm including Spemann's organizer and, although weaker, in the ectoderm. Post-gastrula expression of *DVL1* and *DVL2* largely overlaps and is strongest in the neural tube, premigratory and migrating neural crest, as well as in the otic placode, the presomitic and somitic mesoderm. Notably, *DVL3* was not expressed in the neuroectoderm but restricted to the paraxial mesoderm, the heart, cranial placodes, and at tadpole stages to the branchial arches (Gray et al., 2009).

In chicken embryos, only two *DVL* genes, *DVL1* and *DVL3,* were identified. *DVL3* was already expressed in day 2 embryos and showed broad expression in most embryonic tissues whereas *DVL1* was upregulated only after day 2, i.e., after completion of neurogenesis, and showed a spatially restricted expression in the brain, strongest in the optic stalk, the olfactory bulb, and the ventral forebrain and spinal chord (Gray et al., 2009).

All murine *DVL* genes are maternally expressed in mouse oocytes and pre-implantation embryos, but interestingly individual protein levels differ considerably and dynamically from oocyte to blastocyst (Na et al., 2007). Post-implantation, *DVL1* expression was detected in the mesoderm, but not in the node, at stage E7.5. Post-gastrula, *DVL1* was expressed strongest in the neuroectoderm and later in the CNS, cranial and dorsal root ganglia, somites, the liver, kidney, intestine, and lung (Sussman et al., 1994). For *DVL2*, ubiquitous expression has been reported during embryogenesis in the mouse (Klingensmith et al., 1996). At E 7.5, *DVL3* has also been detected ubiquitously, but shortly after showed elevated expression in the CNS and the somitic mesoderm, the notochord, heart, dorsal root ganglia, and branchial arches and in the limb buds (Tsang et al., 1996; Diez-Roux et al., 2011).

Phylogenetic analyses suggest that *DVL1* separated first from the common ancestor of *DVL2/3*, which split into *DVL2* and *DVL3* in a second round of duplication (Gray et al., 2009). Consistent with corresponding functional divergence, expression of at least one *DVL2/3* paralog in the oocyte and pre-blastula embryo and of *DLV1* in the central nervous system appear to be conserved among vertebrates. Except for these conserved patterns, developmental expression of the three *DVL* genes is highly divergent within a species and among different species with *DVL2* expression showing the highest variability.

Developmental Function and Human Congenital Disease

Both, animal models and human congenital disease provide insights into the developmental function of vertebrate DVL paralogs. To date, DVL has predominantly been studied in the mouse and, to a much lesser extent, in *Xenopus*. Transgenic, single, and compound knock-out mouse models have been discussed comprehensively and in detail in a review by Wynshaw-Boris (2012, see also Supplementary Table 1), therefore we will focus here on human congenital disease and related phenotypes in animal models. In humans, mutations in *DVL* genes have been associated with neural tube closure defects (NTD) and autosomal-dominant Robinow Syndrome (ADRS) (De Marco et al., 2013; Bunn et al., 2015; White et al., 2015, 2016).

Neural Tube Defects

During embryonic development, the neural tube is formed by elevation, convergence and fusion of the lateral neural folds to form a hollow tube. Morphogenesis and closure of the neural tube is affected by nutritional, environmental and genetic factors including Wnt/PCP signaling, which is illustrated by genetic association between NTD in humans and mutations in the PCP genes *VANGL1*, *VANGL2*, *CELSR1*, *FZD6,* and *DVL2* (Cai and Shi, 2014; reviewed in De Marco et al., 2014). Notably, also mutations in *DVL1* or *DVL3* have been identified in humans with NTD (**Figure 2**), although the correlation was not significant (De Marco et al., 2013; Merello et al., 2013; Chen et al., 2016).

Consistently, neural tube closure also requires the same PCP factors in mouse, frog, and zebrafish (Darken et al., 2002; Hamblet et al., 2002; Jessen et al., 2002; Curtin et al., 2003; Formstone and Mason, 2005; Wang et al., 2006). DVL2$^{-/-}$ mice show NTD while single and compound DVL1 and DVL3 deficient mice do not, suggesting that among the three *DVL* genes, *DVL2* is necessary and sufficient to mediate neural tube closure. However, DVL2$^{-/-}$ DVL3$^{+/-}$ and DVL1$^{-/-}$ DVL2$^{-/-}$ mice display much more severe NTD than DVL2$^{-/-}$ mice (Hamblet et al., 2002; Wang et al., 2006; Etheridge et al., 2008), which strongly suggests that DVL1 and DVL3 contribute directly or indirectly to neural tube closure. Along this line, maternal single knock-down of either DVL2 or DVL3 in *Xenopus* caused NTD (Tadjuidje et al., 2011), supporting a contribution of DVL2 and DVL3.

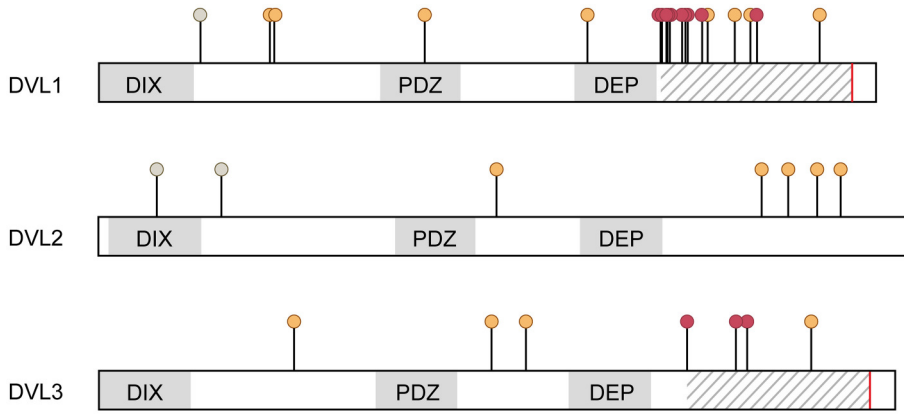

FIGURE 2 | Mutations identified in the three *DVL* genes in humans. Mutations are indicated at the positions of amino acid changes. Changes detected in individuals with NTDs (De Marco et al., 2013; Merello et al., 2013; Chen et al., 2016) are color coded orange (predicted pathogenic) and gray (predicted benign, in all cases A>V). All ADRS mutations are (−1)-frameshift mutations resulting in altered amino acid sequences in the C-terminus and a premature stop (Bunn et al., 2015; White et al., 2015, 2016), which are indicated by hatched area and red line respectively. Positions of individual mutations associated with ADRS are labelled with red dots.

Autosomal-Dominant Robinow Syndrome

Robinow syndrome is a rare genetic disorder characterized by mesomelic limb shortening, short stature, cranio-facial malformations, microgenitalia, and occasional cardiac outflow tract defects with either autosomal dominant or recessive inheritance (reviewed in Robinow et al., 1969; Patton and Afzal, 2002). Notably and despite the multifaceted clinical presentation of affected individuals, NTD have neither been described in ADRS nor the more severe recessive Robinow syndrome (RRS). Two recent studies have identified mutations in exon 14 of *DVL1* and *DVL3* as causative for ADRS (Bunn et al., 2015; White et al., 2015, 2016, **Figure 2**). In addition, ADRS is also associated with *WNT5A* whereas RRS is caused by loss-of-function mutations in *ROR2* (Afzal et al., 2000; van Bokhoven et al., 2000; Person et al., 2010). Thus, the features of Robinow syndrome are generally considered as consequences of defective WNT5A/ROR2-mediated PCP signaling in multiple tissues (Wang et al., 2011) and are partially recapitulated in DVL deficient animal models.

Short stature and defects of the axial skeleton are likely related to impaired convergent extension movements of the paraxial mesoderm and defects in somitogenesis, which have also been reported for DVL2$^{-/-}$, DVL1$^{-/-}$;DVL2$^{-/-}$, and DVL2$^{+/-}$;DVL3$^{-/-}$ mice as well as for *Xenopus* embryos deficient of any DVL paralog (Hamblet et al., 2002; Etheridge et al., 2008; Gray et al., 2009; Gentzel et al., 2015), indicating that all DVL paralogs contribute to the development of the axial skeleton although DVL2 seems of particular importance.

The characteristic cranio-facial deformations seen in ADRS or RRS indicate defective development of the neural crest (NC), which gives rise to the majority of cranial cartilage and bone. In addition, a subpopulation of the NC contributes to the cardiac outflow tract (OFT). Cranio-facial malformations are also visible in DVL1 and DVL2 morphant *Xenopus* embryos and the abovementioned mice. The latter and additionally DVL3$^{-/-}$ animals also show cardiac OFT defects. In *Xenopus*, DVL1 or DVL2 morphant embryos showed normal NC induction but defects in NC migration. The NC is present in DVL3$^{-/-}$ mice whereas the cardiac NC markers PITX2 and PLEXINA2 were decreased in mice lacking DVL2 (Hamblet et al., 2002; Etheridge et al., 2008; Gray et al., 2009), indicating differential roles of DVL2 and DVL3 in NC and cardiac development.

Interestingly, *DVL1* mutations in humans affect predominantly cranio-facial development with little or no aberrations in body height, whereas in *DVL3* and *WNT5A* associated ADRS craniofacial malformations are accompanied by short stature (Person et al., 2010; Bunn et al., 2015; White et al., 2015, 2016). In mouse, *DVL1* is predominantly expressed in the neuroectoderm (Sussman et al., 1994) and as discussed above, knock-out models suggest dominant roles of DVL2 and DVL3 in the development of the axial skeleton and the heart. Although the spatial expression of the three *DVL* isoforms in human embryos is unknown, the differential prevalence of defects in the axial skeleton in DVL1 and DVL3 associated ADRS supports a prevailing role of DVL2/3 in the paraxial mesoderm in mammals.

DVL Signaling in Embryonic Development

The recently characterized mutations in *DVL1* and *DVL3* are frameshift mutations, which alter and shorten the C-termini in the corresponding proteins (White et al., 2015, 2016). This C-terminal domain has been shown to interact with ROR2, a major receptor in β-Catenin independent Wnt signaling and affected in RRS, and is required for ROR2-mediated inhibition of Wnt/β-Catenin signaling (Witte et al., 2010). An initial study suggests a gain of Wnt/β-Catenin activity in ADRS (Bunn et al., 2015), thus it is conceivable that ADRS mutations in *DVL1* and *DVL3* might result in defective β-Catenin independent Wnt signaling and concomitantly upregulate Wnt/β-Catenin activity.

Malformations of the axial skeleton seen in DVL2$^{-/-}$ and DVL1$^{-/-}$;DVL2$^{-/-}$ mice are reminiscent of the phenotypes in ROR2 or WNT5A deficient embryos that can be attributed to aberrant PCP signaling in the paraxial mesoderm (reviewed in Stricker et al., 2017). Genetic interactions between DVL2 and DVL3 with VANGL2 in the mouse further suggest that DVL2 acts in Wnt/PCP signaling in neural tube closure and in cochlea development (Wang et al., 2006; Etheridge et al., 2008). In addition, NTDs and OFT defects in DVL1$^{-/-}$;DVL2$^{-/-}$ mice were similar to defects in VANGL2 mutants and rescued by a DVL2ΔDIX transgene, which is defective in β-Catenin signaling but retains activity in PCP signaling (Wang et al., 2006; Sinha et al., 2012). Notably, also DVL3 KO mice develop OFT defects, but no genetic interaction with VANGL2 in OFT morphogenesis could be demonstrated, indicating a non-redundant function of DVL3 (Etheridge et al., 2008).

Wnt/β-Catenin signaling also contributes to the development of the paraxial mesoderm, heart and neural crest, and patterns the neural tube. However, defective Wnt/β-Catenin signaling results in patterning defects of the dorsal mesoderm and affects proliferation, expansion, or specification of dorsal neural tube progenitors and neural crest (NC) cells (Greco et al., 1996; Ikeya et al., 1997; Pinson et al., 2000; Lou et al., 2008; Seldin et al., 2008; Valenta et al., 2011). By contrast, in either single or compound DVL knock-out mice, Wnt/β-Catenin signaling, and early dorsal mesoderm markers were unaffected. Only in triple knock-out mice a marked downregulation of Wnt/β-catenin signaling has been observed (Etheridge et al., 2008; Hashimoto et al., 2010). Still, defective β-Catenin signaling in smaller cell populations cannot be excluded. One such example might be the cardiac neural crest in DVL2$^{-/-}$ embryos, in which the β-Catenin target PITX2 is downregulated (Hamblet et al., 2002; Kioussi et al., 2002). PITX2 is required for proliferation of cardiac NC, but also for the interaction between cardiac NC and second heart field cells (Kioussi et al., 2002; Ma et al., 2013) and, indirectly, for OFT morphogenesis via its target gene *WNT11* (Zhou et al., 2007). OFT defects in DLV1$^{-/-}$, DVL2$^{-/-}$ embryos were rescued by a DVL2ΔDIX transgene (Sinha et al., 2012), however this does not exclude a role of DVL2 in β-Catenin signaling upstream of PITX2 since the transgene could also restore OFT morphogenesis downstream of WNT11.

Overall it appears that DVL function in β-Catenin-independent Wnt signaling is more sensitive to the loss or dysfunction of one or two paralogs and accounts for most of the developmental phenotypes in knock-out animal models and also for the features of ADRS.

Induced Heart Defects

A number of studies indicate a specific role of DVL1 in cardiac remodeling and regeneration. DVL1 and CamKII are upregulated after induced myocardial infarction and heart failure indicating a role of Wnt/Ca^{2+} signaling in regeneration (Chen et al., 2004; Ai et al., 2005; Bossuyt et al., 2008). Persistent pressure overload induced cardiac hypertrophy was attenuated in *DVL1* knock-out mice, which has been attributed to lower Wnt/β-Catenin activity as well as decreased AKT activation (van de Schans et al., 2007). Consistently, DVL1 gain-of-function induced progressive cardiomyopathy (Malekar et al., 2010). Interestingly, evidence is accumulating that DVL1 is functionally associated with Wnt/Ca^{2+} and CamKII signaling in cardiomyopathy (Malekar et al., 2010; Zhang et al., 2015), in excitory synapses in the rat spinal chord (Ciani et al., 2011) and in convergent extension movements in *Xenopus* gastrulation (Gentzel et al., 2015), indicating a functional specification of DVL1.

PERSPECTIVES

Striking differences between different DVLs and species have been observed in temporal and spatial expression patterns. Loss-of-function phenotypes of each single paralog in mouse as well as ADRS features associated with *DVL1* or *DVL3* mutations also differ, indicating some degree of divergence but also overlapping functions. In addition, expression of transgenes in a single knock-out background further supported partial redundancy and indicated a dose dependency. If the DVL paralogs would be fully redundant in function it might be speculated that the summed abundance of all paralogs is important for cell survival. But even in cell culture models, any single knock-down is effective and the cells apparently do not sense overall "DVL concentration" and do not compensate the down-regulation of one protein by upregulation of the others (Cervenka et al., 2016).

Functional redundancies however, do not connote biochemical identity. The observed differences could reflect differential expression levels, epistatic relations, or differential biochemical properties such as protein-protein interaction affinity and consequently also molecular function, which would also be consistent with dose-dependencies. The developmental phenotypes further indicate that β-Catenin independent Wnt pathways are more sensitive to the dose of individual DVL paralogs than β-Catenin signaling. This hypothesis is further supported by comparison of triple knock-out and triple-RNAi knock-down embryos. Whereas, in triple KO embryos early β-Catenin signaling is strongly reduced resulting in axis and mesodermal mispatterning, in the knock-down, in which ∼25–30% of each paralog were retained, early β-Catenin signaling was not affected, but the embryos showed strong morphogenetic defects in the dorsal mesoderm and neuroectoderm. Consistently, specific and different molecular functionality of the three DVL paralogs has been observed in ciliogenesis and the Wnt/Ca^{2+} pathway, which were revealed in intact tissue or tissue models (Park et al., 2008; Gentzel et al., 2015).

Overall, the currently available data indicate that DVL expression and function have diverged to some degree apparently and consistent with phylogenetic models mostly between DVL1 and DVL2/3. Thus, depending on the cellular context, DVL paralogs exhibit both redundant and distinct functionality.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

The authors' original work was funded by German Research foundation (DFG) grants to AS (grant numbers SCHA965/6-1 and 6-2), MG was funded by the Max Planck Society (MPG).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2017.00059/full#supplementary-material

REFERENCES

Afzal, A. R., Rajab, A., Fenske, C. D., Oldridge, M., Elanko, N., Ternes-Pereira, E., et al. (2000). Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. *Nat. Genet.* 25, 419–422. doi: 10.1038/78107

Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M., and Pogwizd, S. M. (2005). Ca^{2+}/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca^{2+} leak in heart failure. *Circ. Res.* 97, 1314–1322. doi: 10.1161/01.RES.0000194329.41863.89

Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T., and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes Dev.* 12, 2610–2622.

Bossuyt, J., Helmstadter, K., Wu, X., Clements-Jewery, H., Haworth, R. S., Avkiran, M., et al. (2008). Ca^{2+}/calmodulin-dependent protein kinase IIdelta and protein kinase D overexpression reinforce the histone deacetylase 5 redistribution in heart failure. *Circ. Res.* 102, 695–702. doi: 10.1161/CIRCRESAHA.107.169755

Boutros, M., Paricio, N., Strutt, D. I., and Mlodzik, M. (1998). Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* 94, 109–118.

Bunn, K. J., Daniel, P., Rösken, H. S., O'Neill, A. C., Cameron-Christie, S. R., Morgan, T., et al. (2015). Mutations in DVL1 cause an osteosclerotic form of Robinow syndrome. *Am. J. Hum. Genet.* 96, 623–630. doi: 10.1016/j.ajhg.2015.02.010

Cai, C., and Shi, O. (2014). Genetic evidence in planar cell polarity signaling pathway in human neural tube defects. *Front. Med.* 8, 68–78. doi: 10.1007/s11684-014-0308-4

Capelluto, D. G. S., Kutateladze, T. G., Habas, R., Finkielstein, C. V., He, X., and Overduin, M. (2002). The DIX domain targets dishevelled to actin stress fibres and vesicular membranes. *Nature* 419, 726–729. doi: 10.1038/nature01056

Cervenka, I., Valnohova, J., Bernatik, O., Harnoš, J., Radsetoulal, M., Sedová, K., et al. (2016). Dishevelled is a NEK2 kinase substrate controlling dynamics of centrosomal linker proteins. *Proc. Natl. Acad. Sci. U.S.A.* 113, 9304–9309. doi: 10.1073/pnas.1608783113

Chen, L., Wu, Q., Guo, F., Xia, B., and Zuo, J. (2004). Expression of Dishevelled-1 in wound healing after acute myocardial infarction: possible involvement in myofibroblast proliferation and migration. *J. Cell. Mol. Med.* 8, 257–264. doi: 10.1111/j.1582-4934.2004.tb00281.x

Chen, S., Zhang, Q., Bai, B., Ouyang, S., Bao, Y., Li, H., et al. (2016). MARK2/Par1b insufficiency attenuates *DVL* gene transcription via histone deacetylation in lumbosacral spina bifida. *Mol. Neurobiol.* doi: 10.1007/s12035-016-0164-0. [Epub ahead of print].

Ciani, L., Boyle, K. A., Dickins, E., Sahores, M., Anane, D., Lopes, D. M., et al. (2011). Wnt7a signaling promotes dendritic spine growth and synaptic strength through Ca^{2+}/Calmodulin-dependent protein kinase II. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10732–10737. doi: 10.1073/pnas.1018132108

Ciani, L., Krylova, O., Smalley, M. J., Dale, T. C., and Salinas, P. C. (2004). A divergent canonical WNT-signaling pathway regulates microtubule dynamics: dishevelled signals locally to stabilize microtubules. *J. Cell Biol.* 164, 243–253. doi: 10.1083/jcb.200309096

Collu, G. M., and Mlodzik, M. (2015). Planar polarity: converting a morphogen gradient into cellular polarity. *Curr. Biol.* 25, R372–R374. doi: 10.1016/j.cub.2015.03.011

Collu, G. M., Hidalgo-Sastre, A., Acar, A., Bayston, L., Gildea, C., Leverentz, M. K., et al. (2012). Dishevelled limits Notch signalling through inhibition of CSL. *Development* 139, 4405–4415. doi: 10.1242/dev.081885

Curtin, J. A., Quint, E., Tsipouri, V., Arkell, R. M., Cattanach, B., Copp, A. J., et al. (2003). Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. *Curr. Biol.* 13, 1129–1133. doi: 10.1016/S0960-9822(03)00374-9

Darken, R. S., Scola, A. M., Rakeman, A. S., Das, G., Mlodzik, M., and Wilson, P. A. (2002). The planar polarity gene strabismus regulates convergent extension movements in Xenopus. *EMBO J.* 21, 976–985. doi: 10.1093/emboj/21.5.976

De Marco, P., Merello, E., Consales, A., Piatelli, G., Cama, A., Kibar, Z., et al. (2013). Genetic analysis of disheveled 2 and disheveled 3 in human neural tube defects. *J. Mol. Neurosci.* 49, 582–588. doi: 10.1007/s12031-012-9871-9

De Marco, P., Merello, E., Piatelli, G., Cama, A., Kibar, Z., and Capra, V. (2014). Planar cell polarity gene mutations contribute to the etiology of human neural tube defects in our population. *Birth Defects Res. A Clin. Mol. Teratol.* 100, 633–641. doi: 10.1002/bdra.23255

Deng, N., Ye, Y., Wang, W., and Li, L. (2010). Dishevelled interacts with p65 and acts as a repressor of NF-κB-mediated transcription. *Cell Res.* 20, 1117–1127. doi: 10.1038/cr.2010.108

Diez-Roux, G., Banfi, S., Sultan, M., Geffers, L., Anand, S., Rozado, D., et al. (2011). A high-resolution anatomical atlas of the transcriptome in the mouse embryo. *PLoS Biol.* 9:e1000582. doi: 10.1371/journal.pbio.1000582.s018

Dillman, A. R., Minor, P. J., and Sternberg, P. W. (2013). Origin and evolution of dishevelled. *G3 (Bethesda).* 3, 251–262. doi: 10.1534/g3.112.005314

Etheridge, S. L., Ray, S., Li, S., Hamblet, N. S., Lijam, N., Tsang, M., et al. (2008). Murine dishevelled 3 functions in redundant pathways with dishevelled 1 and 2 in normal cardiac outflow tract, cochlea, and neural tube development. *PLoS Genet.* 4:e1000259. doi: 10.1371/journal.pgen.1000259

Fahmy, O. G., and Fahmy, M. (1959). [New mutants report]. *Drosoph. Inf. Serv.* 33, 82–94.

Formstone, C. J., and Mason, I. (2005). Combinatorial activity of Flamingo proteins directs convergence and extension within the early zebrafish embryo via the planar cell polarity pathway. *Dev. Biol.* 282, 320–335. doi: 10.1016/j.ydbio.2005.03.026

Gan, X.-Q., Wang, J.-Y., Xi, Y., Wu, Z.-L., Li, Y.-P., and Li, L. (2008). Nuclear Dvl, c-Jun, β-catenin, and TCF form a complex leading to stabilization of β-catenin-TCF interaction. *J. Cell Biol.* 180, 1087–1100. doi: 10.1083/jcb.200710050

Gao, C., and Chen, Y.-G. (2010). Dishevelled: the hub of Wnt signaling. *Cell. Signal.* 22, 717–727. doi: 10.1016/j.cellsig.2009.11.021

Gentzel, M., Schille, C., Rauschenberger, V., and Schambony, A. (2015). Distinct functionality of dishevelled isoforms on Ca^{2+}/calmodulin-dependent protein kinase 2 (CamKII) in Xenopus gastrulation. *Mol. Biol. Cell* 26, 966–977. doi: 10.1091/mbc.E14-06-1089

Gray, R. S., Bayly, R. D., Green, S. A., Agarwala, S., Lowe, C. J., and Wallingford, J. B. (2009). Diversification of the expression patterns and developmental functions of the dishevelled gene family during chordate evolution. *Dev. Dyn.* 238, 2044–2057. doi: 10.1002/dvdy.22028

Greco, T. L., Takada, S., Newhouse, M. M., McMahon, J. A., McMahon, A. P., and Camper, S. A. (1996). Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. *Genes Dev.* 10, 313–324.

Hamblet, N. S., Lijam, N., Ruiz-Lozano, P., Wang, J., Yang, Y., Luo, Z., et al. (2002). Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. *Development* 129, 5827–5838. doi: 10.1242/dev.00164

Hashimoto, M., Shinohara, K., Wang, J., Ikeuchi, S., Yoshiba, S., Meno, C., et al. (2010). Planar polarization of node cells determines the rotational axis of node cilia. *Nat. Cell Biol.* 12, 170–176. doi: 10.1038/ncb2020

Ikeya, M., Lee, S. M., Johnson, J. E., McMahon, A. P., and Takada, S. (1997). Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* 389, 966–970. doi: 10.1038/40146

Itoh, K., Brott, B. K., Bae, G.-U., Ratcliffe, M. J., and Sokol, S. Y. (2005). Nuclear localization is required for Dishevelled function in Wnt/β-catenin signaling. *J. Biol.* 4, 3. doi: 10.1186/jbiol20

Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A., et al. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat. Cell Biol.* 4, 610–615. doi: 10.1038/ncb828

Jiang, X., Charlat, O., Zamponi, R., Yang, Y., and Cong, F. (2015). Dishevelled promotes Wnt receptor degradation through recruitment of ZNRF3/RNF43 E3 ubiquitin ligases. *Mol. Cell* 58, 522–533. doi: 10.1016/j.molcel.2015.03.015

Kasahara, M. (2007). The 2R hypothesis: an update. *Curr. Opin. Immunol.* 19, 547–552. doi: 10.1016/j.coi.2007.07.009

Kioussi, C., Briata, P., Baek, S. H., Rose, D. W., Hamblet, N. S., Herman, T., et al. (2002). Identification of a Wnt/Dvl/β-Catenin –> Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell* 111, 673–685. doi: 10.1016/S0092-8674(02)01084-X

Klingensmith, J., Yang, Y., Axelrod, J. D., Beier, D. R., Perrimon, N., and Sussman, D. J. (1996). Conservation of dishevelled structure and function between flies and mice: isolation and characterization of Dvl2. *Mech. Dev.* 58, 15–26.

Kohn, A. D., and Moon, R. T. (2005). Wnt and calcium signaling: β-catenin-independent pathways. *Cell Calcium* 38, 439–446. doi: 10.1016/j.ceca.2005.06.022

Kühl, M., Sheldahl, L. C., Park, M., Miller, J. R., and Moon, R. T. (2000). The Wnt/Ca^{2+} pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet.* 16, 279–283. doi: 10.1074/jbc.275.17.12701

Li, L., Yuan, H., Xie, W., Mao, J., Caruso, A. M., McMahon, A., et al. (1999). Dishevelled proteins lead to two signaling pathways. Regulation of LEF-1 and c-Jun N-terminal kinase in mammalian cells. *J. Biol. Chem.* 274, 129–134.

Livingstone, C. D., and Barton, G. J. (1993). Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation. *Comput. Appl. Biosci.* 9, 745–756.

Lou, D. Y., Dominguez, I., Toselli, P., Landesman-Bollag, E., O'Brien, C., and Seldin, D. C. (2008). The alpha catalytic subunit of protein kinase CK2 is required for mouse embryonic development. *Mol. Cell. Biol.* 28, 131–139. doi: 10.1128/MCB.01119-07

Ma, H.-Y., Xu, J., Eng, D., Gross, M. K., and Kioussi, C. (2013). Pitx2-mediated cardiac outflow tract remodeling. *Dev. Dyn.* 242, 456–468. doi: 10.1002/dvdy.23934

Macdonald, B. T., Tamai, K., and He, X. (2009). Wnt/β-catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17, 9–26. doi: 10.1016/j.devcel.2009.06.016

Malekar, P., Hagenmueller, M., Anyanwu, A., Buss, S., Streit, M. R., Weiss, C. S., et al. (2010). Wnt signaling is critical for maladaptive cardiac hypertrophy and accelerates myocardial remodeling. *Hypertension* 55, 939–945. doi: 10.1161/HYPERTENSIONAHA.109.141127

Merello, E., Kibar, Z., Allache, R., Piatelli, G., Cama, A., Capra, V., et al. (2013). Rare missense variants in DVL1, one of the human counterparts of the Drosophila dishevelled gene, do not confer increased risk for neural tube defects. *Birth Defects Res. Clin. Mol. Teratol.* 97, 452–455. doi: 10.1002/bdra.23157

Mlodzik, M. (2016). The dishevelled protein family: still rather a mystery after over 20 years of molecular studies. *Curr. Top. Dev. Biol.* 117, 75–91. doi: 10.1016/bs.ctdb.2015.11.027

Na, J., Lykke-Andersen, K., Torres Padilla, M. E., and Zernicka-Goetz, M. (2007). Dishevelled proteins regulate cell adhesion in mouse blastocyst and serve to monitor changes in Wnt signaling. *Dev. Biol.* 302, 40–49. doi: 10.1016/j.ydbio.2006.08.036

Niehrs, C., and Acebron, S. P. (2012). Mitotic and mitogenic Wnt signalling. *EMBO J.* 31, 2705–2713. doi: 10.1038/emboj.2012.124

Park, T. J., Mitchell, B. J., Abitua, P. B., Kintner, C., and Wallingford, J. B. (2008). Dishevelled controls apical docking and planar polarization of basal bodies in ciliated epithelial cells. *Nat. Genet.* 40, 871–879. doi: 10.1038/ng.104

Patton, M. A., and Afzal, A. R. (2002). Robinow syndrome. *J. Med. Genet.* 39, 305–310. doi: 10.1136/jmg.39.5.305

Penton, A., Wodarz, A., and Nusse, R. (2002). A mutational analysis of dishevelled in Drosophila defines novel domains in the dishevelled protein as well as novel suppressing alleles of axin. *Genetics* 161, 747–762.

Person, A. D., Beiraghi, S., Sieben, C. M., Hermanson, S., Neumann, A. N., Robu, M. E., et al. (2010). WNT5A mutations in patients with autosomal dominant Robinow syndrome. *Dev. Dyn.* 239, 327–337. doi: 10.1002/dvdy.22156

Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J., and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407, 535–538. doi: 10.1038/35035124

Robinow, M., Silverman, F. N., and Smith, H. D. (1969). A newly recognized dwarfing syndrome. *Am. J. Dis. Child.* 117, 645–651.

Seldin, D. C., Lou, D. Y., Toselli, P., Landesman-Bollag, E., and Dominguez, I. (2008). Gene targeting of CK2 catalytic subunits. *Mol. Cell. Biochem.* 316, 141–147. doi: 10.1007/s11010-008-9811-8

Sepich, D. S., Usmani, M., Pawlicki, S., and Solnica-Krezel, L. (2011). Wnt/PCP signaling controls intracellular position of MTOCs during gastrulation convergence and extension movements. *Development* 138, 543–552. doi: 10.1242/dev.053959

Sinha, T., Wang, B., Evans, S., Wynshaw-Boris, A., and Wang, J. (2012). Disheveled mediated planar cell polarity signaling is required in the second heart field lineage for outflow tract morphogenesis. *Dev. Biol.* 370, 135–144. doi: 10.1016/j.ydbio.2012.07.023

Stricker, S., Rauschenberger, V., and Schambony, A. (2017). ROR-family receptor tyrosine kinases. *Curr. Top. Dev. Biol.* 123, 105–142. doi: 10.1016/bs.ctdb.2016.09.003

Sussman, D. J., Klingensmith, J., Salinas, P., Adams, P. S., Nusse, R., and Perrimon, N. (1994). Isolation and characterization of a mouse homolog of the Drosophila segment polarity gene dishevelled. *Dev. Biol.* 166, 73–86. doi: 10.1006/dbio.1994.1297

Tadjuidje, E., Cha, S.-W., Louza, M., Wylie, C., and Heasman, J. (2011). The functions of maternal Dishevelled 2 and 3 in the Early Xenopus embryo. *Dev. Dyn.* 240, 1727–1736. doi: 10.1002/dvdy.22671

Tauriello, D. V. F., Jordens, I., Kirchner, K., Slootstra, J. W., Kruitwagen, T., Bouwman, B. A. M., et al. (2012). Wnt/β-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proc. Natl. Acad. Sci. U.S.A.* 109, E812–E820. doi: 10.1073/pnas.1114802109

Tsang, M., Lijam, N., Yang, Y., Beier, D. R., Wynshaw-Boris, A., and Sussman, D. J. (1996). Isolation and characterization of mouse dishevelled-3. *Dev. Dyn.* 207, 253–262. doi: 10.1002/(SICI)1097-0177(199611)207:3<253::AID-AJA2>3.0.CO;2-G

Valenta, T., Gay, M., Steiner, S., Draganova, K., Zemke, M., Hoffmans, R., et al. (2011). Probing transcription-specific outputs of β-catenin *in vivo*. *Genes Dev.* 25, 2631–2643. doi: 10.1101/gad.181289.111

van Amerongen, R. (2012). Alternative Wnt Pathways and Receptors. *Cold Spring Harb. Perspect. Biol.* 4:a007914. doi: 10.1101/cshperspect.a007914

van Bokhoven, H., Celli, J., Kayserili, H., van Beusekom, E., Balci, S., Brussel, W., et al. (2000). Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. *Nat. Genet.* 25, 423–426. doi: 10.1038/78113

van de Schans, V. A. M., van den Borne, S. W. M., Strzelecka, A. E., Janssen, B. J. A., van der Velden, J. L. J., Langen, R. C. J., et al. (2007). Interruption of Wnt signaling attenuates the onset of pressure overload-induced cardiac hypertrophy. *Hypertension* 49, 473–480. doi: 10.1161/01.HYP.0000255946.55091.24

Vladar, E. K., Antic, D., and Axelrod, J. D. (2009). Planar cell polarity signaling: the developing cell's compass. *Cold Spring Harb. Perspect. Biol.* 1:a002964. doi: 10.1101/cshperspect.a002964

Wang, B., Sinha, T., Jiao, K., Serra, R., and Wang, J. (2011). Disruption of PCP signaling causes limb morphogenesis and skeletal defects and may underlie Robinow syndrome and brachydactyly type B. *Hum. Mol. Genet.* 20, 271–285. doi: 10.1093/hmg/ddq462

Wang, J., Hamblet, N. S., Mark, S., Dickinson, M. E., Brinkman, B. C., Segil, N., et al. (2006). Dishevelled genes mediate a conserved mammalian PCP pathway to regulate convergent extension during neurulation. *Development* 133, 1767–1778. doi: 10.1242/dev.02347

Wang, W., Li, X., Lee, M., Jun, S., Aziz, K. E., Feng, L., et al. (2015). FOXKs promote Wnt/β-catenin signaling by translocating DVL into the nucleus. *Dev. Cell* 32, 707–718. doi: 10.1016/j.devcel.2015.01.031

White, J. J., Mazzeu, J. F., Hoischen, A., Bayram, Y., Withers, M., Gezdirici, A., et al. (2016). DVL3 Alleles resulting in a -1 frameshift of the last exon mediate autosomal-dominant robinow syndrome. *Am. J. Hum. Genet.* 98, 553–561. doi: 10.1016/j.ajhg.2016.01.005

White, J., Mazzeu, J. F., Hoischen, A., Jhangiani, S. N., Gambin, T., Alcino, M. C., et al. (2015). DVL1 frameshift mutations clustering in the penultimate exon cause autosomal-dominant Robinow syndrome. *Am. J. Hum. Genet.* 96, 612–622. doi: 10.1016/j.ajhg.2015.02.015

Witte, F., Bernatik, O., Kirchner, K., Masek, J., Mahl, A., Krejci, P., et al. (2010). Negative regulation of Wnt signaling mediated by CK1-phosphorylated Dishevelled via Ror2. *FASEB J.* 24, 2417–2426. doi: 10.1096/fj.09-150615

Wynshaw-Boris, A. (2012). Dishevelled: *in vivo* roles of a multifunctional gene family during development. *Curr. Top. Dev. Biol.* 101, 213–235. doi: 10.1016/B978-0-12-394592-1.00007-7

Yu, A., Rual, J.-F., Tamai, K., Harada, Y., Vidal, M., He, X., et al. (2007). Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. *Dev. Cell* 12, 129–141. doi: 10.1016/j.devcel.2006.10.015

Zhang, M., Hagenmueller, M., Riffel, J. H., Kreusser, M. M., Bernhold, E., Fan, J., et al. (2015). Calcium/calmodulin-dependent protein kinase II couples Wnt signaling with histone deacetylase 4 and mediates dishevelled-induced cardiomyopathy. *Hypertension* 65, 335–344. doi: 10.1161/HYPERTENSIONAHA.114.04467

Zhou, W., Lin, L., Majumdar, A., Li, X., Zhang, X., Liu, W., et al. (2007). Modulation of morphogenesis by noncanonical Wnt signaling requires ATF/CREB family-mediated transcriptional activation of TGFβ2. *Nat. Genet.* 39, 1225–1234. doi: 10.1038/ng2112

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 26 May 2017
doi: 10.3389/fcell.2017.00056

Autophagy in the Vertebrate Inner Ear

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Autophagy is a conserved catabolic process that results in the lysosomal degradation of cell components. During development, autophagy is associated with tissue and organ remodeling, and under physiological conditions it is tightly regulated as it plays a housekeeping role in removing misfolded proteins and damaged organelles. The vertebrate inner ear is a complex sensory organ responsible for the perception of sound and for balance. Cell survival, death and proliferation, as well as cell fate specification and differentiation, are processes that are strictly coordinated during the development of the inner ear in order to generate the more than a dozen specialized cell types that constitute this structure. Here, we review the existing evidence that implicates autophagy in the generation of the vertebrate inner ear. At early stages of chicken otic development, inhibiting autophagy impairs neurogenesis and causes aberrant otocyst morphogenesis. Autophagy provides energy for the clearing of dying cells and it favors neuronal differentiation. Moreover, autophagy is required for proper vestibular development in the mouse inner ear. The autophagy-related genes *Becn1*, *Atg4g*, *Atg5,* and *Atg9*, are expressed in the inner ear from late developmental stages to adulthood, and *Atg4b* mutants show impaired vestibular behavior associated to defects in otoconial biogenesis that are also common to *Atg5* mutants. Autophagic flux appears to be age-regulated, augmenting from perinatal stages to young adulthood in mice. This up-regulation is concomitant with the functional maturation of the hearing receptor. Hence, autophagy can be considered an intracellular pathway fundamental for in vertebrate inner ear development and maturation.

Keywords: Atg4, Atg5, Beclin-1, cochlea, LC3, otic development, vestibular system

OPEN ACCESS

Edited by:
Andrea Erika Münsterberg, University of East Anglia, United Kingdom

Reviewed by:
Markus A. N. Hartl, University of Innsbruck, Austria
Mai Har Sham, University of Hong Kong, Hong Kong

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *23 January 2017*
Accepted: *09 May 2017*
Published: *26 May 2017*

Citation:
Magariños M, Pulido S, Aburto MR, de Iriarte Rodríguez R and Varela-Nieto I (2017) Autophagy in the Vertebrate Inner Ear. Front. Cell Dev. Biol. 5:56. doi: 10.3389/fcell.2017.00056

AN INTRODUCTION TO AUTOPHAGY

Autophagy is a catabolic process that degrades the cytoplasmic content of a cell in lysosomes, returning energy, and molecular building bricks to the cell. Indeed, autophagy has a housekeeping role in cells as it is a way to eliminate damaged macromolecules, organelles, and pathogens. Since the initial description of autophagy by Christian de Duve in 1963, it has become more and more relevant as it has become implicated in a variety of physiological and pathological situations (Jiang and Mizushima, 2014). Indeed, three different types of autophagy are now recognized: (1) Macroautophagy (herein autophagy), where a double-membrane autophagosome forms and engulfs cytoplasmic content, subsequently fusing with the lysosome to form an autolysosome and releasing the autophagosome cargo into the lysosome lumen to be degraded by hydrolases; (2) Microautophagy, in which the cargo reaches the lumen by invagination of the lysosomal membrane;

and (3) Chaperone-mediated autophagy, exclusive to mammals, where proteins associated to chaperones bind to the LAMP2A lysosomal receptor and are delivered directly to the lumen (Tasset and Cuervo, 2016).

The formation of the autophagosome requires the activity of a set of proteins, most of them encoded by the autophagy related genes (*ATG*; **Figure 1A**). The formation of the autophagosome involves induction, nucleation, elongation, and completion. A specific subset of ATG proteins has been associated to each of these stages (Ariosa and Klionsky, 2016). As such, the ULK1/2 complex (ATG13, ATG101, FIP200) participates in induction and ULK1 activates the phosphatidylinositol 3-kinase complex (PI3KC: Beclin-1, Vsp34, Vps15, ATG14) to promote nucleation. Two ubiquitin-like conjugation systems contribute to elongate the phagophore: ATG12 (ATG12, ATG7, ATG10, ATG5, and ATG16L) and ATG8 (LC3, the mammalian homolog of ATG8). Both these complexes regulate the formation of LC3-II, the relative levels of which serve as a readout of the autophagic flux, along with SQSTM1/p62 that facilitates the entry of the cargo into the autophagosome. Accordingly, the SQSTM1/p62 levels are inversely correlated with those of LC3-II (Katsuragi et al., 2015; Klionsky et al., 2016). Finally, the ATG9 cycling system incorporates membranes from cell donor locations (Pavel and Rubinsztein, 2016). Following the completion of the autophagosome, its fusion with lysosomes requires the activity of proteins involved in other vesicular fusion events, such as the SNARE (soluble NSF attachment protein receptor) and HOPS (homotypic fusion and vacuole sorting proteins) complexes (Zhen and Li, 2015).

Autophagy can be induced by starvation, growth factor deprivation, hypoxia, or infections. These stimuli elicit an immediate response and long-term gene expression responses mediated by specific transcription factors like TFEB (transcription factor EB). TFEB acts as a master regulator of autophagy by up-regulating the expression of autophagy genes. Under nutrient-rich conditions, TFEB is phosphorylated by mTORC1 (mammalian target of rapamycin complex (1) and kept inactivate in the cytosol, mTORC1 also inhibiting autophagy by phosphorylation of ATG13, Füllgrabe et al., 2016; Napolitano and Ballabio, 2016).

Autophagy is a housekeeping mechanism that removes damaged molecules and organelles from the cell's cytoplasm, yet it also participates in the immune response, and it provides energy and molecules as building blocks when needed. Autophagy is essential during development, as it contributes to organ and tissue sculpting in *Drosophila* by facilitating cell death (Denton et al., 2012). Indeed, autophagy may promote largescale cytosolic self-digestion and the removal of certain pro-survival proteins (Yu et al., 2006). Thus, the final output of autophagy could be either positive or negative for the cell, and this depends on the intensity and duration of its induction.

DEVELOPMENTAL AUTOPHAGY

Autophagy contributes to developmental tissue remodeling, responding to specific extrinsic, and intrinsic stimuli. For example, following fertilization of the mouse egg, autophagy removes maternal mRNA and proteins, allowing the egg to initiate its zygotic program (Tsukamoto et al., 2008; Yamamoto et al., 2014). Later on in development, autophagy drives the development of the nervous system, adipose tissue, osseous tissue, hematopoietic system, and the heart (Aburto et al., 2012a). The study of genetically modified mice has shed light on the roles played by the genes involved in autophagy. *Ambra1* is an essential gene for the development of the mouse central nervous system, the deficiency of which impairs autophagy and induces aberrant neuronal proliferation (Fimia et al., 2007; Antonioli et al., 2015). Different mutations in genes that participate in the autophagy machinery have shown that autophagy is needed for terminal neuronal differentiation, and specifically for axonal outgrowth and guidance. For example, axon formation is disturbed in the cerebellar granule neurons of $Ulk1^{-/-}$ mice (Zhou et al., 2007) and more recently, ALFY, an adaptor protein between the cargo and the ATG proteins, was seen to be required for axon outgrowth in the brain and to establish neuronal connectivity (Dragich et al., 2016).

Atg mutants have provided evidence that autophagy is needed for the correct development of adipose, osseous and cardiac tissues, as well as for the differentiation of hematopoietic cells. *Atg5 and Atg7* deficiency is associated with a reduction in thymocytes and B-lymphocytes (Pua et al., 2007), as well as reduced levels of adipocyte differentiation factors and decreased lipid adipose mass (Singh et al., 2009). In addition, *Atg5* and *Atg7* deficiency in the embryonic P19CL6 cells inhibited cardiac cell differentiation (Jia et al., 2014). However, in the analysis of the phenotypes associated to these mutations it should be considered that ATG proteins also fulfill functions that are not related to autophagy (Mauthe and Reggiori, 2016).

During development, autophagy facilitates rapid changes in intracellular composition, promoting the turnover of specific proteins, receptors, cytoskeletal components, or transcription factors necessary to define the different cell fates. It is also essential for the temporal dynamics of cell organelles, controlling their number, and quality (e.g., mitochondria). Finally, after birth and before the initiation of suckling behavior, the up-regulation of autophagy protects newborns from death by starvation (Kuma et al., 2004). Autophagy may not only supply energy at this stage but it may also help control oxidative stress (Schiaffino et al., 2008).

AN INTRODUCTION TO INNER EAR ANATOMY

The mammalian inner ear is a complex sensory structure within the temporal bone that is composed of the cochlea and the vestibule, structures that are responsible for the senses of hearing and balance, respectively (**Figure 1B**). The auditory and vestibular organs contain the mechanosensory receptors that transduce mechanical stimuli into electrochemical signals that are transmitted to the brain by the fibers of the VIIIth cranial nerve. The auditory receptor is the organ of Corti in the scala media of the cochlea (Magariños et al., 2012, 2014), which is

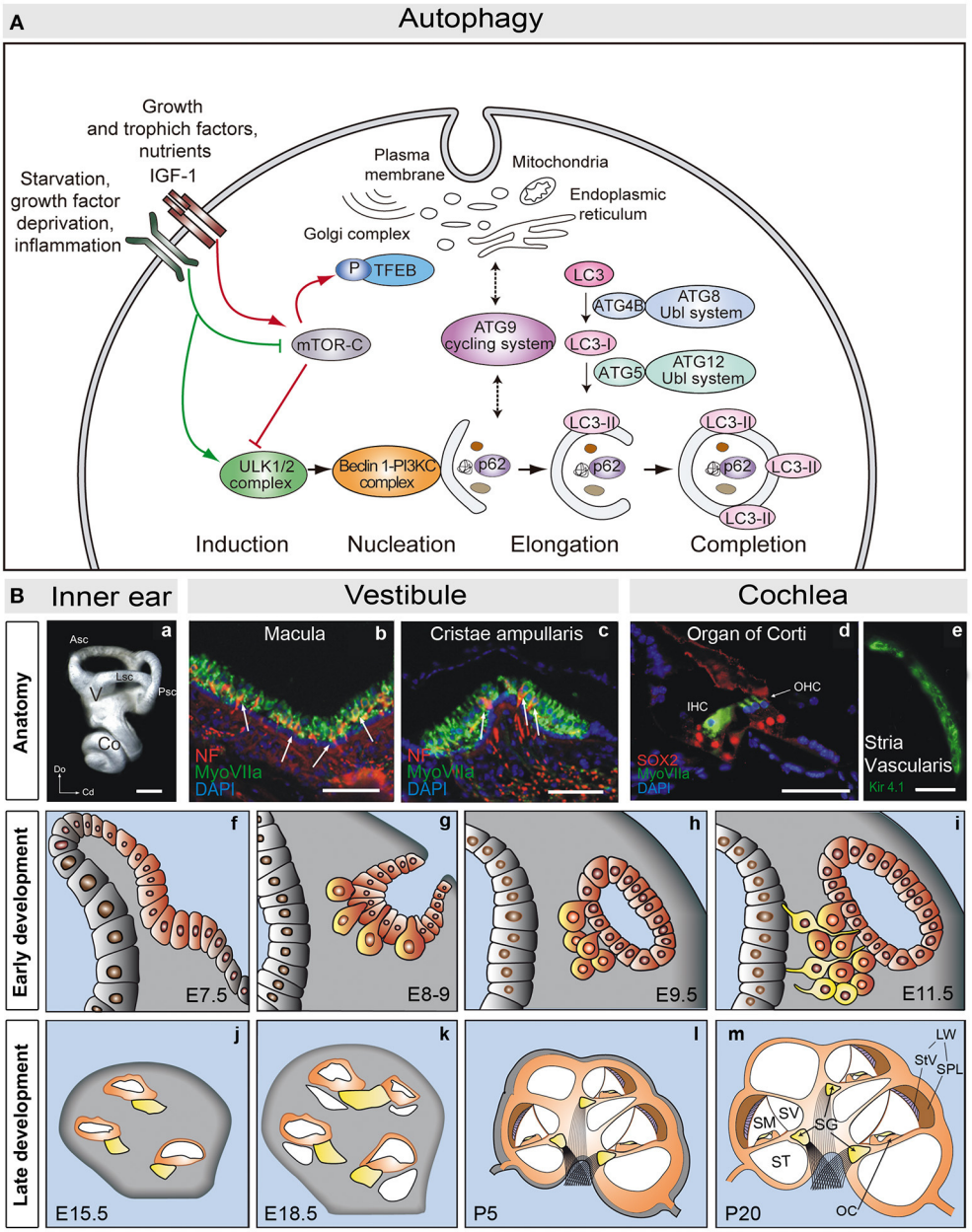

FIGURE 1 | (A) Schematic view of the molecular steps of macroautophagy. Growth factors and nutrient-rich conditions activate mTORC1, a negative regulator of the ULK1/2 complex and TEFB. In turn, growth factor deprivation, inflammation, or nutrient starvation, activate the ULK1/2 complex, which phosphorylates and activates the PI3K complex III (PI3KC). The ATG9 cycling system provides membranes to form the autophagosome from different donor sources. Autophagosome formation also requires the action of two ubiquitin-like (Ubl) systems, ATG8-Ubl and ATG12-Ubl, required for the elongation and completion of the autophagosome. LC3 is converted into the cytosolic form, LC3-I, by cleavage of ATG4B, and into the membrane associated form, LC3-II, by conjugation with phosphoethanolamine via ATG5 (and the remaining components of the ATG12-Ubl system). SQSTM1/p62 (p62) binds to ubiquitinated proteins and carries them to the autophagosome (adapted from de Iriarte Rodríguez et al., 2015). **(B)** Anatomy of the adult mouse inner ear. **(a)** Lateral view showing a mammalian inner ear. **(b,c)** Detail of the vestibular macula **(b)** and cristae ampullaris **(c)**, where sensory hair cells are labeled for myosin VIIa (green) and neurofilament (red). **(d)** Detail of the organ of Corti showing myosin VIIa positive hair cells (green) and SOX2 positive supporting cells (red). **(e)** The stria vascularis is visualized by labeling for Kir4.1 (green). Development of the mouse inner ear. The inner ear develops from the otic placode (**f**, E7.5). The otic placode invaginates to form the otic cup (**g**, E8-9), which later pinches off to form the otic vesicle or otocyst **(h,i)**. Neural precursors delaminate from the ventral otocyst epithelium to form the acoustic-vestibular ganglion (AVG: **g–i**). The cochlear duct evaginates from the ventromedial region of the otic vesicle, and it will be innervated by the acoustic portion of the AVG, also known as the spiral ganglion (SG: yellow, **j–m**). The cochlear duct elongates and grows to form a coiled tube, the membranous labyrinth, which includes the primordium of the scalas media, vestibularis, and tympanic **(j–m)**. At the cochlear duct the prosensory patch will become the primitive organ of Corti. Scale bars: **(a)** 0.5 mm; **(b–e)** 50 μm. Co, cochlea; V, vestibule; Asc, Lsc and Psc, anterior, lateral and posterior semicircular canals; Do, dorsal; Cd, caudal; IHC, inner hair cells; OHC, outer hair cells; StV, stria vasculari; SpL, spiral ligament; SV, scala vestibule; SM, scala media; ST, scala tympani; LW, lateral wall; OC, Organ of Corti (adapted from Magariños et al., 2014).

formed by sensory hair cells and by non-sensory support cells (Deiters', Hensen's and Claudius') that maintain the ionic and metabolic cochlear homeostasis (Forge and Wright, 2002). There are two functional types of hair cells arranged in a stereotypic manner: one row of inner hair cells (IHC) and three rows of outer hair cells (OHC; Forge and Wright, 2002; Magariños et al., 2012). The IHC cells connect to bipolar auditory type I neurons of the spiral ganglion, whilst the OHC are innervated by type II neurons (Nayagam et al., 2011; Fritzsch et al., 2015). The axons of these neurons leave the spiral ganglion to form the cochlear division of the acoustic-vestibular nerve, which is responsible for transmitting the auditory information through a multisynaptic, ascendant pathway from the cochlea to the auditory cortex (Demanez and Demanez, 2003). HC stereocilia are bathed by endolymph, which maintains the unique ionic concentration required for mechanotransduction. The stria vascularis is located in the lateral wall of the scala media. This three-layered structure regulates cochlear ion transport and maintains the endocochlear potential (Patuzzi, 2011).

The vestibular system is formed by five sensory structures, three cristae located at the base of the semicircular canals and two maculae. Each of these structures has a similar organization, with sensory HC and non-sensory support cells innervated by the vestibular ganglion axons. The vestibule is responsible for sensing equilibrium, and for the perception of linear and angular acceleration, and of gravity (Highstein and Fay, 2004; Ekdale, 2016).

THE REGULATION OF INNER EAR DEVELOPMENT BY EXTRACELLULAR FACTORS AND INTRACELLULAR SIGNALING NETWORKS

The development of the inner ear is initiated by the induction of the otic placode from the ectoderm lying between the rhombomeres 5 and 6 (Magariños et al., 2014; Whitfield, 2015). Otic placode induction is orchestrated from mesoderm signals that coordinate with intrinsic factors in the ectoderm. FGFs, Notch and WNT signaling play a key role during these initial events (Ohyama et al., 2006, 2007; Jayasena et al., 2008). The otic placode then invaginates to form the otic cup that will later detach and close to form the otocyst or otic vesicle. The otocyst is transient embryonic round structure whose multipotent cells will differentiate to produce most adult inner ear cell types (Bissonnette and Fekete, 1996; Sanchez-Calderon et al., 2007). The ventral region of the otocyst is specified by the Sonic hedgehog (Shh) secreted from the floor plate and notochord (Riccomagno et al., 2002, 2005), as well as through repression by the WNT signaling pathway (Groves and Fekete, 2012). Significantly, it is this region that will form the auditory portion of the inner ear. The vestibule develops from the dorsal otocyst, instructed by signals from the bone morphogenetic protein BMP4 (Chang et al., 2008) that antagonize Shh. Sensory HC, non-sensory support cells, plus the acoustic and vestibular neurons that contribute to the acoustic-vestibular ganglion (AVG) also arise from the otocyst. Finally, Notch signaling helps specify the prosensory domain (Daudet and Lewis, 2005; Hartman et al., 2010) and in combination with *Atoh1* expression, it is involved in determining the HC and supporting cells (Mizutari et al., 2013).

Otic vesicle development requires the coordinated response to apoptosis, survival and proliferation signals. IGF-1 signaling, mainly through the RAF-MEK-ERK and PI3K/AKT pathways, fulfills a critical role in regulating these processes. In the chicken embryo, PI3K/AKT signaling regulates the number of otic neurons and it determines the timing of their generation (Aburto et al., 2012b). Moreover, both the RAF-MEK-ERK and PI3K/AKT pathways modulate AVG neuritogenesis (Magariños et al., 2010; Aburto et al., 2012b). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is required to define the size of the neuroblast population (Kim et al., 2013) and it negatively regulates the AKT signaling pathway, as well as interacting with the WNT, Notch, and BMP pathways.

Cell cycle regulation is also essential for correct inner ear organogenesis. IGF-1, Notch, and WNT are among the signaling pathways involved in regulating the proliferation of otic progenitors (Magariños et al., 2014). Through the RAF-MEK-ERK pathway, IGF-1 promotes the cell cycle progression of otic progenitors (Sanz et al., 1999b; Magariños et al., 2010). Finally, the otocyst must undergo the morphogenetic changes that transform the simple pseudostratified otic vesicle epithelia into an extremely complex three-dimensional membranous labyrinth (Kelly and Chen, 2009). The neighboring mesenchymal cells will be responsible for generating the bony labyrinth (Chang et al., 2002).

DEVELOPMENTAL AUTOPHAGY IN THE EMBRYONIC CHICKEN INNER EAR

Beclin-1 and Atg5 transcripts are expressed throughout the developmental stages in the chick when otic vesicles can be explanted and studied in organotypic cultures. Indeed, the Beclin-1 and LC3B proteins are present in the otic epithelium and the AVG (Aburto et al., 2012c; summarized in **Figure 2A**). Chemical and genetic inhibitors of autophagy demonstrate the importance of the autophagic flux for the development and cellular dynamics of the otocyst (Aburto et al., 2012c; **Figure 2A**). Inhibiting autophagy shows that it is required for the clearance of apoptotic cells and for cell cycle progression. Developmental apoptosis is an essential process during inner ear development (Fekete et al., 1997; Sanz et al., 1999a; Frago et al., 2003; León et al., 2004; Magariños et al., 2012), and both this cell death and the elimination of apoptotic cells require energy (Qu et al., 2007; Mellén et al., 2008). During development, autophagy provides ATP by degrading intracellular components and it thereby facilitates apoptosis. Impaired autophagy causes an accumulation of apoptotic cells that cannot be eliminated from the otic vesicle, a failing that can be reverted by adding ATP. The region where otic neural progenitors originate is the neurogenic zone, where the extracellular matrix is degraded to detach cells and the migrating detached cells accumulate

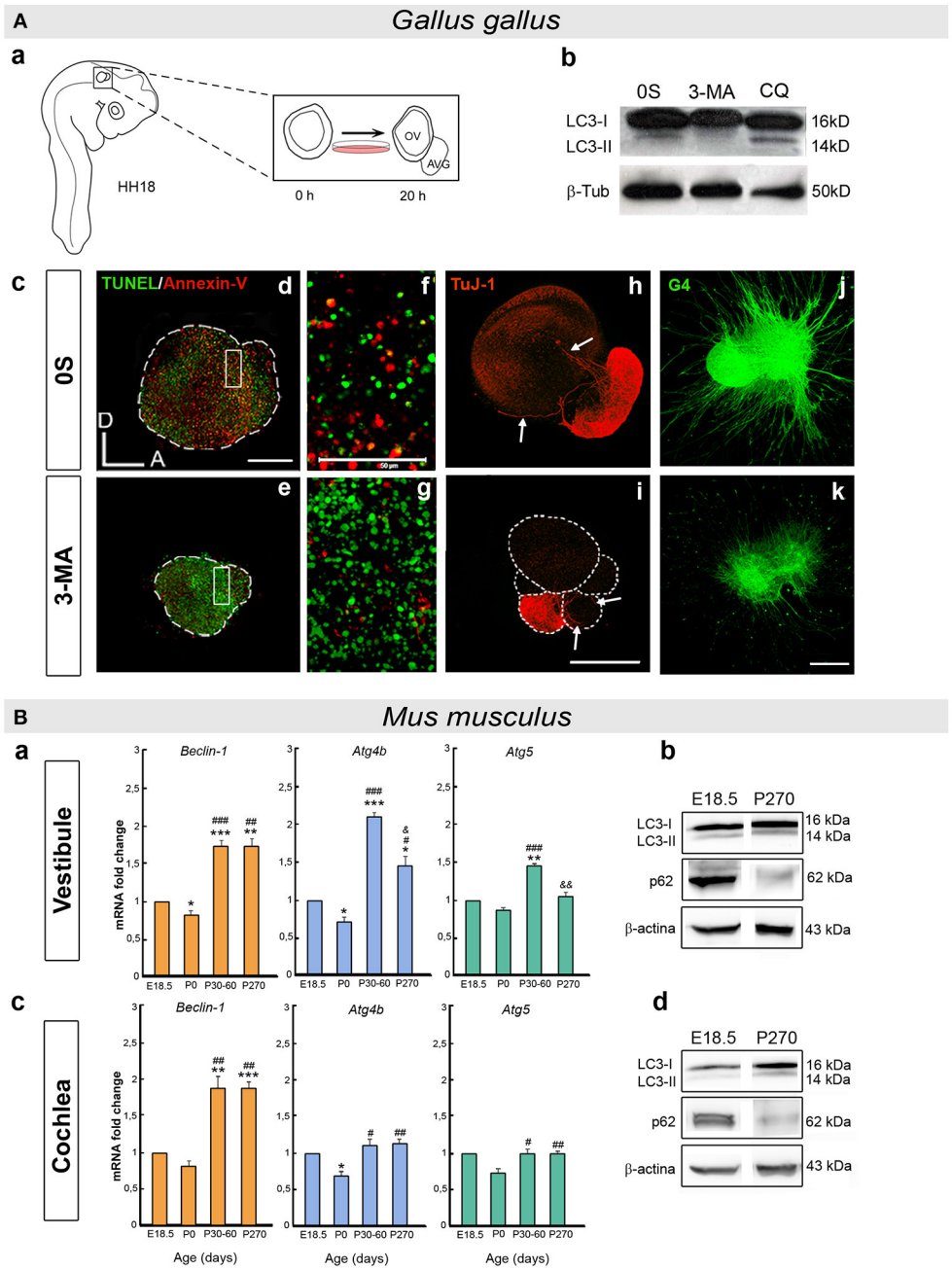

FIGURE 2 | (A) Developmental autophagy in the chicken otocyst. **(a)** Scheme showing the *ex vivo* culture of otic vesicles from HH18 embryos. The acoustic-vestibular ganglion (AVG) develops from the cultured otic vesicle after 20 h in serum-free culture medium (0S). **(b)** Autophagic flux is typically measured in Western blots to determine the LC3 ratio in the presence or absence of chemical inhibitors of autophagy (3-MA and CQ). **(c)** Otic vesicles incubated with an inhibitor of autophagy accumulate apoptotic cells, as evident by reduced staining for An-V in red and by increased TUNEL green spots **(d–g)**. Aberrant AVG development is also seen **(h,i)**, with fewer neuroblasts (TuJ-1, red), and **(j,k)** altered neurite outgrowth and pathfinding (G4, green). **(f,g)** Higher magnification of the boxed regions in **(d)** and **(e)**, respectively. annexin-V, An-V; 3-methyladenin, 3-MA; chloroquine, CQ. Scale bars: **(d–i)**, 150 μm; **(f)**, **(g)**, 50 μm; **(j)**, **(k)** 300 μm (adapted from Aburto et al., 2012c). **(B)** Autophagy in the postnatal and adult mouse inner ear. **(a,c)** Histograms showing the changes in Beclin-1, Atg4b, and Atg5 expression with age in the mouse vestibule **(a)** and cochlea **(c)**, as determined by RT-qPCR. **(b,d)** Autophagic flux increases with age in the mouse inner ear. The LC3-II/LC3-I and SQSTM1/p62 (p62/β-actin) ratios were determined in Western blots of the vestibule **(b)** and cochlea **(d)** at E18.5 and P270. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. E18.5; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. P0; and $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ vs. P30–60. E, embryonic day and P, postnatal day (adapted from de Iriarte Rodríguez et al., 2015).

autophagic vacuoles. Conversely, the inhibition of autophagy results in aberrant AVG phenotypes (Aburto et al., 2012c; **Figure 2A**). Therefore, autophagy is required for the migration of the epithelial neuroblasts from the neurogenic zone to form the AVG. In summary, the early development of the inner ear is one example of many where developmental autophagy plays a supporting role to apoptosis and migration (Di Bartolomeo et al., 2010; Wada et al., 2014; Lorda-Diez et al., 2015; Boya et al., 2016).

AUTOPHAGY IN THE MOUSE INNER EAR

Autophagy is required for the development of the vestibular system in the mouse. *Atg4* deficient mice have impaired balance, with different phenotypic penetrance from severe to mild vestibular alterations. The biogenesis of the otoconia is defective in both $Atg4b^{-/-}$ and $Atg5^{-/-}$ mice (Mariño et al., 2010), and otoconial impairment occurs in mice exposed to streptomycin ototoxicity (Takumida et al., 1997), which can inhibit autophagy (Levano and Bodmer, 2015) and increase cell damage in the inner ear due to oxidative stress (Guthrie, 2008). In fact, autophagy reduces the reactive oxygen species (ROS) in mice subjected to noise-induced hearing loss (Yuan et al., 2015). This crucial role of autophagy in eliminating ROS could explain the similarities between autophagy gene mutants and streptomycin-treated animals. However, increased ROS are not the only consequence of inhibiting autophagy during vestibular development, as otoconial biogenesis requires the secretion and assembly of specific proteins that are also affected by inhibiting autophagy (Mariño et al., 2010).

Autophagy plays a key role in newborn mice, and *Atg5*, *Atg7*, *Atg9,* and *Atg16* null mice die soon after birth (Mizushima and Levine, 2010). The transcriptome of the E18.5 mouse cochlea shows that a wide variety of *Atg* genes are expressed, underlining the relevance of autophagy at perinatal stages (de Iriarte Rodríguez et al., 2015). Furthermore, several key genes of the autophagic molecular machinery (*Becn1*, *Atg4b*, *Atg5,* and *Atg9*) are expressed in the mouse vestibule and cochlea throughout development and adulthood (de Iriarte Rodríguez et al., 2015; summarized in **Figure 2B**). The expression of these genes is significantly enhanced from the perinatal stages (E18.5 and P0) to adulthood (P30) as the inner ear acquires its complete functionality (Rueda et al., 1996). A temporal analysis of autophagic proteolysis in the cochlea and vestibule confirms the induction of autophagy in adults rather than E18.5 embryos. Moreover, there is significantly less SQSTM1/p62 at P270 than at E.18.5, whilst the relative LC3-II levels increase in the cochlea and vestibule (de Iriarte Rodríguez et al., 2015; **Figure 2B**). Indeed, autophagosomes are clearly visible in adult neurons of the spiral ganglion but not at earlier stages. LC3B forms granular structures in the neuronal soma at P30 and onwards, yet not at E18.5 (de Iriarte Rodríguez et al., 2015). Autophagy is essential in neurons because they do not dilute their damaged molecules or organelles by proliferation. Thus, autophagy is required for detoxification and to manage damage (Son et al., 2012; Damme et al., 2015; He et al., 2016). Accordingly, the postnatal onset of hearing and the concomitant increase in neuronal activity is correlated with the induction of autophagy in the cochlea.

THE INFLUENCE OF AUTOPHAGY ON INNER EAR HOMEOSTASIS AND AGING

Autophagy plays an additional role in inner ear homeostasis once development is concluded. Otic injury caused by a combination of aminoglycoside and loop diuretics augments aspects of autophagy (Taylor et al., 2008). Moreover, autophagy is activated by rapamycin alleviated ototoxicity in cisplatin-treated rats (Fang and Xiao, 2014) and in mice exposed to an auditory insult (Yuan et al., 2015). Thus, autophagy helps maintain adult hearing in response to stress. Proteostasis is impaired during aging (López-Otín et al., 2013) and the stabilization of proteic events that is mostly provided by molecular chaperones also declines with age (Rodriguez et al., 2016). In addition, protein degradation systems control the levels of misfolded or aggregated proteins, the accumulation of which drives age-related neurodegenerative diseases like Parkinson's or Alzheimer's disease (Balchin et al., 2016). Thus, it is not surprising that the senescence-accelerated mouse prone 8 (SAMP8) mutant mice exhibit age-related hearing loss and autophagy stress (Menardo et al., 2012).

Our studies of 9 month-old $Igf1^{-/-}$ mice show they suffer defects in the proteostasis associated with aging. These $Igf1^{-/-}$ mice suffer a loss of hearing and a reduced lifespan, among other traits (Varela-Nieto et al., 2013). Hearing loss in $Igf1^{-/-}$ deficient mice is accompanied by a general failure of the hearing receptor (Riquelme et al., 2010), although the weaker autophagy gene expression in one-year-old cochlea may also contribute to this auditory phenotype (de Iriarte Rodríguez et al., 2015). However, the vestibular defects in the $Igf1^{-/-}$ mouse are milder than those found in the cochlea (Rodríguez-de la Rosa et al., 2015). *Becn1*, *Atg4b,* and *Atg5* are more strongly expressed in 9-month-old $Igf1^{-/-}$ vestibules compared to those of wild-type mice. Thus, the induction of autophagy might provide $Igf1^{-/-}$ vestibules with some protection, as it does in $Igf1^{-/-}$ retinas (Arroba et al., 2016). After differentiation, hair cells do not regenerate in the mammalian cochlea, whilst vestibular hair cells do to a limited extent (Burns and Stone, 2016). The up-regulation of autophagy might be partially responsible for the different potentiality of vestibular and cochlear hair cells.

CONCLUSIONS

During the development of the vertebrate inner ear, autophagy participates in cell remodeling and dynamics, and it contributes to the biogenesis of the vestibular otoconia. In the postnatal cochlea, the autophagy machinery is upregulated concomitant with the increase in neuronal activity at the onset of hearing. Autophagy becomes a housekeeping process in the adult inner ear, and it is a means to protect hearing during aging and in response to injury. Further work is needed to fully understand

the role of autophagy in the inner ear and to explore the potential of modulating autophagy as a novel strategy to combat inner ear diseases.

AUTHOR CONTRIBUTIONS

MM and IV designed and drafted the work and wrote the manuscript; MM, IV, SP, MA, and RdIR analyzed and interpreted the data, revised the manuscript critically and approved the version to be published; SP designed and performed the figures. All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENTS

We warmly thank our present and former colleagues of the Neurobiology of Hearing group for sharing information and for useful discussions. SP is supported by a FPI predoctoral fellowship (BES-2015-071311) funded by MINECO and the European Social Fund. This work was supported by the European Commission FP7-PEOPLE-2013-IAPP TARGEAR and Spanish MINECO/FEDER SAF2014-53979-R to IV.

REFERENCES

Aburto, M. R., Hurlé, J. M., Varela-Nieto, I., and Magariños, M. (2012a). Autophagy during vertebrate development. *Cells* 1, 428–448. doi: 10.3390/cells1030428

Aburto, M. R., Magariños, M., Leon, Y., Varela-Nieto, I., and Sanchez-Calderon, H. (2012b). AKT signaling mediates IGF-I survival actions on otic neural progenitors. *PLoS ONE* 7:e30790. doi: 10.1371/journal.pone.0030790

Aburto, M. R., Sánchez-Calderón, H., Hurlé, J. M., Varela-Nieto, I., and Magariños, M. (2012c). Early otic development depends on autophagy for apoptotic cell clearance and neural differentiation. *Cell Death Dis.* 3:e394. doi: 10.1038/cddis.2012.132

Antonioli, M., Albiero, F., Fimia, G. M., and Piacentini, M. (2015). AMBRA1-regulated autophagy in vertebrate development. *Int. J. Dev. Biol.* 59, 109–117. doi: 10.1387/ijdb.150057mp

Ariosa, A. R., and Klionsky, D. J. (2016). Autophagy core machinery: overcoming spatial barriers in neurons. *J. Mol. Med. Berl. Ger.* 94, 1217–1227. doi: 10.1007/s00109-016-1461-9

Arroba, A. I., Rodríguez-de la Rosa, L., Murillo-Cuesta, S., Vaquero-Villanueva, L., Hurlé, J. M., Varela-Nieto, I., et al. (2016). Autophagy resolves early retinal inflammation in Igf1-deficient mice. *Dis. Model. Mech.* 9, 965–974. doi: 10.1242/dmm.026344

Balchin, D., Hayer-Hartl, M., and Hartl, F. U. (2016). *In vivo* aspects of protein folding and quality control. *Science* 353:aac4354. doi: 10.1126/science.aac4354

Bissonnette, J. P., and Fekete, D. M. (1996). Standard atlas of the gross anatomy of the developing inner ear of the chicken. *J. Comp. Neurol.* 368, 620–630.

Boya, P., Esteban-Martínez, L., Serrano-Puebla, A., Gómez-Sintes, R., and Villarejo-Zori, B. (2016). Autophagy in the eye: development, degeneration, and aging. *Prog. Retin. Eye Res.* 55, 206–245. doi: 10.1016/j.preteyeres.2016.08.001

Burns, J. C., and Stone, J. S. (2016). Development and regeneration of vestibular hair cells in mammals. *Semin. Cell Dev. Biol.* 65, 96–105. doi: 10.1016/j.semcdb.2016.11.001

Chang, W., Lin, Z., Kulessa, H., Hebert, J., Hogan, B. L. M., and Wu, D. K. (2008). Bmp4 is essential for the formation of the vestibular apparatus that detects angular head movements. *PLoS Genet.* 4:e1000050. doi: 10.1371/journal.pgen.1000050

Chang, W., ten Dijke, P., and Wu, D. K. (2002). BMP pathways are involved in otic capsule formation and epithelial-mesenchymal signaling in the developing chicken inner ear. *Dev. Biol.* 251, 380–394. doi: 10.1006/dbio.2002.0822

Damme, M., Suntio, T., Saftig, P., and Eskelinen, E.-L. (2015). Autophagy in neuronal cells: general principles and physiological and pathological functions. *Acta Neuropathol. (Berl.)* 129, 337–362. doi: 10.1007/s00401-014-1361-4

Daudet, N., and Lewis, J. (2005). Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Dev. Camb. Engl.* 132, 541–551. doi: 10.1242/dev.01589

de Iriarte Rodríguez, R., Pulido, S., Rodríguez-de la Rosa, L., Magariños, M., and Varela-Nieto, I. (2015). Age-regulated function of autophagy in the mouse inner ear. *Hear. Res.* 330, 39–50. doi: 10.1016/j.heares.2015.07.020

Demanez, J. P., and Demanez, L. (2003). Anatomophysiology of the central auditory nervous system: basic concepts. *Acta Otorhinolaryngol. Belg.* 57, 227–236.

Denton, D., Nicolson, S., and Kumar, S. (2012). Cell death by autophagy: facts and apparent artefacts. *Cell Death Differ.* 19, 87–95. doi: 10.1038/cdd.2011.146

Di Bartolomeo, S., Nazio, F., and Cecconi, F. (2010). The role of autophagy during development in higher eukaryotes. *Traffic Cph. Den.* 11, 1280–1289. doi: 10.1111/j.1600-0854.2010.01103.x

Dragich, J. M., Kuwajima, T., Hirose-Ikeda, M., Yoon, M. S., Eenjes, E., Bosco, J. R., et al. (2016). Autophagy linked FYVE (Alfy/WDFY3) is required for establishing neuronal connectivity in the mammalian brain. *eLife* 5:e14810. doi: 10.7554/eLife.14810

Ekdale, E. G. (2016). Form and function of the mammalian inner ear. *J. Anat.* 228, 324–337. doi: 10.1111/joa.12308

Fang, B., and Xiao, H. (2014). Rapamycin alleviates cisplatin-induced ototoxicity *in vivo*. *Biochem. Biophys. Res. Commun.* 448, 443–447. doi: 10.1016/j.bbrc.2014.04.123

Fekete, D. M., Homburger, S. A., Waring, M. T., Riedl, A. E., and Garcia, L. F. (1997). Involvement of programmed cell death in morphogenesis of the vertebrate inner ear. *Dev. Camb. Engl.* 124, 2451–2461.

Fimia, G. M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R., et al. (2007). Ambra1 regulates autophagy and development of the nervous system. *Nature* 447, 1121–1125. doi: 10.1371/journal.pone.0090151

Forge, A., and Wright, T. (2002). The molecular architecture of the inner ear. *Br. Med. Bull.* 63, 5–24. doi: 10.1093/bmb/63.1.5

Frago, L. M., Cañón, S., de la Rosa, E. J., León, Y., and Varela-Nieto, I. (2003). Programmed cell death in the developing inner ear is balanced by nerve growth factor and insulin-like growth factor I. *J. Cell Sci.* 116, 475–486. doi: 10.1242/jcs.00223

Fritzsch, B., Pan, N., Jahan, I., and Elliott, K. L. (2015). Inner ear development: building a spiral ganglion and an organ of Corti out of unspecified ectoderm. *Cell Tissue Res.* 361, 7–24. doi: 10.1007/s00441-014-2031-5

Füllgrabe, J., Ghislat, G., Cho, D.-H., and Rubinsztein, D. C. (2016). Transcriptional regulation of mammalian autophagy at a glance. *J. Cell Sci.* 129, 3059–3066. doi: 10.1242/jcs.188920

Groves, A. K., and Fekete, D. M. (2012). Shaping sound in space: the regulation of inner ear patterning. *Dev. Camb. Engl.* 139, 245–257. doi: 10.1242/dev.067074

Guthrie, O. W. (2008). Aminoglycoside induced ototoxicity. *Toxicology* 249, 91–96. doi: 10.1016/j.tox.2008.04.015

Hartman, B. H., Reh, T. A., and Bermingham-McDonogh, O. (2010). Notch signaling specifies prosensory domains via lateral induction in the developing mammalian inner ear. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15792–15797. doi: 10.1073/pnas.1002827107

He, M., Ding, Y., Chu, C., Tang, J., Xiao, Q., and Luo, Z.-G. (2016). Autophagy induction stabilizes microtubules and promotes axon regeneration after spinal cord injury. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11324–11329. doi: 10.1073/pnas.1611282113

Highstein, S. M., and Fay, R. (2004). *The Vestibular System. (Vol. 19)*. New York, NY: Springer Handbook of Auditory Research.

Jayasena, C. S., Ohyama, T., Segil, N., and Groves, A. K. (2008). Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode. *Dev. Camb. Engl.* 135, 2251–2261. doi: 10.1242/dev.017905

Jia, Z., Wang, J., Wang, W., Tian, Y., XiangWei, W., Chen, P., et al. (2014). Autophagy eliminates cytoplasmic β-catenin and NICD to promote the cardiac differentiation of P19CL6 cells. *Cell. Signal.* 26, 2299–2305. doi: 10.1016/j.cellsig.2014.07.028

Jiang, P., and Mizushima, N. (2014). Autophagy and human diseases. *Cell Res.* 24, 69–79. doi: 10.1038/cr.2013.161

Katsuragi, Y., Ichimura, Y., and Komatsu, M. (2015). p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J.* 282, 4672–4678. doi: 10.1111/febs.13540

Kelly, M. C., and Chen, P. (2009). Development of form and function in the mammalian cochlea. *Curr. Opin. Neurobiol.* 19, 395–401. doi: 10.1016/j.conb.2009.07.010

Kim, H. J., Woo, H.-M., Ryu, J., Bok, J., Kim, J. W., Choi, S. B., et al. (2013). Conditional deletion of pten leads to defects in nerve innervation and neuronal survival in inner ear development. *PLoS ONE* 8:e55609. doi: 10.1371/journal.pone.0055609

Klionsky, D. J., Abdelmohsen, K., Abe, A., Abedin, M. J., Abeliovich, H., Acevedo Arozena, A., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12, 1–222. doi: 10.1080/15548627.2015.1100356

Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., et al. (2004). The role of autophagy during the early neonatal starvation period. *Nature* 432, 1032–1036. doi: 10.1038/nature03029

León, Y., Sánchez-Galiano, S., and Gorospe, I. (2004). Programmed cell death in the development of the vertebrate inner ear. *Apoptosis* 9, 255–264. doi: 10.1023/B:APPT.0000025802.07676.a5

Levano, S., and Bodmer, D. (2015). Loss of STAT1 protects hair cells from ototoxicity through modulation of STAT3, c-Jun, Akt, and autophagy factors. *Cell Death Dis.* 6:e2019. doi: 10.1038/cddis.2015.362

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194–1217. doi: 10.1016/j.cell.2013.05.039

Lorda-Diez, C. I., Montero, J. A., Garcia-Porrero, J. A., and Hurle, J. M. (2015). Interdigital tissue regression in the developing limb of vertebrates. *Int. J. Dev. Biol.* 59, 55–62. doi: 10.1387/ijdb.150065jh

Magariños, M., Aburto, M. R., Sánchez-Calderón, H., Muñoz-Agudo, C., Rapp, U. R., and Varela-Nieto, I. (2010). RAF kinase activity regulates neuroepithelial cell proliferation and neuronal progenitor cell differentiation during early inner ear development. *PLoS ONE* 5:e14435. doi: 10.1371/journal.pone.0014435.

Magariños, M., Contreras, J., Aburto, M. R., and Varela-Nieto, I. (2012). Early development of the vertebrate inner ear. *Anat. Rec. Hoboken NJ* 2007 295, 1775–1790. doi: 10.1002/ar.22575

Magariños, M., Contreras, J., and Varela-Nieto, I. (2014). "Early development of the vertebrate inner ear," in *Development of Auditory and Vestibular Systems 4th Edn.*, eds R. Romand, and I. Varela-Nieto (Oxford: Elsevier), 1–30.

Mariño, G., Fernández, A. F., Cabrera, S., Lundberg, Y. W., Cabanillas, R., Rodríguez, F., et al. (2010). Autophagy is essential for mouse sense of balance. *J. Clin. Invest.* 120, 2331–2344. doi: 10.1172/JCI42601

Mauthe, M., and Reggiori, F. (2016). ATG proteins: are we always looking at autophagy? *Autophagy* 12, 2502–2503. doi: 10.1080/15548627.2016.1236878

Mellén, M. A., de la Rosa, E. J., and Boya, P. (2008). The autophagic machinery is necessary for removal of cell corpses from the developing retinal neuroepithelium. *Cell Death Differ.* 15, 1279–1290. doi: 10.1038/cdd.2008.40

Menardo, J., Tang, Y., Ladrech, S., Lenoir, M., Casas, F., Michel, C., et al. (2012). Oxidative stress, inflammation, and autophagic stress as the key mechanisms of premature age-related hearing loss in SAMP8 mouse Cochlea. *Antioxid. Redox Signal.* 16, 263–274. doi: 10.1089/ars.2011.4037

Mizushima, N., and Levine, B. (2010). Autophagy in mammalian development and differentiation. *Nat. Cell Biol.* 12, 823–830. doi: 10.1038/ncb0910-823

Mizutari, K., Fujioka, M., Hosoya, M., Bramhall, N., Okano, H. J., Okano, H., et al. (2013). Notch inhibition induces cochlear hair cell regeneration and recovery of hearing after acoustic trauma. *Neuron* 77, 58–69. doi: 10.1016/j.neuron.2012.10.032

Napolitano, G., and Ballabio, A. (2016). TFEB at a glance. *J. Cell Sci.* 129, 2475–2481. doi: 10.1242/jcs.146365

Nayagam, B. A., Muniak, M. A., and Ryugo, D. K. (2011). The spiral ganglion: connecting the peripheral and central auditory systems. *Hear. Res.* 278, 2–20. doi: 10.1016/j.heares.2011.04.003

Ohyama, T., Groves, A. K., and Martin, K. (2007). The first steps towards hearing: mechanisms of otic placode induction. *Int. J. Dev. Biol.* 51, 463–472. doi: 10.1387/ijdb.072320to

Ohyama, T., Mohamed, O. A., Taketo, M. M., Dufort, D., and Groves, A. K. (2006). Wnt signals mediate a fate decision between otic placode and epidermis. *Dev. Camb. Engl.* 133, 865–875. doi: 10.1242/dev.02271

Patuzzi, R. (2011). Ion flow in stria vascularis and the production and regulation of cochlear endolymph and the endolymphatic potential. *Hear. Res.* 277, 4–19. doi: 10.1016/j.heares.2011.01.010

Pavel, M., and Rubinsztein, D. C. (2016). Mammalian autophagy and the plasma membrane. *FEBS J.* 284, 672-679. doi: 10.1111/febs.13931

Pua, H. H., Dzhagalov, I., Chuck, M., Mizushima, N., and He, Y.-W. (2007). A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J. Exp. Med.* 204, 25–31. doi: 10.1084/jem.20061303

Qu, X., Zou, Z., Sun, Q., Luby-Phelps, K., Cheng, P., Hogan, R. N., et al. (2007). Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell* 128, 931–946. doi: 10.1016/j.cell.2006.12.044

Riccomagno, M. M., Martinu, L., Mulheisen, M., Wu, D. K., and Epstein, D. J. (2002). Specification of the mammalian cochlea is dependent on Sonic hedgehog. *Genes Dev.* 16, 2365–2378. doi: 10.1101/gad.1013302

Riccomagno, M. M., Takada, S., and Epstein, D. J. (2005). Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh. *Genes Dev.* 19, 1612–1623. doi: 10.1101/gad.1303905

Riquelme, R., Cediel, R., Contreras, J., la Rosa Lourdes, R., Murillo-Cuesta, S., Hernandez-Sanchez, C., et al. (2010). A comparative study of age-related hearing loss in wild type and insulin-like growth factor I deficient mice. *Front. Neuroanat.* 4:27. doi: 10.3389/fnana.2010.00027

Rodríguez-de la Rosa, L., Sánchez-Calderón, H., Contreras, J., Murillo-Cuesta, S., Falagan, S., Avendaño, C., et al. (2015). Comparative gene expression study of the vestibular organ of the Igf1 deficient mouse using whole-transcript arrays. *Hear. Res.* 330, 62–77. doi: 10.1016/j.heares.2015.08.016.

Rodriguez, K. A., Valentine, J. M., Kramer, D. A., Gelfond, J. A., Kristan, D. M., Nevo, E., et al. (2016). Determinants of rodent longevity in the chaperone-protein degradation network. *Cell Stress Chaperones* 21, 453–466. doi: 10.1007/s12192-016-0672-x

Rueda, J., Cantos, R., and Lim, D. J. (1996). Tectorial membrane-organ of Corti relationship during cochlear development. *Anat. Embryol. (Berl.)* 194, 501–514. doi: 10.1007/BF00185996

Sanchez-Calderon, H., Milo, M., Leon, Y., and Varela-Nieto, I. (2007). A network of growth and transcription factors controls neuronal differentation and survival in the developing ear. *Int. J. Dev. Biol.* 51, 557–570. doi: 10.1387/ijdb.072373hs

Sanz, C., León, Y., Cañón, S., Alvarez, L., Giraldez, F., and Varela-Nieto, I. (1999a). Pattern of expression of the jun family of transcription factors during the early development of the inner ear: implications in apoptosis. *J. Cell Sci.* 112 (Pt 22), 3967–3974.

Sanz, C., León, Y., Troppmair, J., Rapp, U. R., and Varela-Nieto, I. (1999b). Strict regulation of c-Raf kinase levels is required for early organogenesis of the vertebrate inner ear. *Oncogene* 18, 429–437. doi: 10.1038/sj.onc.1202312

Schiaffino, S., Mammucari, C., and Sandri, M. (2008). The role of autophagy in neonatal tissues: just a response to amino acid starvation? *Autophagy* 4, 727–730. doi: 10.4161/auto.6143

Singh, R., Xiang, Y., Wang, Y., Baikati, K., Cuervo, A. M., Luu, Y. K., et al. (2009). Autophagy regulates adipose mass and differentiation in mice. *J. Clin. Invest.* 119, 3329–3339. doi: 10.1172/jci39228

Son, J. H., Shim, J. H., Kim, K. H., Ha, J. Y., and Han, J. Y. (2012). Neuronal autophagy and neurodegenerative diseases. *Exp. Mol. Med.* 44, 89–98. doi: 10.3858/emm.2012.44.2.031

Takumida, M., Zhang, D. M., Yajin, K., and Harada, Y. (1997). Formation and fate of giant otoconia of the guinea pig following streptomycin intoxication. *Acta Otolaryngol. (Stockh.)* 117, 538–544. doi: 10.3109/00016489709113434

Tasset, I., and Cuervo, A. M. (2016). Role of chaperone-mediated autophagy in metabolism. *FEBS J.* 283, 2403–2413. doi: 10.1111/febs.13677

Taylor, R. R., Nevill, G., and Forge, A. (2008). Rapid hair cell loss: a mouse model for cochlear lesions. *J. Assoc. Res. Otolaryngol. JARO* 9, 44–64. doi: 10.1007/s10162-007-0105-8

Tsukamoto, S., Kuma, A., and Mizushima, N. (2008). The role of autophagy during the oocyte-to-embryo transition. *Autophagy* 4, 1076–1078. doi: 10.4161/auto.7065

Varela-Nieto, I., Murillo-Cuesta, S., Rodríguez-de la Rosa, L., Lassatetta, L., and Contreras, J. (2013). IGF-I deficiency and hearing loss: molecular clues and clinical implications. *Pediatr. Endocrinol. Rev.* 10, 460–472.

Wada, Y., Sun-Wada, G.-H., Kawamura, N., and Aoyama, M. (2014). Role of autophagy in embryogenesis. *Curr. Opin. Genet. Dev.* 27, 60–66. doi: 10.1016/j.gde.2014.03.010

Whitfield, T. T. (2015). Development of the inner ear. *Curr. Opin. Genet. Dev.* 32, 112–118. doi: 10.1016/j.gde.2015.02.006

Yamamoto, A., Mizushima, N., and Tsukamoto, S. (2014). Fertilization-induced autophagy in mouse embryos is independent of mTORC1. *Biol. Reprod.* 91:7. doi: 10.1095/biolreprod.113.115816

Yu, L., Wan, F., Dutta, S., Welsh, S., Liu, Z., Freundt, E., et al. (2006). Autophagic programmed cell death by selective catalase degradation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 4952–4957. doi: 10.1073/pnas.0511288103

Yuan, H., Wang, X., Hill, K., Chen, J., Lemasters, J., Yang, S.-M., et al. (2015). Autophagy attenuates noise-induced hearing loss by reducing oxidative stress. *Antioxid. Redox Signal.* 22, 1308–1324. doi: 10.1089/ars.2014.6004

Zhen, Y., and Li, W. (2015). Impairment of autophagosome-lysosome fusion in the buff mutant mice with the VPS33A(D251E) mutation. *Autophagy* 11, 1608–1622. doi: 10.1080/15548627.2015.1072669

Zhou, X., Babu, J. R., da Silva, S., Shu, Q., Graef, I. A., Oliver, T., et al. (2007). Unc-51-like kinase 1/2-mediated endocytic processes regulate filopodia extension and branching of sensory axons. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5842–5847. doi: 10.1073/pnas.0701402104

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 01 May 2017
doi: 10.3389/fcell.2017.00044

More than Just a Simple Cardiac Envelope; Cellular Contributions of the Epicardium

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The adult pumping heart is formed by distinct tissue layers. From inside to outside, the heart is composed by an internal endothelial layer, dubbed the endocardium, a thick myocardial component which supports the pumping capacity of the heart and exteriorly covered by a thin mesothelial layer named the epicardium. Cardiac insults such as coronary artery obstruction lead to ischemia and thus to an irreversible damage of the myocardial layer, provoking in many cases heart failure and death. Thus, searching for new pathways to regenerate the myocardium is an urgent biomedical need. Interestingly, the capacity of heart regeneration is present in other species, ranging from fishes to neonatal mammals. In this context, several lines of evidences demonstrated a key regulatory role for the epicardial layer. In this manuscript, we provide a state-of-the-art review on the developmental process leading to the formation of the epicardium, the distinct pathways controlling epicardial precursor cell specification and determination and current evidences on the regenerative potential of the epicardium to heal the injured heart.

Keywords: epicardium, proepicardium, non-coding RNAs, heart development, regeneration

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Consejo Superior de Investigaciones Científicas, Spain

Reviewed by:
Nicola Smart, University of Oxford, UK
Manvendra K. Singh, DUKE-NUS Medical School, Singapore
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *31 January 2017*
Accepted: *10 April 2017*
Published: *01 May 2017*

Citation:
Dueñas A, Aranega AE and Franco D (2017) More than Just a Simple Cardiac Envelope; Cellular Contributions of the Epicardium. Front. Cell Dev. Biol. 5:44. doi: 10.3389/fcell.2017.00044

The development of the heart is a complex process. The primitive heart tube is formed from cardiogenic mesoderm of the cardiac crescents, i.e., first heart field (FHF), while anterior and venous poles are derived from a subsequent subset of cardiogenic cells located medial to the cardiac crescents, dubbed second heart field (SHF; Kelly et al., 2001; Kelly and Buckingham, 2002). In addition, external cellular contributions to the developing heart will take place from this stage onwards. On the one hand, cardiac neural crest will colonize the most anterior parts of the heart playing a pivotal role on aortico-pulmonary septation (Kirby and Waldo, 1990, 1995). On the other hand, cell originating from the proepicardium (PE) will cover and infiltrate into the developing heart leading to distinct cellular subpopulations, such as endothelial and smooth muscle cells forming the coronary vasculature, endocardial cushion mesenchyme, cardiac fibroblasts, and of

Abbreviations: Bmp, Bone morphogenetic protein; CXCR4, Chemokine (C-X-C motif) receptor 4; Cre, Cre recombinase; CXCL12, Chemokine (C-X-C motif) ligand 12; Dkk1, Dickkopf-related protein 1; Dkk2, Dickkopf-related protein 2; EMT, Epitelial-to-mesenchymal transition; EPDCs, Epicardial derived cells; Fabp4, Fatty acid binding protein 4; Fgf, Fibroblast growth factor; Fgf10, Fibroblast growth factor 10; Fgfr2b, Fibroblast growth factor receptor 2b; FHF, First heart field; lcnRNA, Long non-coding RNA; MAPK, Mitogen-Activated Protein Kinase; Nfatc1, Nuclear factor of activated T-cells, cytoplasmic 1; NF-κB, Nuclear Factor κB; Nrg1, Neuregulin 1; Pcd4, Programmed Cell Death 4; PCP, Planar cell polarity; PE, Proepicardium; Pod1/Tcf21, Podocyte-expressed 1/Transcription factor 21; Raldh2, Aldehyde dehydrogenase family 1, subfamily A2; SHF, Second heart field; Tbx18, T-box homeobox 18; Tcf21/Pod1, Transcription factor 21/Podocyte-expressed 1; VEGF, Vascular endothelial growth factor; Wt1, Wilms tumor protein; Yap/Taz, Yes associated protein 1/Transcriptional coactivator with PDZ-binding motif.

course the adult epicardial lining (Winter and Gittenberger-de Groot, 2007; Gittenberger-de Groot et al., 2012). In this manuscript we will provide a state-of-the-art review on the developmental process leading to the formation of the PE/epicardium, the signaling pathways providing cell specification and fate determination to those epicardial precursor cells including the upcoming role of non-coding RNAs, and current evidences on the regenerative role of the epicardium as to heal the injured heart.

INITIAL PHASES OF THE PROEPICARDIAL (PE) AND EPICARDIAL FORMATION; A JOURNEY TO THE DEVELOPING EMBRYONIC HEART

The proepicardium (PE) is a small protuberance that progressively develops within limiting boundaries between the hepatic and cardiac primordia. It is composed of an external epithelial lining configured as a cauliflower structure and an internal mesenchymal component (Virágh et al., 1993; Kálmán et al., 1995; Ratajska et al., 2008). A single PE anlage is observed at early developmental stages in zebrafish (Serluca, 2008) while in the sturgeon and in mice bilateral PE buds are formed subsequently merging into a single midline structure (Schulte et al., 2007; Icardo et al., 2009). Curiously, in chicken two PE primordia are formed, but interestingly the right PE anlage develops before the left one is visible (Schulte et al., 2007). These data suggest divergent evolutionary trends on the formation of the PE primordia and furthermore advocate that embryonic left-right signaling might play a role controlling PE formation (Schlueter and Brand, 2012).

Transcriptional heterogeneity is widely documented for the PE anlage, and in addition, cell specific markers for several of the PE/epicardial cell derivatives, such as endothelial (Poelmann et al., 1993; Mikawa and Gourdie, 1996; Cossette and Misra, 2011; Niderla-Bielińska et al., 2015) and smooth muscle (Valder and Olson, 1994) cells have also been documented, suggesting an early heterogeneous compartmentalization. Subsequently after the formation of the PE a process of delamination and migration of the proepicardial cells occurs. This process will lead to external covering of the atrioventricular canal and the entire atrial and ventricular myocardial chambers as demonstrated by seminal studies using quail-chicken embryos (Pérez-Pomares et al., 1998, 2002; Vrancken Peeters et al., 1999; **Figure 1**). In zebrafish, this process is dependent on the pericardial fluid currents (Peralta et al., 2013, 2014; Plavicki et al., 2013, 2014). In mice, proepicardial cells are detached from the PE forming cysts that migrate to the developing cardiac chambers through the pericardial cavity (Männer et al., 2001; Hirose et al., 2006). These cysts randomly attach to the ventricular and atrial chambers and progressively expand until the final full coverage of the cardiac chambers is completed.

Once the PE cells migrate and cover the surface of the developing embryonic myocardium an epicardial-myocardial signaling crosstalk is initiated. This process is crucial for the correct development of both cardiac tissue layers. The epicardium is instructed to initiate an epithelial-to-mesenchymal transformation (EMT), detaching from the epithelial epicardial layer and migrating first into the subepicardial space. These cells subsequently invade the myocardial walls, giving rise to the epicardial derived cells (EPDCs) (Dettman et al., 1998). An additional source of subepicardial cells of hematopoietic origin is provided during embryonic development which further contributes to the heterogeneity of the embryonic and postnatal epicardium (Balmer et al., 2014). In the following chapters we provide a state-of-the-art review on the differential contribution of the embryonic epicardium in cardiovascular development and disease.

CELL FATE AND CONTRIBUTION OF THE EMBRYONIC EPICARDIUM TO THE MATURE HEART

Epicardial derived cells once they go through the subepicardial space continue their journey into the developing heart. Seminal approaches using quail-chick chimeras demonstrated that quail EPDCs contribute to distinct cardiac cell lineages, such as endothelial and smooth muscle cells in the coronary vasculature, endocardial mesenchymal cells in the atrioventricular cushions and also cardiac fibroblasts (Poelmann et al., 1993; Dettman et al., 1998; **Figure 1**). Since the experimental model used was a heterologous chimera, multiple criticisms were arising as which was indeed the real contribution of these cells. Supporting evidences were generated using retroviral-defective cell lineage tracing experiments in chicken hearts providing similar results (Mikawa and Gourdie, 1996); i.e., vascular endothelial, smooth muscle, and cardiac fibroblasts. Contribution to endocardial cushions is scarce, although it has been proposed that these cells are important for the correct development of the atrioventricular junction and the annulus fibrosus (Lie-Venema et al., 2008; Zhou et al., 2010; Lockhart et al., 2014). More recently, a contribution to cardiac resident stem cells (mesenchymal-like) has also been reported (Chong et al., 2011). In all cases, contribution to the developing myocardium was never observed (Poelmann et al., 1993; Mikawa and Gourdie, 1996; Pérez-Pomares et al., 2002). Surprisingly, *in vitro* PE culture experiments demonstrated that cardiomyocytes could be derived from these precursor cell pools (Kruithof et al., 2006).

With the advent of the molecular era, genetic lineage tracing in mice assaulted the quest to understand the contribution of the PE/embryonic epicardium to the mature murine heart. Several lineage tracing approaches were documented, in most cases, using Cre/loxP conditional activation of the reporter genes. In this setting, *Tbx18*-lineage tracing demonstrated a contribution to all the previously reported EPDC-derived lineages but surprisingly, also to the cardiomyocyte lineage. Whereas, these studies claimed that epicardial Tbx18+ cells contributed *in vivo* to ventricular cardiomyocytes (Cai et al., 2008), it was previously reported that fetal cardiomyocytes also expressed *Tbx18* (Franco et al., 2006; Christoffels et al., 2009; Zeng et al., 2011) and thus those Tbx18+ epicardial lineage tracing experiments were dubious.

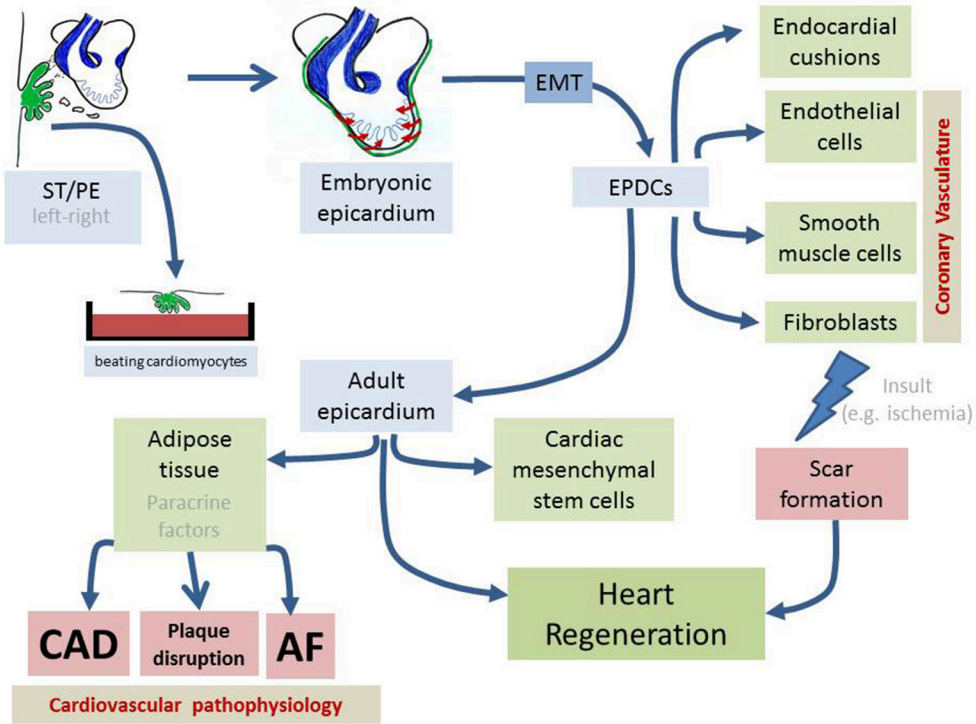

FIGURE 1 | Schematic representation of the distinct developmental stages of the proepicardium (PE)/septum transversum (ST) formation as well as on the distinct lineage contribution of the embryonic and adult epicardium.

Epicardial Wt1+ derived cells have also been reported to contribute to endothelial cells and to the myocardium (Zhou et al., 2008; Zhou and Pu, 2011). Evidence for Wt1+ cells in the embryonic heart has also been reported but excluding cardiomyocytes (Zeng et al., 2011) yet more recent evidence demonstrated that *Wt1*-derived cardiomyoctes can be traced in the developing heart before PE/epicardial formation (Rudat and Kispert, 2012; Cano et al., 2016), thus questioning the epicardial contribution to the developing cardiac muscle. On the other hand, prove that epicardial cells do not contribute to myocardium in zebrafish comes from *Tcf21*-tracing (Kikuchi et al., 2011) and transplant experiments (González-Rosa et al., 2012), in which a contribution to the perivascular beds is reported. While these data might support the notion that epicardial cells can contribute to the formation of cardiomyocytes *in vivo*, yet these evidences remain controversial, mainly because the limitation on the use of Cre-based techniques as a *bone fide* fate mapping approach (Christoffels et al., 2009).

Additional controversies have also arisen regarding the contribution of EPDCs to other vascular components. To date, it seems clear that EPDCs mostly contribute to cardiac fibroblasts and vascular smooth muscle cells, but their contribution to vascular endothelial cells have also been challenged by additional Cre-based fate mapping experiments. In fact, epicardial-derived Cre based lineage tracing in mice failed to provide substantial contribution to the developing vascular endothelium in mice (Merki et al., 2005; Cai et al., 2008; Zhou et al., 2008). Red-Horse et al. (2010) described that coronary endothelial lining was mostly entirely derived from the sinus venosus endothelium as revealed by an *Apelin-Cre* mice (Red-Horse et al., 2010; Tian et al., 2013), a process that is VEGF-dependent (Chen et al., 2014). However, additional evidences reported that ventricular endocardial cells also can contribute to the coronary vasculature (Wu et al., 2012) as revealed by *Nfatc1-Cre* lineage tracing. Furthermore, by the usage of novel proepicardial lineage tracing markers such as *Scleraxis-Cre*, *Semaphorin-3D-Cre,* and *Fabp4-CreER* drivers (Katz et al., 2012; He et al., 2014) a contribution to the coronary vasculature was also reported. In fact, reconciling evidences reported by Chen et al. (2014) determined that sinus-venous (SV) derived coronary vasculature mostly contributed to the dorsal and lateral coronary vasculature (∼70%) whereas the ventral aspects were mostly endocardial derived (∼70–80%), with just a small (∼20%) but uniform contribution from the epicardium. These data are in line with a recent report that similarly estimated a 20% contribution from the proepicardium (Cano et al., 2016). Interestingly, a significant proportion of SV-derived and endocardial-derived cells displayed overlapping patterns with PE-derived cells, suggesting a common lineage origin. These data support the notion that multiple precursor cell populations contribute to the formation of the cardiac vasculature in mice, in contrast to avian hearts, in which the epicardial-derived contribution is large and undisputed. Lineage relationships between these three distinct coronary vasculature components remain nonetheless to be fully elucidated in mice.

Over the last decade our understanding of the molecular regulation of epicardial derived cells has largely increased with

TABLE 1 | List of transcription factors, growth factors are other distinct molecules involved in distinct phases of proepicardium/epicardium development.

	PE formation	EMT	Cell differentiation	References
TRANSCRIPTION FACTORS				
wt1	Specification	Cell migration	Endothelial and myocardial cells	Zhou et al., 2008; Zhou and Pu, 2011; Rudat and Kispert, 2012; Cano et al., 2016
tbx5	Specification			Liu and Stainier, 2010; Diman et al., 2014
tbx18		Cell migration		Takeichi et al., 2013; Wu et al., 2013
tcf21/pod1			Inhibits SM cells; promotes fibroblasts	Braitsch et al., 2012
nkx2.5	Specification			Zhou et al., 2008
islet-1	Specification		Fibroblasts formation	Zhou et al., 2008; Brønnum et al., 2013a
gata-4	Specification			Watt et al., 2004; Kolander et al., 2014
Coup-tfII		Cell migration		Lin et al., 2012
Mrtf1/Mrtf2		Cell migration		Trembley et al., 2015
Nf1		Cell migration		Baek and Tallquist, 2012
GROWTH FACTORS				
Tgfb1	Tgf b signaling	Cell migration		Craig et al., 2010a
Tgfb2	Tgf b signaling	Cell migration		Craig et al., 2010a
Tgfbr3	Tgf b signaling	Cell migration		Sánchez and Barnett, 2012
fgf10	Fgf signaling		Fibroblasts	Guadix et al., 2006; Vega-Hernández et al., 2011
fgfr2b	Fgf signaling		Fibroblasts	Guadix et al., 2006; Vega-Hernández et al., 2011
dkk1	Wnt signaling			Phillips et al., 2011
dkk2	Wnt signaling			Phillips et al., 2011
cxcl12			Coronary vasculature contribution	Cavallero et al., 2015
ccr4			Coronary vasculature contribution	Cavallero et al., 2015
yap	Hippo signaling		Coronary vasculature contribution	Singh et al., 2016
taz	Hippo signaling		Coronary vasculature contribution	Singh et al., 2016
pdgfrbeta	PDGF signaling	Cell migration	SM cells	Mellgren et al., 2008; Bax et al., 2009; Smith et al., 2011
vegf		Cell migration	Endothelial cells	Guadix et al., 2006; Tomanek et al., 2006; Azambuja et al., 2010
OTHERS				
ra			Endothelial cells	Guadix et al., 2006; Tomanek et al., 2006; Azambuja et al., 2010
MEKK1	MAPK signaling	Cell migration		Craig et al., 2010b
tenascin c			SM cell recruitment	Ando et al., 2011
nephrin			SM cell recruitment	Wagner et al., 2011
Par6/Smurf/RhoA	Wnt signaling	Cell migration		Sánchez and Barnett, 2012
Vcam/RhoA		Cell migration		Dokic and Dettman, 2006

the usage of conditional spatio-temporal deletion of discrete signaling pathways. Epicardial cells display distinct divergent and overlapping expression patterns of *Wt1, Nfatc1, Tbx18,* and *Pod1* in the chicken and murine hearts (Braitsch et al., 2012), providing a heterogeneous panel of potentially distinct cardiac stem cells. Whereas, to date it remains elusive when and how epicardial cells becomes specific to their prospective lineage, it is increasing clear that multiple factors play pivotal roles in this process as summarized in **Table 1**. In particular, PDGFRβ is important for epicardial migration and for the development of coronary vascular smooth muscle cells (Mellgren et al., 2008; Bax et al., 2009; Smith et al., 2011), retinoic acid and VEGF primes endothelial vs. smooth muscle differentiation (Guadix et al., 2006; Tomanek et al., 2006; Azambuja et al., 2010) and Fgf signaling (Guadix et al., 2006), mainly through Fgf10 and Fgfr2b are essential for cardiac fibroblast formation (Vega-Hernández et al., 2011). In addition, *Pod1/Tcf21* is regulated by retinoic acid and inhibits differentiation of EPDCs into smooth muscle cells in chicken and mice (Braitsch et al., 2012) while Wnt signaling is also important for epicardial specification, as *Dkk1* and *Dkk2* mouse mutants display impaired epicardial development (Phillips et al., 2011). Similarly PCP disruption is also critical in this context (Phillips et al., 2008) as well as MAPK kinase genetic inactivation (Liberatore and Yutzey, 2004; Craig et al., 2010a,b). Other signaling pathways, such as *CXCL12/CXCR4* are also crucial for cardiac vascular development (Cavallero et al., 2015). Furthermore, Hippo signaling, mediated by *Yap/Taz* modulates *Tbx18* and *Wt1* expression in the epicardium controlling their contribution to the coronary vasculature (Singh et al., 2016). Several other molecules have also been reported to be critical for coronary artery formation, such as tenascin C (Ando et al., 2011) and nephrin (Wagner et al., 2011) particularly for smooth muscle recruitment to those cardiac vessels. Overall these findings highlight the complexity of distinct signaling pathways and molecules governing the coronary vasculature development.

THE ROLE OF THE POSTNATAL EPICARDIUM IN THE INJURED HEART

Within the adult heart, the epicardium represents the outermost layer, which is a simple epithelial layer. For many years, the functional role of this layer has been neglected as it was considered as an external cover devoid of any functional meaning. The discovery that the epicardial precursors can differentiate to beating cardiomyocytes has branded the epicardium as a source of cardiac stem cells with great therapeutic potential (Wessels and Pérez-Pomares, 2004; Pérez-Pomares et al., 2006; Winter et al., 2009). In addition, it has been reported that the adult epicardium plays a pivotal role in cardiac regeneration (Bollini et al., 2011, 2015; Schlueter and Brand, 2012; Masters and Riley, 2014; Kennedy-Lydon and Rosenthal, 2015; **Figure 1**) as detailed below.

Seminal work by Kruithof et al. (2006) described that the embryonic chicken PE if placed in appropriate cell culture conditions, was capable of giving rise to beating cardiomyocytes. Such *in vitro* conditions could be further promoted by Bmp administration and blocked by Fgf signaling. Thus, these data opened out the possibility that the epicardium could serve as an *in vivo* source of potential cardiomyocytes if the appropriate signals would be instructed *in vivo*. Importantly, Smart et al. (2011) demonstrated that adult epicardial derived cells, if previously primed with thymosinβ4, eventually generated functionally beating cardiomyocytes in an ischemic heart, yet the proportion of *de novo* integrated cells was rather spurious and its instructive mechanism remains rather obscure (Gajzer et al., 2013). Nonetheless, as a proof of principle approach it was highly valuable. This work introduced a novel concept of an activated epicardium, a condition by which embryonic epicardial markers such as *Wt1* and *Tbx18* are re-expressed in the adult epicardium (Huang et al., 2012; van Wijk et al., 2012; Braitsch et al., 2013; Bollini et al., 2014; Aguiar and Brunt, 2015) in response to distinct biological stimuli such as thymosinβ4 (Smart et al., 2012; Smart and Riley, 2012), stem cell factor (SCF; Xiang et al., 2014), and prokineticins (Urayama et al., 2008) among others. In addition, this activated epicardium secretes paracrine factors that modulate myocardial injury response (Zhou et al., 2011; Foglio et al., 2015).

While it is documented that the human heart has a limited capacity to regenerate (Bergmann et al., 2009), it is also highly acknowledged that the newt heart can also widely regenerate by other means (Becker et al., 1974; Oberpriller and Oberpriller, 1974). Furthermore, the adult zebrafish heart can also regenerate (Poss et al., 2002) and the epicardium provides a pivotal role during this regeneration process (Gemberling et al., 2015; Wang et al., 2015). Molecular analyses have demonstrated that the epicardium becomes activated as soon as the heart is injured and such activation provides instructive signals that promote cardiomyocyte proliferation, revascularization, and tissue repair (Lien et al., 2006, 2012; Marín-Juez et al., 2016). During this process a transitory scar stage occurs and is subsequently replaced by fully functional and integrated cardiomyocytes (González-Rosa et al., 2011; Mercer et al., 2013; Itou et al., 2014; Marro et al., 2016).

Further analyses in this front identified that Wnt1/β-catenin is crucial promoting formation of cardiac fibroblasts and hence cardiac repair (Duan et al., 2012). Several studies have identified key molecules modulating this regeneration capacity. For example, Nrg1 acts as a mitogenic agent in cardiomyocytes following injury during cardiac zebrafish regeneration (Gemberling et al., 2015). Notch (Zhao et al., 2014), Raldh2 (Itou et al., 2014), and myocardial NF-κB (Karra et al., 2015) are also essential for heart regeneration in zebrafish. Hydrogen peroxide (Han et al., 2014) has been reported to prime heart regeneration and telomerase has been identified as instrumental for zebrafish regeneration (Bednarek et al., 2015), but still it remains to be established if these factors are modulated by the epicardium. More recently, it has been demonstrated that epicardial regeneration is guided by the cardiac outflow tract and hedgehog signaling (Wang et al., 2015) and single cell transcriptome of the epicardium has identified caveolin1 as an essential factor in regenerating zebrafish heart (Cao and Poss, 2016). Moreover, re-expression of epicardial developmental genes and enhanced EMT in response to injury has been widely demonstrated (Lepilina et al., 2006; Kim et al., 2010; González-Rosa et al., 2011; Schnabel et al., 2011). These data suggest that complex regulatory networks control zebrafish regeneration (Rodius et al., 2016) positioning the epicardium as a key tissue layer for regeneration. Thus, these data will be highly instrumental to search for novel ways to heal the injured heart.

In adult mice, the regenerative capacity is lost and the injured heart responds by generating a fibrous scar which is derived from pre-existing epicardial cells (Zhou et al., 2008; Duan et al., 2012) as well as *de novo* recruited bone marrow-borne circulating cells (Ruiz-Villalba et al., 2015). Interestingly, full regeneration is achieved at early developmental stages, i.e., on the first week of life, in which the epicardium (Porrello et al., 2011) is also a highly instructive player and thymosinβ4 priming increases the time window for mammalian heart regeneration (Rui et al., 2014). In addition a role for Wnt signaling has also been identified in the regenerating heart in mice (Mizutani et al., 2016). Recent evidence demonstrated that exosomal signaling from the epicardium is essential for myocardial maturation highlighting a pivotal role for clustering in this process (Foglio et al., 2015). All these efforts have provided the bases of heart regeneration. A giant step was recently reported by Wei et al. (2015) whom used reconstitution of epicardial follistatin-like1 expression in biomaterial patches to heal the adult injured heart, opening a novel way to regenerate the adult mammalian heart.

AN UNEXPECTED EPICARDIAL DERIVATIVE WITH PARACRINE SIGNALING LEADING TO CAD AND AF

While it is highly acknowledged that the epicardial precursor cells, within the PE, and subsequently the EPDCs will give rise to distinct cardiovascular embryonic cell lineages, it has remained unexplored if the adult epicardium can generate additional cellular subpopulations. Recent evidences have demonstrated that intramyocardial adipose tissue is derived from

the endocardium (Zhang et al., 2016), whereas adipose tissue around the heart, mainly at the venous, arterial connections, and atrial appendages is an adult epicardium derivative (Yamaguchi et al., 2015). Furthermore, cardiac adipose tissue deposition has recently been associated to distinct cardiovascular pathologies (**Figure 1**), such as coronary arteries diseases (Iwayama et al., 2014), atherosclerosis plaque disruption (Talman et al., 2014; Yamashita et al., 2014), and atrial fibrillation (Batal et al., 2010; Nakanishi et al., 2012; Gaborit et al., 2013). Although, these are early days to fully understand the molecular mechanisms linking epicardium, adipose tissue deposition, and cardiovascular pathologies, supporting evidences suggest that these cells can act as paracrine signaling center that, if impaired, can be the source of cardiovascular diseases (Langlois et al., 2010; Greulich et al., 2012).

NON-CODING RNAs IN THE PE/EPICARDIUM

Over the last decade we have witnessed a revolution in the concept of the control of gene expression with the discovery of non-coding RNAs. Non-coding RNAs can be broadly classified according to the transcript size into long non-coding RNAs (lncRNAs) and small non-coding RNAs. Our current understanding of lncRNAs is still in its infancy with just a limited number of reports in the developing heart (Grote et al., 2013; Klattenhoff et al., 2013; Sauvageau et al., 2013; Zhu et al., 2014; Kurian et al., 2015). On the other hand, our knowledge on the functional role of small non-coding RNAs, in particular microRNAs, has been largely increased (Callis and Wang, 2008; Chen and Wang, 2012; Bonet et al., 2013; Philippen et al., 2015; Yan and Jiao, 2016). microRNAs are small non-coding RNA of 18–24 nt in length that by homologous base-priming are capable of blocking translation or degrading mRNA transcripts. microRNAs are transcribed by RNA polymerase II, 5′ capped and 3′ polyadenylated leading to mature microRNA by RNA endonucleases such as Drosha and Dicer (Aranega and Franco, 2015; Towler et al., 2015). Mature microRNAs are loaded into the RISC complex which can thereafter search for mRNA transcript base complementarity (Hammond, 2015; Shen and Hung, 2015). To date more than a 1,000 distinct microRNAs have been identified in humans, which are quite conserved among evolution. A seminal study by Singh et al. (2011) demonstrated that conditional ablation of Dicer, an RNAse processing enzyme, in the epicardium provoked impaired epicardial formation, thin-walled myocardium, and aberrant coronary vasculature formation. Thus, this study demonstrated a pivotal role for microRNAs in PE/epicardium development. A large array of studies have been reported in key developmental processes by which the PE/epicardium is formed, such as epithelial-to-mesenchymal transition in cancer (see for recent reviews; Behbahani et al., 2016; Peng et al., 2016; Sulaiman et al., 2016; Zou et al., 2016) and also within the heart (Stankunas et al., 2010; Bonet et al., 2015) and cardiac regeneration (Porrello et al., 2013) but surprisingly only a short list of studies have been reported in PE/epicardium formation. miR-21 has been reported in numerous studies promoting fibrogenesis both during cardiac development and disease (Thum et al., 2008; Adam et al., 2012; Derda et al., 2015; Gupta et al., 2016). Brønnum et al. (2013a) has recently reported that miR-21 promotes fibrogenic EMT in epicardial cells by modulating *Pcd4* and *Sprouty-1* and these authors (Brønnum et al., 2013b) have also reported that islet-1 can influence miR-21 expression and therefore modulate cardiac fibrogenic EMT. Seeger et al. (2016) demonstrated that *let-7* inhibition enhances the recruitment of epicardial cells after myocardial infarction promoting an improved cardiac function. Overall these studies demonstrate a nascent role for microRNAs in PE/epicardium formation, which might provide novel approaches to activate and prime epicardial cells for cardiac regeneration.

CONCLUSIONS AND PERSPECTIVES

Over the last decade our understanding of the cellular contribution of the PE/epicardium has largely increased. Seminal works using quail-chick chimeras demonstrated a large plasticity for the EPCDs, contributing to the cardiac fibrous skeleton, the coronary vasculature and the developing atrioventricular valves (Poelmann et al., 1993; Wessels and Pérez-Pomares, 2004; **Figure 1**). However, with the advent of molecular tracing tools, multiple evidences demonstrated a rather more complex contribution and architecture to the coronary vasculature in mice. Cre-driven fate mapping can be pervasive and promiscuous tools, deriving in complex and in many cases controversial findings. We hope that either retrospective clonal analysis as previously reported for myocardial components (Meilhac et al., 2003, 2004a,b) or genuine prospective lineage tracing would serve to reconcile these findings in the PE/epicardial context. With no doubt one of the seminal work that prompted the interest of the epicardial lining in the context of cardiac stem cell and cardiac regeneration was reported by Kruithof et al. (2006) demonstrating that PE/epicardial cells could be generating cardiomyocyte *in vitro* opening the possibilities to unlock the myocardial lineage commitment *in vivo*. Thymosin beta4 was the first of these unlocking tools, providing an entry site to regenerate the heart using the epicardium as a cell source (Smart et al., 2011; Smart and Riley, 2012). In addition, bridging epicardial activation by follistatin-like1 into biomaterials provided additional convincing evidences on the feasibility of these approaches (Wei et al., 2015). New tools will be discovered in the near future.

In recent years a novel link between the epicardium and epicardial derived structures is emerging (**Figure 1**). Intriguingly, adipose fat deposition within the pericardiac regions has been linked to cardiac pathophysiologies such as coronary artery atherosclerosis and atrial fibrillation. To date the casual relationship remains enigmatic, yet a plausible embryonic link might be present since epicardial cells can differentiate into adipose tissue (Zhang et al., 2016) and epicardial cells contribute to both endothelial and smooth muscle components of the coronary vessels (Pérez-Pomares et al., 2002; Cano et al., 2016). However, our current understanding is still in its infancy and for

sure we will witness additional cellular and molecular evidences deciphering the interplay between these rather apparent distinct cardiovascular entities.

While our cellular and molecular understanding of PE/epicardium/EPDC has greatly advanced in recently years, the discovery of novel levels of gene regulations, in particular those exerted by the non-coding RNAs, is called to change our molecular and signaling pathways schemes. The discovery that microRNAs are crucial to epicardial development is simply demonstrating the equally pivotal roles of these tiny molecules in other cardiovascular developmental contexts (Cordes and Srivastava, 2009; Chinchilla et al., 2011; Bonet et al., 2015). In addition to microRNAs, long-non-coding RNAs are also called to play pivotal role in cardiogenesis (Grote et al., 2013; Klattenhoff et al., 2013) and thus similarly in epicardial development. In coming years, additional routes would be discovered demonstrating the essential role of these new players in epicardial biology both during development and disease.

AUTHOR CONTRIBUTIONS

AD searched for literature records and wrote part of the manuscript. DF wrote part of the manuscript, editing, and approved the final manuscript version. AA read and suggested editing comments to the manuscript.

FUNDING

DF and AA are partially funding by a grant of the Ministerio de Economia y Competitividad (BFU2015-67131-P) of the Spanish Government and the Consejeria de IEconomia y Conocimiento (CTS-446) of the Junta de Andalucia Regional Council.

REFERENCES

Adam, O., Löhfelm, B., Thum, T., Gupta, S. K., Puhl, S. L., Schäfers, H. J., et al. (2012). Role of miR-21 in the pathogenesis of atrial fibrosis. *Basic Res. Cardiol.* 107:278. doi: 10.1007/s00395-012-0278-0

Aguiar, C., and Brunt, K. R. (2015). Wilms' tumor 1 (re)activation in evidence for both epicardial progenitor and endothelial cells for cardiovascular regeneration. *J. Mol. Cell. Cardiol.* 84, 112–115. doi: 10.1016/j.yjmcc.2015.04.001

Ando, K., Takahashi, M., Yamagishi, T., Miyagawa-Tomita, S., Imanaka-Yoshida, K., Yoshida, T., et al. (2011). Tenascin C may regulate the recruitment of smooth muscle cells during coronary artery development. *Differentiation* 81, 299–306. doi: 10.1016/j.diff.2011.03.002

Aranega, A. E., and Franco, D. (2015). "Post-transcriptional regulation by proteins and non-coding RNAs," in *Congenital Heart Diseases: The Broken Heart*, eds S. Rickert-Sperling, R. G. Kelly, D. J. Driscoll (Wien: Springer), 153–172.

Azambuja, A. P., Portillo-Sánchez, V., Rodrigues, M. V., Omae, S. V., Schechtman, D., Strauss, B. E., et al. (2010). Retinoic acid and VEGF delay smooth muscle relative to endothelial differentiation to coordinate inner and outer coronary vessel wall morphogenesis. *Circ. Res.* 107, 204–216. doi: 10.1161/circresaha.109.214650

Baek, S. T., and Tallquist, M. D. (2012). Nf1 limits epicardial derivative expansion by regulating epithelial to mesenchymal transition and proliferation. *Development* 139, 2040–2049. doi: 10.1242/dev.074054

Balmer, G. M., Bollini, S., Dubé, K. N., Martinez-Barbera, J. P., Williams, O., and Riley, P. R. (2014). Dynamic haematopoietic cell contribution to the developing and adult epicardium. *Nat. Commun.* 5:4054. doi: 10.1038/ncomms5054

Batal, O., Schoenhagen, P., Shao, M., Ayyad, A. E., Van Wagoner, D. R., Halliburton, S. S., et al. (2010). Left atrial epicardial adiposity and atrial fibrillation. *Circ. Arrhythm. Electrophysiol.* 3, 230–236. doi: 10.1161/CIRCEP.110.957241

Bax, N. A., Lie-Venema, H., Vicente-Steijn, R., Bleyl, S. B., Van Den Akker, N. M., Maas, S., et al. (2009). Platelet-derived growth factor is involved in the differentiation of second heart field-derived cardiac structures in chicken embryos. *Dev. Dyn.* 238, 2658–2669. doi: 10.1002/dvdy.22073

Becker, R. O., Chapin, S., and Sherry, R. (1974). Regeneration of the ventricular myocardium in amphibians. *Nature* 248, 145–147.

Bednarek, D., González-Rosa, J. M., Guzmán-Martínez, G., Gutiérrez-Gutiérrez, Ó., Aguado, T., Sánchez-Ferrer, C., et al. (2015). Telomerase is essential for zebrafish heart regeneration. *Cell Rep.* 12, 1691–1703. doi: 10.1016/j.celrep.2015.07.064

Behbahani, G. D., Ghahhari, N. M., Javidi, M. A., Molan, A. F., Feizi, N., and Babashah, S. (2016). MicroRNA-mediated post-transcriptional regulation of epithelial to mesenchymal transition in cancer. *Pathol. Oncol. Res.* 23, 1–12. doi: 10.1007/s12253-016-0101-6

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., et al. (2009). Evidence for cardiomyocyte renewal in humans. *Science* 324, 98–102. doi: 10.1126/science.1164680

Bollini, S., Riley, P. R., and Smart, N. (2015). Thymosin β4: multiple functions in protection, repair and regeneration of the mammalian heart. *Expert Opin. Biol. Ther.* 15(Suppl. 1), S163–S174. doi: 10.1517/14712598.2015.1022526

Bollini, S., Smart, N., and Riley, P. R. (2011). Resident cardiac progenitor cells: at the heart of regeneration. *J. Mol. Cell. Cardiol.* 50, 296–303. doi: 10.1016/j.yjmcc.2010.07.006

Bollini, S., Vieira, J. M., Howard, S., Dubè, K. N., Balmer, G. M., Smart, N., et al. (2014). Re-activated adult epicardial progenitor cells are a heterogeneous population molecularly distinct from their embryonic counterparts. *Stem Cells Dev.* 23, 1719–1730. doi: 10.1089/scd.2014.0019

Bonet, F., Dueñas, Á., López-Sánchez, C., García-Martínez, V., Aránega, A. E., and Franco, D. (2015). MiR-23b and miR-199a impair epithelial-to-mesenchymal transition during atrioventricular endocardial cushion formation. *Dev. Dyn.* 244, 1259–1275. doi: 10.1002/dvdy.24309

Bonet, F., Hernandez-Torres, F., Esteban, F. J., Aranega, A., and Franco, D. (2013). Comparative analyses of microRNA microarrays during cardiogenesis: functional perspectives. *Microarrays* 2, 81–96. doi: 10.3390/microarrays2020081

Braitsch, C. M., Combs, M. D., Quaggin, S. E., and Yutzey, K. E. (2012). Pod1/Tcf21 is regulated by retinoic acid signaling and inhibits differentiation of epicardium-derived cells into smooth muscle in the developing heart. *Dev. Biol.* 368, 345–357. doi: 10.1016/j.ydbio.2012.06.002

Braitsch, C. M., Kanisicak, O., van Berlo, J. H., Molkentin, J. D., and Yutzey, K. E. (2013). Differential expression of embryonic epicardial progenitor markers and localization of cardiac fibrosis in adult ischemic injury and hypertensive heart disease. *J. Mol. Cell. Cardiol.* 65, 108–119. doi: 10.1016/j.yjmcc.2013.10.005

Brønnum, H., Andersen, D. C., Schneider, M., Nossent, A. Y., Nielsen, S. B., and Sheikh, S. P. (2013a). Islet-1 is a dual regulator of fibrogenic epithelial-to-mesenchymal transition in epicardial mesothelial cells. *Exp. Cell Res.* 319, 424–435. doi: 10.1016/j.yexcr.2012.12.019

Brønnum, H., Andersen, D. C., Schneider, M., Sandberg, M. B., Eskildsen, T., Nielsen, S. B., et al. (2013b). miR-21 promotes fibrogenic epithelial-to-mesenchymal transition of epicardial mesothelial cells involving Programmed Cell Death 4 and Sprouty-1. *PLoS ONE* 8:e56280. doi: 10.1371/journal.pone.0056280

Cai, C. L., Martin, J. C., Sun, Y., Cui, L., Wang, L., Ouyang, K., et al. (2008). A myocardial lineage derives from Tbx18 epicardial cells. *Nature* 454, 104–108. doi: 10.1038/nature06969

Callis, T. E., and Wang, D. Z. (2008). Taking microRNAs to heart. *Trends Mol. Med.* 14, 254–260. doi: 10.1016/j.molmed.2008.03.006

Cano, E., Carmona, R., Ruiz-Villalba, A., Rojas, A., Chau, Y. Y., Wagner, K. D., et al. (2016). Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arterio-venous connections. *Proc. Natl. Acad. Sci. U.S.A.* 113, 656–661. doi: 10.1073/pnas.1509834113

Cao, J., and Poss, K. D. (2016). Explant culture of adult zebrafish hearts for epicardial regeneration studies. *Nat. Protoc.* 11, 872–881. doi: 10.1038/nprot.2016.049

Cavallero, S., Shen, H., Yi, C., Lien, C. L., Kumar, S. R., and Sucov, H. M. (2015). CXCL12 signaling is essential for maturation of the ventricular coronary endothelial plexus and establishment of functional coronary circulation. *Dev. Cell* 33, 469–477. doi: 10.1016/j.devcel.2015.03.018

Chen, H. I., Sharma, B., Akerberg, B. N., Numi, H. J., Kivelä, R., Saharinen, P., et al. (2014). The sinus venosus contributes to coronary vasculature through VEGFC-stimulated angiogenesis. *Development* 141, 4500–4512. doi: 10.1242/dev.113639

Chen, J., and Wang, D. Z. (2012). microRNAs in cardiovascular development. *J. Mol. Cell. Cardiol.* 52, 949–957. doi: 10.1016/j.yjmcc.2012.01.012

Chinchilla, A., Lozano, E., Daimi, H., Esteban, F. J., Crist, C., Aranega, A. E., et al. (2011). MicroRNA profiling during mouse ventricular maturation: a role for miR-27 modulating Mef2c expression. *Cardiovasc. Res.* 89, 98–108. doi: 10.1093/cvr/cvq264

Chong, J. J., Chandrakanthan, V., Xaymardan, M., Asli, N. S., Li, J., Ahmed, I., et al. (2011). Adult cardiac-resident MSC-like stem cells with a proepicardial origin. *Cell Stem Cell* 9, 527–540. doi: 10.1016/j.stem.2011.10.002

Christoffels, V. M., Grieskamp, T., Norden, J., Mommersteeg, M. T., Rudat, C., and Kispert, A. (2009). Tbx18 and the fate of epicardial progenitors. *Nature* 458, E8–E9; discussion: E9–E10. doi: 10.1038/nature07916

Cordes, K. R., and Srivastava, D. (2009). MicroRNA regulation of cardiovascular development. *Circ. Res.* 104, 724–732. doi: 10.1161/CIRCRESAHA.108.192872

Cossette, S., and Misra, R. (2011). The identification of different endothelial cell populations within the mouse proepicardium. *Dev. Dyn.* 240, 2344–2353. doi: 10.1002/dvdy.22724

Craig, E. A., Austin, A. F., Vaillancourt, R. R., Barnett, J. V., and Camenisch, T. D. (2010a). TGFβ2-mediated production of hyaluronan is important for the induction of epicardial cell differentiation and invasion. *Exp. Cell Res.* 316, 3397–3405. doi: 10.1016/j.yexcr.2010.07.006

Craig, E. A., Parker, P., Austin, A. F., Barnett, J. V., and Camenisch, T. D. (2010b). Involvement of the MEKK1 signaling pathway in the regulation of epicardial cell behavior by hyaluronan. *Cell. Signal.* 22, 968–976. doi: 10.1016/j.cellsig.2010.02.004

Derda, A. A., Thum, S., Lorenzen, J. M., Bavendiek, U., Heineke, J., Keyser, B., et al. (2015). Blood-based microRNA signatures differentiate various forms of cardiac hypertrophy. *Int. J. Cardiol.* 196, 115–122. doi: 10.1016/j.ijcard.2015.05.185

Dettman, R. W., Denetclaw, W. Jr., Ordahl, C. P., and Bristow, J. (1998). Common epicardial origin of coronary vascular smooth muscle, perivascular fibroblasts, and intermyocardial fibroblasts in the avian heart. *Dev. Biol.* 193, 169–181. doi: 10.1006/dbio.1997.8801

Diman, N. Y., Brooks, G., Kruithof, B. P., Elemento, O., Seidman, J. G., Seidman, C. E., et al. (2014). Tbx5 is required for avian and Mammalian epicardial formation and coronary vasculogenesis. *Circ. Res.* 115, 834–844. doi: 10.1161/CIRCRESAHA.115.304379

Dokic, D., and Dettman, R. W. (2006). VCAM-1 inhibits TGFbeta stimulated epithelial-mesenchymal transformation by modulating Rho activity and stabilizing intercellular adhesion in epicardial mesothelial cells. *Dev. Biol.* 299, 489–504. doi: 10.1016/j.ydbio.2006.08.054

Duan, J., Gherghe, C., Liu, D., Hamlett, E., Srikantha, L., Rodgers, L., et al. (2012). Wnt1/βcatenin injury response activates the epicardium and cardiac fibroblasts to promote cardiac repair. *EMBO J.* 31, 429–442. doi: 10.1038/emboj.2011.418

Foglio, E., Puddighinu, G., Fasanaro, P., D'Arcangelo, D., Perrone, G. A., Mocini, D., et al. (2015). Exosomal clusterin, identified in the pericardial fluid, improves myocardial performance following MI through epicardial activation, enhanced arteriogenesis and reduced apoptosis. *Int. J. Cardiol.* 197, 333–347. doi: 10.1016/j.ijcard.2015.06.008

Franco, D., Meilhac, S. M., Christoffels, V. M., Kispert, A., Buckingham, M., and Kelly, R. G. (2006). Left and right ventricular contributions to the formation of the interventricular septum in the mouse heart. *Dev. Biol.* 294, 366–375. doi: 10.1016/j.ydbio.2006.02.045

Gaborit, B., Abdesselam, I., and Dutour, A. (2013). Epicardial fat: more than just an "epi" phenomenon? *Horm. Metab. Res.* 45, 991–1001. doi: 10.1055/s-0033-1358669

Gajzer, D. C., Balbin, J., and Chaudhry, H. W. (2013). Thymosin β4 and cardiac regeneration: are we missing a beat? *Stem Cell Rev.* 9, 303–312. doi: 10.1007/s12015-012-9378-3

Gemberling, M., Karra, R., Dickson, A. L., and Poss, K. D. (2015). Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *elife* 4:e05871. doi: 10.7554/eLife.05871

Gittenberger-de Groot, A. C., Winter, E. M., Bartelings, M. M., Goumans, M. J., DeRuiter, M. C., and Poelmann, R. E. (2012). The arterial and cardiac epicardium in development, disease and repair. *Differentiation* 84, 41–53. doi: 10.1016/j.diff.2012.05.002

González-Rosa, J. M., Martín, V., Peralta, M., Torres, M., and Mercader, N. (2011). Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* 138, 1663–1674. doi: 10.1242/dev.060897

González-Rosa, J. M., Peralta, M., and Mercader, N. (2012). Pan-epicardial lineage tracing reveals that epicardium derived cells give rise to myofibroblasts and perivascular cells during zebrafish heart regeneration. *Dev. Biol.* 370, 173–186. doi: 10.1016/j.ydbio.2012.07.007

Greulich, F., Farin, H. F., Schuster-Gossler, K., and Kispert, A. (2012). Tbx18 function in epicardial development. *Cardiovasc. Res.* 96, 476–483. doi: 10.1093/cvr/cvs277

Grote, P., Wittler, L., Hendrix, D., Koch, F., Währisch, S., Beisaw, A., et al. (2013). The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. *Dev. Cell* 24, 206–214. doi: 10.1016/j.devcel.2012.12.012

Guadix, J. A., Carmona, R., Muñoz-Chápuli, R., and Pérez-Pomares, J. M. (2006). *In vivo* and *in vitro* analysis of the vasculogenic potential of avian proepicardial and epicardial cells. *Dev. Dyn.* 235, 1014–1026. doi: 10.1002/dvdy.20685

Gupta, S. K., Itagaki, R., Zheng, X., Batkai, S., Thum, S., Ahmad, F., et al. (2016). miR-21 promotes fibrosis in an acute cardiac allograftransplantation model. *Cardiovasc. Res.* 110, 215–226. doi: 10.1093/cvr/cvw030

Hammond, S. M. (2015). An overview of microRNAs. *Adv. Drug Deliv. Rev.* 87, 3–14. doi: 10.1016/j.addr.2015.05.001

Han, P., Zhou, X. H., Chang, N., Xiao, C. L., Yan, S., Ren, H., et al. (2014). Hydrogen peroxide primes heart regeneration with a derepression mechanism. *Cell Res.* 24, 1091–1107. doi: 10.1038/cr.2014.108

He, L., Tian, X., Zhang, H., Wythe, J. D., and Zhou, B. (2014). Fabp4-CreER lineage tracing reveals two distinctive coronary vascular populations. *J. Cell. Mol. Med.* 18, 2152–2156. doi: 10.1111/jcmm.12415

Hirose, T., Karasawa, M., Sugitani, Y., Fujisawa, M., Akimoto, K., Ohno, S., et al. (2006). PAR3 is essential for cyst-mediated epicardial development by establishing apical cortical domains. *Development* 133, 1389–1398. doi: 10.1242/dev.02294

Huang, G. N., Thatcher, J. E., McAnally, J., Kong, Y., Qi, X., Tan, W., et al. (2012). C/EBP transcription factors mediate epicardial activation during heart development and injury. *Science* 338, 1599–1603. doi: 10.1126/science.1229765

Icardo, J. M., Guerrero, A., Durán, A. C., Colvee, E., Domezain, A., and Sans-Coma, V. (2009). The development of the epicardium in the sturgeon *Acipenser naccarii*. *Anat. Rec. (Hoboken).* 292, 1593–1601. doi: 10.1002/ar.20939

Itou, J., Akiyama, R., Pehoski, S., Yu, X., Kawakami, H., and Kawakami, Y. (2014). Regenerative responses after mild heart injuries for cardiomyocyte proliferation in zebrafish. *Dev. Dyn.* 243, 1477–1486. doi: 10.1002/dvdy.24171

Iwayama, T., Nitobe, J., Watanabe, T., Ishino, M., Tamura, H., Nishiyama, S., et al. (2014). Role of epicardial adipose tissue in coronary artery disease in non-obese patients. *J. Cardiol.* 63, 344–349. doi: 10.1016/j.jjcc.2013.10.002

Kálmán, F., Virágh, S., and Módis, L. (1995). Cell surface glycoconjugates and theextracellular matrix of the developing mouse embryo epicardium. *Anat. Embryol.* 191, 451–464. doi: 10.1007/BF00304430

Karra, R., Knecht, A. K., Kikuchi, K., and Poss, K. D. (2015). Myocardial NF-κB activation is essential for zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13255–13260. doi: 10.1073/pnas.1511209112

Katz, T. C., Singh, M. K., Degenhardt, K., Rivera-Feliciano, J., Johnson, R. L., Epstein, J. A., et al. (2012). Distinct compartments of the proepicardial

organ give rise to coronary vascular endothelial cells. *Dev. Cell* 22, 639–650. doi: 10.1016/j.devcel.2012.01.012

Kelly, R. G., and Buckingham, M. E. (2002). The anterior heart-forming field: voyage to the arterial pole of the heart. *Trends Genet.* 18, 210–216. doi: 10.1016/S0168-9525(02)02642-2

Kelly, R. G., Brown, N. A., and Buckingham, M. E. (2001). The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. *Dev. Cell* 1, 435–440. doi: 10.1016/S1534-5807(01)00040-5

Kennedy-Lydon, T., and Rosenthal, N. (2015). Cardiac regeneration: epicardial mediated repair. *Proc. Biol. Sci.* 282:20152147. doi: 10.1098/rspb.2015.2147

Kikuchi, K., Gupta, V., Wang, J., Holdway, J. E., Wills, A. A., Fang, Y., et al. (2011). tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* 138, 2895–2902. doi: 10.1242/dev.067041

Kim, J., Wu, Q., Zhang, Y., Wiens, K. M., Huang, Y., Rubin, N., et al. (2010). PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17206–17210. doi: 10.1073/pnas.0915016107

Kirby, M. L., and Waldo, K. L. (1990). Role of neural crest in congenital heart disease. *Circulation* 82, 332–340. doi: 10.1161/01.CIR.82.2.332

Kirby, M. L., and Waldo, K. L. (1995). Neural crest and cardiovascular patterning. *Circ. Res.* 77, 211–215. doi: 10.1161/01.RES.77.2.211

Klattenhoff, C. A., Scheuermann, J. C., Surface, L. E., Bradley, R. K., Fields, P. A., Steinhauser, M. L., et al. (2013). Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. *Cell* 152, 570–583. doi: 10.1016/j.cell.2013.01.003

Kolander, K. D., Holtz, M. L., Cossette, S. M., Duncan, S. A., and Misra, R. P. (2014). Epicardial GATA factors regulate early coronary vascular plexus formation. *Dev. Biol.* 386, 204–215. doi: 10.1016/j.ydbio.2013.12.033

Kruithof, B. P., van Wijk, B., Somi, S., Kruithof-de Julio, M., Pérez Pomares, J. M., Weesie, F., et al. (2006). BMP and FGF regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. *Dev. Biol.* 295, 507–522. doi: 10.1016/j.ydbio.2006.03.033

Kurian, L., Aguirre, A., Sancho-Martinez, I., Benner, C., Hishida, T., Nguyen, T. B., et al. (2015). Identification of novel long noncoding RNAs underlying vertebrate cardiovascular development. *Circulation* 131, 1278–1290. doi: 10.1161/CIRCULATIONAHA.114.013303

Langlois, D., Hneino, M., Bouazza, L., Parlakian, A., Sasaki, T., Bricca, G., et al. (2010). Conditional inactivation of TGF-β type II receptor in smooth muscle cells and epicardium causes lethal aortic and cardiac defects. *Transgenic Res.* 19, 1069–1082. doi: 10.1007/s11248-010-9379-4

Lepilina, A., Coon, A. N., Kikuchi, K., Holdway, J. E., Roberts, R. W., Burns, C. G., et al. (2006). A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* 127, 607–619. doi: 10.1016/j.cell.2006.08.052

Liberatore, C. M., and Yutzey, K. E. (2004). MAP kinase activation in avian cardiovascular development. *Dev. Dyn.* 230, 773–780. doi: 10.1002/dvdy.20092

Lien, C. L., Harrison, M. R., Tuan, T. L., and Starnes, V. A. (2012). Heart repair and regeneration: recent insights from zebrafish studies. *Wound Repair Regen.* 20, 638–646. doi: 10.1111/j.1524-475X.2012.00814.x

Lien, C. L., Schebesta, M., Makino, S., Weber, G. J., and Keating, M. T. (2006). Gene expression analysis of zebrafish heart regeneration. *PLoS Biol.* 4:e260. doi: 10.1371/journal.pbio.0040260

Lie-Venema, H., Eralp, I., Markwald, R. R., van den Akker, N. M., Wijffels, M. C., Kolditz, D. P., et al. (2008). Periostin expression by epicardium-derived cells is involved in the development of the atrioventricular valves and fibrous heart skeleton. *Differentiation* 76, 809–819. doi: 10.1111/j.1432-0436.2007.00262.x

Lin, F. J., You, L. R., Yu, C. T., Hsu, W. H., Tsai, M. J., and Tsai, S. Y. (2012). Endocardial cushion morphogenesis and coronary vessel development require chicken ovalbumin upstream promoter-transcription factor II. *Arterioscler. Thromb. Vasc. Biol.* 32, e135–e146. doi: 10.1161/ATVBAHA.112.300255

Liu, J., and Stainier, D. Y. (2010). Tbx5 and Bmp signaling are essential for proepicardium specification in zebrafish. *Circ. Res.* 106, 1818–1828. doi: 10.1161/CIRCRESAHA.110.217950

Lockhart, M. M., Phelps, A. L., van den Hoff, M. J., and Wessels, A. (2014). The epicardium and the development of the atrioventricular junction in the murine heart. *J. Dev. Biol.* 2, 1–17. doi: 10.3390/jdb2010001

Männer, J., Pérez-Pomares, J. M., Macías, D., and Muñoz-Chápuli, R. (2001). The origin, formation and developmental significance of the epicardium: a review. *Cells Tissues Organs* 169, 89–103. doi: 10.1159/000047867

Marín-Juez, R., Marass, M., Gauvrit, S., Rossi, A., Lai, S. L., Materna, S. C., et al. (2016). Fast revascularization of the injured area is essential to support zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11237–11242. doi: 10.1073/pnas.1605431113

Marro, J., Pfefferli, C., de Preux Charles, A. S., Bise, T., and Jaźwińska, A. (2016). Collagen XII contributes to epicardial and connective tissues in the zebrafish heart during ontogenesis and regeneration. *PLoS ONE* 11:e0165497. doi: 10.1371/journal.pone.0165497

Masters, M., and Riley, P. R. (2014). The epicardium signals the way towards heart regeneration. *Stem Cell Res.* 13(3 Pt B), 683–692. doi: 10.1016/j.scr.2014.04.007

Meilhac, S. M., Esner, M., Kelly, R. G., Nicolas, J. F., and Buckingham, M. E. (2004a). The clonal origin of myocardial cells in different regions of the embryonic mouse heart. *Dev. Cell* 6, 685–698. doi: 10.1016/S1534-5807(04)00133-9

Meilhac, S. M., Esner, M., Kerszberg, M., Moss, J. E., and Buckingham, M. E. (2004b). Oriented clonal cell growth in the developing mouse myocardium underlies cardiac morphogenesis. *J. Cell Biol.* 164, 97–109. doi: 10.1083/jcb.200309160

Meilhac, S. M., Kelly, R. G., Rocancourt, D., Eloy-Trinquet, S., Nicolas, J. F., and Buckingham, M. E. (2003). A retrospective clonal analysis of the myocardium reveals two phases of clonal growth in the developing mouse heart. *Development* 130, 3877–3889. doi: 10.1242/dev.00580

Mellgren, A. M., Smith, C. L., Olsen, G. S., Eskiocak, B., Zhou, B., Kazi, M. N., et al. (2008). Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. *Circ. Res.* 103, 1393–1401. doi: 10.1161/CIRCRESAHA.108.176768

Mercer, S. E., Odelberg, S. J., and Simon, H. G. (2013). A dynamic spatiotemporal extracellular matrix facilitates epicardial-mediated vertebrate heart regeneration. *Dev. Biol.* 382, 457–469. doi: 10.1016/j.ydbio.2013.08.002

Merki, E., Zamora, M., Raya, A., Kawakami, Y., Wang, J., Zhang, X., et al. (2005). Epicardial retinoid X receptor alpha is required for myocardial growth and coronary artery formation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18455–18460. doi: 10.1073/pnas.0504343102

Mikawa, T., and Gourdie, R. G. (1996). Pericardial mesoderm generates a population of coronary smooth muscle cells migrating into the heart along with ingrowth of the epicardial organ. *Dev. Biol.* 174, 221–232. doi: 10.1006/dbio.1996.0068

Mizutani, M., Wu, J. C., and Nusse, R. (2016). Fibrosis of the neonatal mouse heart after cryoinjury is accompanied by wnt signaling activation and epicardial-to-mesenchymal transition. *J. Am. Heart Assoc.* 5:e002457. doi: 10.1161/JAHA.115.002457

Nakanishi, K., Fukuda, S., Tanaka, A., Otsuka, K., Sakamoto, M., Taguchi, H., et al. (2012). Peri-atrial epicardial adipose tissue is associated with new-onset nonvalvular atrial fibrillation. *Circ. J.* 76, 2748–2754. doi: 10.1253/circj.CJ-12-0637

Niderla-Bielińska, J., Gula, G., Flaht-Zabost, A., Jankowska-Steifer, E., Czarnowska, E., Radomska-Leśniewska, D. M., et al. (2015). 3-D reconstruction and multiple marker analysis of mouse proepicardial endothelial cell population. *Microvasc. Res.* 102, 54–69. doi: 10.1016/j.mvr.2015.08.007

Oberpriller, J. O., and Oberpriller, J. C. (1974). Response of the adult newt ventricle to injury. *J. Exp. Zool.* 187, 249–253. doi: 10.1002/jez.1401870208

Peng, F., Xiong, L., Tang, H., Peng, C., and Chen, J. (2016). Regulation of epithelial-mesenchymal transition through microRNAs: clinical and biological significance of microRNAs in breast cancer. *Tumour Biol.* 37, 14463–14477. doi: 10.1007/s13277-016-5334-1

Peralta, M., González-Rosa, J. M., Marques, I. J., and Mercader, N. (2014). The epicardium in the embryonic and adult zebrafish. *J. Dev. Biol.* 2, 101–116. doi: 10.3390/jdb2020101

Peralta, M., Steed, E., Harlepp, S., González-Rosa, J. M., Monduc, F., Ariza-Cosano, A., et al. (2013). Heartbeat-driven pericardiac fluid forces contribute to epicardium morphogenesis. *Curr. Biol.* 23, 1726–1735. doi: 10.1016/j.cub.2013.07.005

Pérez-Pomares, J. M., Macías, D., García-Garrido, L., and Muñoz-Chápuli, R. (1998). The origin of the subepicardial mesenchyme in the avian embryo: an

immunohistochemical and quail-chick chimera study. *Dev. Biol.* 200, 57–68. doi: 10.1006/dbio.1998.8949

Pérez-Pomares, J. M., Mironov, V., Guadix, J. A., Macías, D., Markwald, R. R., and Muñoz-Chápuli, R. (2006). *In vitro* self-assembly of proepicardial cell aggregates: an embryonic vasculogenic model for vascular tissue engineering. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* 288, 700–713. doi: 10.1002/ar.a.20338

Pérez-Pomares, J. M., Phelps, A., Sedmerova, M., Carmona, R., González-Iriarte, M., Muñoz-Chápuli, R., et al. (2002). Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs). *Dev. Biol.* 247, 307–326. doi: 10.1006/dbio.2002.0706

Philippen, L. E., Dirkx, E., da Costa-Martins, P. A., and De Windt, L. J. (2015). Non-coding RNA in control of gene regulatory programs in cardiac development and disease. *J. Mol. Cell. Cardiol.* 89(Pt A), 51–58. doi: 10.1016/j.yjmcc.2015.03.014

Phillips, H. M., Hildreth, V., Peat, J. D., Murdoch, J. N., Kobayashi, K., Chaudhry, B., et al. (2008). Non-cell-autonomous roles for the planar cell polarity gene Vangl2 in development of the coronary circulation. *Circ. Res.* 102, 615–623. doi: 10.1161/CIRCRESAHA.107.160861

Phillips, M. D., Mukhopadhyay, M., Poscablo, C., and Westphal, H. (2011). Dkk1 and Dkk2 regulate epicardial specification during mouse heart development. *Int. J. Cardiol.* 150, 186–192. doi: 10.1016/j.ijcard.2010.04.007

Plavicki, J., Hofsteen, P., Peterson, R. E., and Heideman, W. (2013). Dioxin inhibits zebrafish epicardium and proepicardium development. *Toxicol. Sci.* 131, 558–567. doi: 10.1093/toxsci/kfs301

Plavicki, J. S., Hofsteen, P., Yue, M. S., Lanham, K. A., Peterson, R. E., and Heideman, W. (2014). Multiple modes of proepicardial cell migration require heartbeat. *BMC Dev. Biol.* 14:18. doi: 10.1186/1471-213X-14-18

Poelmann, R. E., Gittenberger-de Groot, A. C., Mentink, M. M., Bökenkamp, R., and Hogers, B. (1993). Development of the cardiac coronary vascular endothelium, studied with antiendothelial antibodies, in chicken-quail chimeras. *Circ. Res.* 73, 559–568. doi: 10.1161/01.RES.73.3.559

Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., et al. (2011). Transient regenerative potential of the neonatal mouse heart. *Science* 331, 1078–1080. doi: 10.1126/science.1200708

Porrello, E. R., Mahmoud, A. I., Simpson, E., Johnson, B. A., Grinsfelder, D., Canseco, D., et al. (2013). Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc. Natl. Acad. Sci. U.S.A.* 110, 187–192. doi: 10.1073/pnas.1208863110

Poss, K. D., Wilson, L. G., and Keating, M. T. (2002). Heart regeneration in zebrafish. *Science* 298, 2188–2190. doi: 10.1126/science.1077857

Ratajska, A., Czarnowska, E., and Ciszek, B. (2008). Embryonic development of the proepicardium and coronary vessels. *Int. J. Dev. Biol.* 52, 229–236. doi: 10.1387/ijdb.072340ar

Red-Horse, K., Ueno, H., Weissman, I. L., and Krasnow, M. A. (2010). Coronary arteries form by developmental reprogramming of venous cells. *Nature* 464, 549–553. doi: 10.1038/nature08873

Rodius, S., Androsova, G., Götz, L., Liechti, R., Crespo, I., Merz, S., et al. (2016). Analysis of the dynamic co-expression network of heart regeneration in the zebrafish. *Sci. Rep.* 6:26822. doi: 10.1038/srep26822

Rudat, C., and Kispert, A. (2012). Wt1 and epicardial fate mapping. *Circ. Res.* 111, 165–169. doi: 10.1161/CIRCRESAHA.112.273946

Rui, L., Yu, N., Hong, L., Feng, H., Chunyong, H., Jian, M., et al. (2014). Extending the time window of mammalian heart regeneration by thymosin beta 4. *J. Cell. Mol. Med.* 18, 2417–2424. doi: 10.1111/jcmm.12421

Ruiz-Villalba, A., Simón, A. M., Pogontke, C., Castillo, M. I., Abizanda, G., Pelacho, B., et al. (2015). Interacting resident epicardium-derived fibroblasts and recruited bone marrow cells form myocardial infarction scar. *J. Am. Coll. Cardiol.* 65, 2057–2066. doi: 10.1016/j.jacc.2015.03.520

Sánchez, N. S., and Barnett, J. V. (2012). TGFβ and BMP-2 regulate epicardial cell invasion via TGFβR3 activation of the Par6/Smurf1/RhoA pathway. *Cell. Signal.* 24, 539–548. doi: 10.1016/j.cellsig.2011.10.006

Sauvageau, M., Goff, L. A., Lodato, S., Bonev, B., Groff, A. F., Gerhardinger, C., et al. (2013). Multiple knockout mouse models reveal lincRNAs are required for life and brain development. *elife* 2:e01749. doi: 10.7554/eLife.01749

Schlueter, J., and Brand, T. (2012). Epicardial progenitor cells in cardiac development and regeneration. *J. Cardiovasc. Transl. Res.* 5, 641–653. doi: 10.1007/s12265-012-9377-4

Schnabel, K., Wu, C. C., Kurth, T., and Weidinger, G. (2011). Regeneration of cryoinjury induced necrotic heart lesions in zebrafish is associated with epicardial activation and cardiomyocyte proliferation. *PLoS ONE* 6:e18503. doi: 10.1371/journal.pone.0018503

Schulte, I., Schlueter, J., Abu-Issa, R., Brand, T., and Männer, J. (2007). Morphological and molecular left-right asymmetries in the development of the proepicardium: a comparative analysis on mouse and chick embryos. *Dev. Dyn.* 236, 684–695. doi: 10.1002/dvdy.21065

Seeger, T., Xu, Q. F., Muhly-Reinholz, M., Fischer, A., Kremp, E. M., Zeiher, A. M., et al. (2016). Inhibition of let-7 augments the recruitment of epicardial cells and improves cardiac function after myocardial infarction. *J. Mol. Cell. Cardiol.* 94, 145–152. doi: 10.1016/j.yjmcc.2016.04.002

Serluca, F. C. (2008). Development of the proepicardial organ in the zebrafish. *Dev. Biol.* 315, 18–27. doi: 10.1016/j.ydbio.2007.10.007

Shen, J., and Hung, M. C. (2015). Signaling-mediated regulation of MicroRNA processing. *Cancer Res.* 75, 783–791. doi: 10.1158/0008-5472.CAN-14-2568

Singh, A., Ramesh, S., Cibi, D. M., Yun, L. S., Li, J., Li, L., et al. (2016). Hippo signaling mediators yap and taz are required in the epicardium for coronary vasculature development. *Cell Rep.* 15, 1384–1393. doi: 10.1016/j.celrep.2016.04.027

Singh, M. K., Lu, M. M., Massera, D., and Epstein, J. A. (2011). MicroRNA-processing enzyme Dicer is required in epicardium for coronary vasculature development. *J. Biol. Chem.* 286, 41036–41045. doi: 10.1074/jbc.M111.268573

Smart, N., and Riley, P. R. (2012). The epicardium as a candidate for heart regeneration. *Future Cardiol.* 8, 53–69. doi: 10.2217/fca.11.87

Smart, N., Bollini, S., Dubé, K. N., Vieira, J. M., Zhou, B., Davidson, S., et al. (2011). *De novo* cardiomyocytes from within the activated adult heart after injury. *Nature* 474, 640–644. doi: 10.1038/nature10188

Smart, N., Bollini, S., Dubé, K. N., Vieira, J. M., Zhou, B., Riegler, J., et al. (2012). Myocardial regeneration: expanding the repertoire of thymosin β4 in the ischemic heart. *Ann. N.Y. Acad. Sci.* 1269, 92–101. doi: 10.1111/j.1749-6632.2012.06708.x

Smith, C. L., Baek, S. T., Sung, C. Y., and Tallquist, M. D. (2011). Epicardial-derived cell epithelial-to-mesenchymal transition and fate specification require PDGF receptor signaling. *Circ. Res.* 108, e15–e26. doi: 10.1161/CIRCRESAHA.110.235531

Stankunas, K., Ma, G. K., Kuhnert, F. J., Kuo, C. J., and Chang, C. P. (2010). VEGF signaling has distinct spatiotemporal roles during heart valve development. *Dev. Biol.* 347, 325–336. doi: 10.1016/j.ydbio.2010.08.030

Sulaiman, S. A., Ab Mutalib, N. S., and Jamal, R. (2016). miR-200c regulation of metastases in ovarian cancer: potential role in epithelial and mesenchymal transition. *Front. Pharmacol.* 7:271. doi: 10.3389/fphar.2016.00271

Takeichi, M., Nimura, K., Mori, M., Nakagami, H., and Kaneda, Y. (2013). The transcription factors Tbx18 and Wt1 control the epicardial epithelial-mesenchymal transition through bi-directional regulation of Slug in murine primary epicardial cells. *PLoS ONE* 8:e57829. doi: 10.1371/journal.pone.0057829

Talman, A. H., Psaltis, P. J., Cameron, J. D., Meredith, I. T., Seneviratne, S. K., and Wong, D. T. (2014). Epicardial adipose tissue: far more than a fat depot. *Cardiovasc. Diagn. Ther.* 4, 416–429. doi: 10.3978/j.issn.2223-3652.2014.11.05

Thum, T., Gross, C., Fiedler, J., Fischer, T., Kissler, S., Bussen, M., et al. (2008). MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature* 456, 980–984. doi: 10.1038/nature07511

Tian, X., Hu, T., Zhang, H., He, L., Huang, X., Liu, Q., et al. (2013). Subepicardial endothelial cells invade the embryonic ventricle wall to form coronary arteries. *Cell Res.* 23, 1075–1090. doi: 10.1038/cr.2013.83

Tomanek, R. J., Ishii, Y., Holifield, J. S., Sjogren, C. L., Hansen, H. K., and Mikawa, T. (2006). VEGF family members regulate myocardial tubulogenesis and coronary artery formation in the embryo. *Circ. Res.* 98, 947–953. doi: 10.1161/01.RES.0000216974.75994.da

Towler, B. P., Jones, C. I., and Newbury, S. F. (2015). Mechanisms of regulation of mature miRNAs. *Biochem. Soc. Trans.* 43, 1208–1214. doi: 10.1042/BST20150157

Trembley, M. A., Velasquez, L. S., de Mesy Bentley, K. L., and Small, E. M. (2015). Myocardin-related transcription factors control the motility of epicardium-derived cells and the maturation of coronary vessels. *Development* 142, 21–30. doi: 10.1242/dev.116418

Urayama, K., Guilini, C., Turkeri, G., Takir, S., Kurose, H., Messaddeq, N., et al. (2008). Prokineticin receptor-1 induces neovascularization and epicardial-derived progenitor cell differentiation. *Arterioscler. Thromb. Vasc. Biol.* 28, 841–849. doi: 10.1161/ATVBAHA.108.162404

Valder, A. M., and Olson, M. D. (1994). Immuno localization of vascular smooth muscle precursors during coronary vasculogenesis in the embryonic quail heart. *FASEB J.* 8:A394.

van Wijk, B., Gunst, Q. D., Moorman, A. F., and van den Hoff, M. J. (2012). Cardiac regeneration from activated epicardium. *PLoS ONE* 7:e44692. doi: 10.1371/journal.pone.0044692

Vega-Hernández, M., Kovacs, A., De Langhe, S., and Ornitz, D. M. (2011). FGF10/FGFR2b signaling is essential for cardiac fibroblast development and growth of the myocardium. *Development* 138, 3331–3340. doi: 10.1242/dev.064410

Virágh, S., Gittenberger-de Groot, A. C., Poelmann, R. E., and Kálmán, F. (1993). Early development of quail heart epicardium and associated vascular and glandular structures. *Anat. Embryol.* 188, 381–393. doi: 10.1007/BF00185947

Vrancken Peeters, M. P., Gittenberger-de Groot, A. C., Mentink, M. M., and Poelmann, R. E. (1999). Smooth muscle cells and fibroblasts of the coronary arteries derive from epithelial-mesenchymal transformation of the epicardium. *Anat. Embryol.* 199, 367–378. doi: 10.1007/s004290050235

Wagner, N., Morrison, H., Pagnotta, S., Michiels, J. F., Schwab, Y., Tryggvason, K., et al. (2011). The podocyte protein nephrin is required for cardiac vessel formation. *Hum. Mol. Genet.* 20, 2182–2194. doi: 10.1093/hmg/ddr106

Wang, J., Cao, J., Dickson, A. L., and Poss, K. D. (2015). Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling. *Nature* 522, 226–230. doi: 10.1038/nature14325

Watt, A. J., Battle, M. A., Li, J., and Duncan, S. A. (2004). GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12573–12578. doi: 10.1073/pnas.0400752101

Wei, K., Serpooshan, V., Hurtado, C., Diez-Cuñado, M., Zhao, M., Maruyama, S., et al. (2015). Epicardial FSTL1 reconstitution regenerates the adult mammalian heart. *Nature* 525, 479–485. doi: 10.1038/nature15372

Wessels, A., and Pérez-Pomares, J. M. (2004). The epicardium and epicardially derived cells (EPDCs) as cardiac stem cells. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* 276, 43–57. doi: 10.1002/ar.a.10129

Winter, E. M., and Gittenberger-de Groot, A. C. (2007). Epicardium-derived cells in cardiogenesis and cardiac regeneration. *Cell. Mol. Life Sci.* 64, 692–703. doi: 10.1007/s00018-007-6522-3

Winter, E. M., van Oorschot, A. A., Hogers, B., van der Graaf, L. M., Doevendans, P. A., Poelmann, R. E., et al. (2009). A new direction for cardiac regeneration therapy: application of synergistically acting epicardium-derived cells and cardiomyocyte progenitor cells. *Circ. Heart Fail.* 2, 643–653. doi: 10.1161/CIRCHEARTFAILURE.108.843722

Wu, S. P., Dong, X. R., Regan, J. N., Su, C., and Majesky, M. W. (2013). Tbx18 regulates development of the epicardium and coronary vessels. *Dev. Biol.* 383, 307–320. doi: 10.1016/j.ydbio.2013.08.019

Wu, Y. C., Liu, C. Y., Chen, Y. H., Chen, R. F., Huang, C. J., and Wang, I. J. (2012). Blood vessel epicardial substance (Bves) regulates epidermal tight junction integrity through atypical protein kinase C. *J. Biol. Chem.* 287, 39887–39897. doi: 10.1074/jbc.M112.372078

Xiang, F. L., Liu, Y., Lu, X., Jones, D. L., and Feng, Q. (2014). Cardiac-specific overexpression of human stem cell factor promotes epicardial activation and arteriogenesis after myocardial infarction. *Circ. Heart Fail.* 7, 831–842. doi: 10.1161/CIRCHEARTFAILURE.114.001423

Yamaguchi, Y., Cavallero, S., Patterson, M., Shen, H., Xu, J., Kumar, S. R., et al. (2015). Adipogenesis and epicardial adipose tissue: a novel fate of the epicardium induced by mesenchymal transformation and PPARγ activation. *Proc. Natl. Acad. Sci. U.S.A.* 112, 2070–2075. doi: 10.1073/pnas.1417232112

Yamashita, K., Yamamoto, M. H., Ebara, S., Okabe, T., Saito, S., Hoshimoto, K., et al. (2014). Association between increased epicardial adipose tissue volume and coronary plaque composition. *Heart Vessels* 29, 569–577. doi: 10.1007/s00380-013-0398-y

Yan, S., and Jiao, K. (2016). Functions of miRNAs during mammalian heart development. *Int. J. Mol. Sci.* 17:789. doi: 10.3390/ijms17050789

Zeng, B., Ren, X. F., Cao, F., Zhou, X. Y., and Zhang, J. (2011). Developmental patterns and characteristics of epicardial cell markers Tbx18 and Wt1 in murine embryonic heart. *J. Biomed. Sci.* 18:67. doi: 10.1186/1423-0127-18-67

Zhang, H., Pu, W., Liu, Q., He, L., Huang, X., Tian, X., et al. (2016). Endocardium contributes to cardiac fat. *Circ. Res.* 118, 254–265. doi: 10.1161/CIRCRESAHA.115.307202

Zhao, L., Borikova, A. L., Ben-Yair, R., Guner-Ataman, B., MacRae, C. A., Lee, R. T., et al. (2014). Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 111, 1403–1408. doi: 10.1073/pnas.1311705111

Zhou, B., Honor, L. B., He, H., Ma, Q., Oh, J. H., Butterfield, C., et al. (2011). Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J. Clin. Invest.* 121, 1894–1904. doi: 10.1172/JCI45529

Zhou, B., Ma, Q., Rajagopal, S., Wu, S. M., Domian, I., Rivera-Feliciano, J., et al. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* 454, 109–113. doi: 10.1038/nature07060

Zhou, B., and Pu, W. T. (2011). Epicardial epithelial-to-mesenchymal transition in injured heart. *J. Cell. Mol. Med.* 15, 2781–2783. doi: 10.1111/j.1582-4934.2011.01450.x

Zhou, B., von Gise, A., Ma, Q., Hu, Y. W., and Pu, W. T. (2010). Genetic fate mapping demonstrates contribution of epicardium-derived cells to the annulus fibrosis of the mammalian heart. *Dev. Biol.* 338, 251–261. doi: 10.1016/j.ydbio.2009.12.007

Zhu, J. G., Shen, Y. H., Liu, H. L., Liu, M., Shen, Y. Q., Kong, X. Q., et al. (2014). Long noncoding RNAs expression profile of the developing mouse heart. *J. Cell. Biochem.* 115, 910–918. doi: 10.1002/jcb.24733

Zou, X. Z., Liu, T., Gong, Z. C., Hu, C. P., and Zhang, Z. (2016). MicroRNAs-mediated epithelial-mesenchymal transition in fibrotic diseases. *Eur. J. Pharmacol.* 796, 190–206. doi: 10.1016/j.ejphar.2016.12.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 23 March 2017
doi: 10.3389/fcell.2017.00022

Non-myogenic Contribution to Muscle Development and Homeostasis: The Role of Connective Tissues

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Skeletal muscles belong to the musculoskeletal system, which is composed of bone, tendon, ligament and irregular connective tissue, and closely associated with motor nerves and blood vessels. The intrinsic molecular signals regulating myogenesis have been extensively investigated. However, muscle development, homeostasis and regeneration require interactions with surrounding tissues and the cellular and molecular aspects of this dialogue have not been completely elucidated. During development and adult life, myogenic cells are closely associated with the different types of connective tissue. Connective tissues are defined as specialized (bone and cartilage), dense regular (tendon and ligament) and dense irregular connective tissue. The role of connective tissue in muscle morphogenesis has been investigated, thanks to the identification of transcription factors that characterize the different types of connective tissues. Here, we review the development of the various connective tissues in the context of the musculoskeletal system and highlight their important role in delivering information necessary for correct muscle morphogenesis, from the early step of myoblast differentiation to the late stage of muscle maturation. Interactions between muscle and connective tissue are also critical in the adult during muscle regeneration, as impairment of the regenerative potential after injury or in neuromuscular diseases results in the progressive replacement of the muscle mass by fibrotic tissue. We conclude that bi-directional communication between muscle and connective tissue is critical for a correct assembly of the musculoskeletal system during development as well as to maintain its homeostasis in the adult.

Keywords: connective tissue, muscles, bones, tendons, development, regeneration

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Peleg Hasson, Technion – Israel Institute of Technology, Israel
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *20 December 2016*
Accepted: *07 March 2017*
Published: *23 March 2017*

Citation:
Nassari S, Duprez D and Fournier-Thibault C (2017) Non-myogenic Contribution to Muscle Development and Homeostasis: The Role of Connective Tissues. Front. Cell Dev. Biol. 5:22. doi: 10.3389/fcell.2017.00022

INTRODUCTION

Skeletal muscle forms a highly complex and heterogeneous structure, which is part of the musculoskeletal system of the body. The process of generating muscles is defined as "myogenesis." This mechanism occurring during development is an important step in the establishment of the musculoskeletal system allowing its essential functions, for instance body motion or the ability to breath. Myogenesis occurs through successive and overlapping phases that ultimately give rise to correctly patterned muscles. In the first phase of myogenesis, which is called embryonic

myogenesis, embryonic progenitors cells form primary muscle fibers, which constitute the scaffold of the muscles. During the second phase of myogenesis named fetal myogenesis, fetal progenitors fuse between themselves and with primary fibers to form secondary fibers and allow muscle growth. Both waves of myogenesis occur during embryonic development, and involve specific types of muscle progenitors cells. After birth, a third wave of myogenesis can be activated during muscle regeneration, which occurs after muscle damage. This step involves muscle stem cells, so-called muscle satellite cells, which contribute to muscle reconstruction by fusing with the existing muscle fibers or generating new muscle fibers (Stockdale, 1992; Tajbakhsh, 2009; Tedesco et al., 2010). Studies suggest that embryonic myogenesis is largely exhausted at the end of embryonic development, while fetal and perinatal phases of myogenesis persist to contribute to the majority of adult muscle stem cells (reviewed in Tajbakhsh, 2009).

The intrinsic molecular signals regulating the different waves of myogenesis have been well described in the literature. However, the interactions between muscles and adjacent tissues during development are not completely elucidated. During development and adult life, as part of the musculoskeletal system, muscles are closely associated with the other components of this system: bone, cartilage, tendon, ligament and irregular connective tissue, all of them emerging from the family of connective tissues. Although the interactions between the different components of the musculoskeletal system during development has been highlighted from the 1980's, more recent work has begun to decipher the molecular mechanisms underlying the importance of connective tissue in the regulation of developmental and regenerative myogenesis.

The scope of this review is to synthesize the data supporting the process of connective tissue-mediated muscle development and regeneration and to point out the active role of this so-called "supporting tissue" in muscle formation and repair. Indeed, defect in connective tissue-muscle interactions can lead to human pathology, as congenital diaphragmatic hernias, a birth defect of the diaphragm muscle (Merrell et al., 2015), or the Holt-Oram syndrome characterized by skeletal defects of the upper limbs and heart anomalies (Hasson et al., 2010). In addition, in skeletal muscle regenerative disorders (muscular dystrophies) as well as in aging (sarcopenia), the impairment of the muscle regenerative potential correlates with a progressive replacement of contractile mass by fibrotic and adipose tissues (reviewed in Farup et al., 2015). It is therefore necessary to better understand the interactions occurring between the different components of the musculoskeletal system. This would allow us to decipher the molecular mechanisms underlying muscle disorders not related to the impairment of intrinsic regulation of myogenesis.

CONNECTIVE TISSUE DEVELOPMENT

Different Types of Connective Tissues

In the body, the main role of **connective tissues (CTs)** is to support and connect organs together. CTs are primarily composed of fibroblasts and extracellular matrix consisting of amorphous gel-like and matrix fibers. The amorphous gel-like, named ground substance, mostly contains glycoproteins and proteoglycans, while the fibrous network is made of collagen and elastic fibers (Omelyanenko and Slutsky, 2013). Among the **supportive** CTs, two main types can be distinguished: the **specialized CT** and the **dense CT**. The specialized CT refers to bones and cartilage elements. The dense CT is further divided into two subtypes: the **dense regular CT** and the **dense irregular CT**, which refer respectively to tendon/ligament structures and to CT embedding organs (**Table 1**). The nature and function of these different CTs are predominately determined by the composition and organization of the extracellular matrix. In dense regular CT, fibroblasts produce a significant amount of collagen fibers that display a spatial organization, while in the dense irregular CT, fibroblasts produce collagen fibers that do not present any specific organization (Omelyanenko and Slutsky, 2013).

Connective Tissue Formation

During embryonic development, undifferentiated mesenchymal cells, derived from mesodermal and mesectodermal (neural crest cells) origins, give rise to the different forms of CT: bones, cartilage, tendons, ligaments, and irregular CT (Wachtler et al., 1981). Head CTs originate from neural crest cells, while CTs of the body originate from paraxial or lateral plate mesoderm (**Figure 1**). The specification and differentiation processes of the different types of CTs is controlled by specific key transcription factors or signaling molecules. Irrespective to their embryological origins, transcription factors have been identified for the specification of each type of CT from undifferentiated mesenchymal cells (**Figure 2**).

Specialized Connective Tissue (Bone and Cartilage)

The embryonic origins of cartilage and bone are multiple. Indeed, elements of the trunk, head and limb skeleton arise from three distinct embryonic structures, somites, neural crest cells and lateral plate mesoderm (**Figure 1**, Wachtler et al., 1981; Christ and Wilting, 1992; Noden and Trainor, 2005). The process of skeleton formation, which corresponds to the development of cartilage and bone elements, is initiated by the condensation of undifferentiated mesenchymal cells at the future sites of bones. Following condensation, mesenchymal precursors undergo either chondrocyte or osteoblast differentiation, giving rise respectively to cartilage or bone. Osteogenesis characterizes the process of ossification, which occurs through two different mechanisms. The process of intramembranous ossification corresponds to a direct transition from condensed undifferentiated-mesenchymal cells into osteoblasts (as described above). The second mechanism of bone formation is called endochondral ossification. It defines the replacement of cartilage by bone. In this case, chondrogenesis is the first step in a process that ultimately gives rise to bones. Intramembranous ossification occurs in bones of the skull, while other bones form by endochondral ossification (reviewed in Karsenty and Wagner, 2002).

Molecular mechanisms involved in cartilage and bone specifications are well understood (**Figure 2**). Members of the

TABLE 1 | Classification of the different types of connective tissues.

Connective tissue types			
	Proper		Specialized
Soft	Dense		
	Regular	Irregular	
Adipose tissue: brown, beige and white adipose tissue **Areolar** tissue: sub-cutaneous, around blood vessels, and nerves **Reticular tissue**: into the liver, pancreas, lymph nodes, spleen, bone marrow	**Tendons:** direct tendons, wrap-around tendons **Ligaments:** intra articular and extraarticular, synovial joints	Dermis Capsules of organs (periosteum, epimysium) Walls of tubular organs Muscle connective tissue (endomysium, perimysium)	**Bones cartilage**

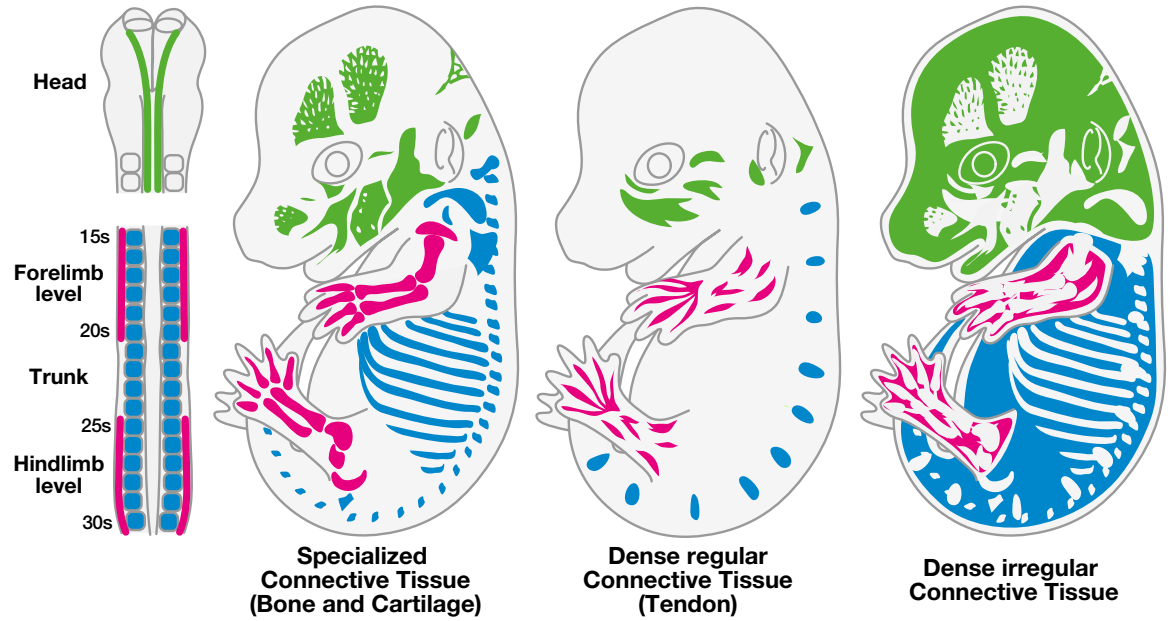

FIGURE 1 | Embryonic origins of the different types of connective tissues. The different types of connective tissues, specialized (bone and cartilage), dense (tendon) and irregular connective tissues are depicted in three mouse E14.5 embryos. The color code corresponds to the embryological origins of the different types of connective tissues, which differ depending on their location in the body. Connective tissues of the head derive from neural crest cells (green), while trunk connective tissues arise from the somites (blue) and limb connective tissues arise from the lateral plate mesoderm (pink).

SOX (SRY-related HMG-box) transcription factor family are key players in the regulation of cartilage specification (Lefebvre et al., 1998). During mouse embryonic development, *Sox9* presents a similar expression pattern to *Col2a1*, the main collagen in the cartilage extracellular matrix (Zhao et al., 1997). In mouse mutant embryos for *Sox9*, cartilage development fails. The complete absence of cartilage elements in *Sox9* mutant mice highlights a role for *Sox9* in the regulation of mesenchymal cell condensation and differentiation toward a cartilage fate (Bi et al., 1999; Akiyama et al., 2002). Moreover, it has been shown that *Sox9* is required for the expression of two additional *Sox* genes, *Sox5*, and *Sox6* that are co-expressed with *Sox9* in committed cartilage cells (chondrocytes), (Lefebvre et al., 1998, 2001). Both *Sox5* and *Sox6* mutant mice show skeletal abnormalities, with no modification of *Sox9* expression, demonstrating that *Sox9* acts upstream of *Sox5* and *Sox6* (Smits et al., 2001).

Runx2 (Runt-related transcription factor 2) is a master gene for osteogenesis (Komori et al., 1997; Ducy et al., 1999). This transcription factor is specific to bone progenitor cell lineage (Ducy et al., 1999). Knockout mice for *Runx2* show no osteogenesis. While cartilage elements are still present in *Runx2* $^{-/-}$ mouse, all bones are missing, demonstrating the importance of *Runx2* in bone specification (Komori et al., 1997). In contrast to *Sox9,* which is required for cartilage differentiation in addition to specification (Akiyama et al., 2002), *Runx2* is not required for bone differentiation (Takarada et al., 2013). After osteogenic cell commitment, *Runx2* activity has to be shut down to allow immature committed bone cells to become fully mature and to differentiate (Yoshida et al., 2004; Takarada et al., 2013; Adhami et al., 2014). *Osterix* (*Osx*) is also a key transcription factor in bone formation (Nakashima et al., 2002). *Osx* is specifically expressed in all bones (Nakashima et al., 2002) and is required for differentiation of bone progenitor cells. In mutant mouse

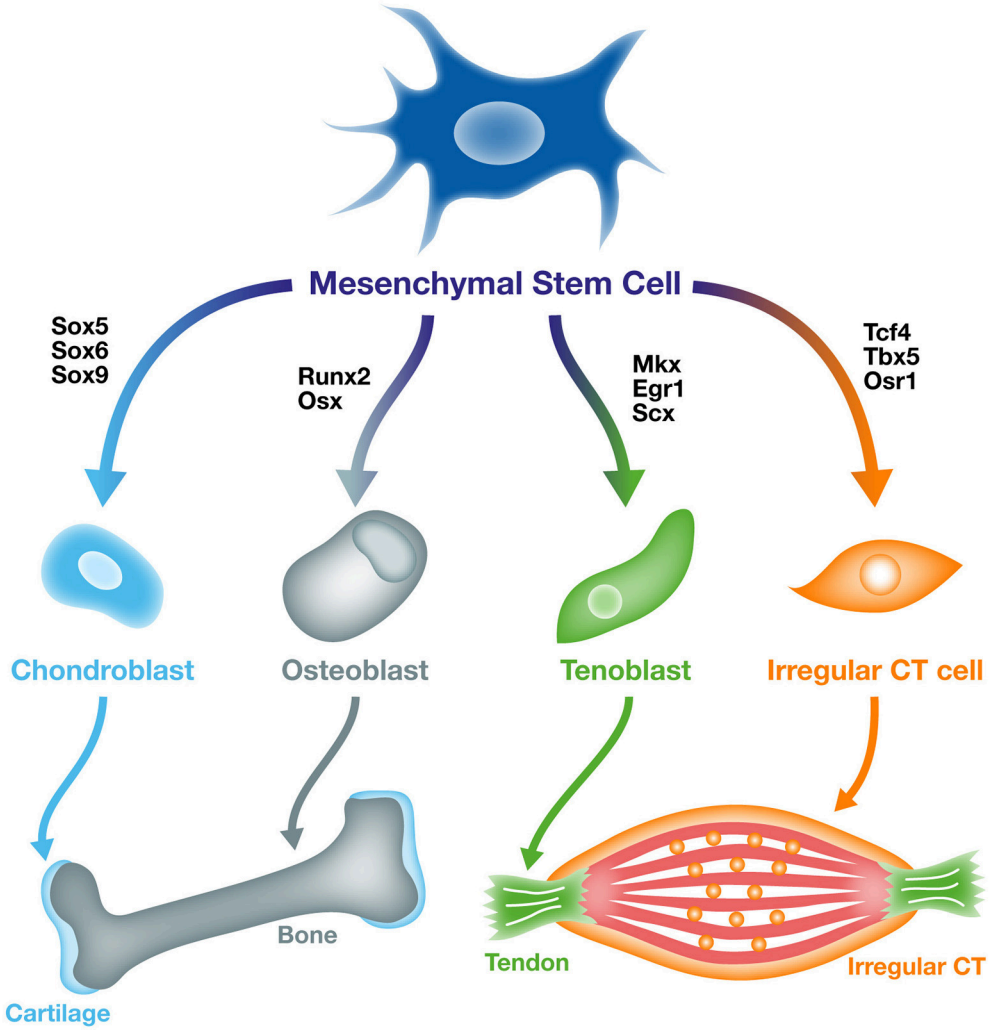

FIGURE 2 | From mesenchymal stem cells to specific connective tissue cell types. Undifferentiated mesoderm-derived cells or mesenchymal stem cells are able to differentiate into different types of connective tissues including, bone, cartilage, tendon, and irregular connective tissue. Specific transcription factors have been identified as able to induce mesenchymal stem cell differentiation toward the different types of connective tissue cells. The *Sox5/6/9*, *Runx2/Osx*, *Scx/Mkx/Egr1,* and *Tcf4/Tbx5/Osr1* genes drive undifferentiated cells to differentiate into cartilage, bone, tendon and irregular connective tissue, respectively.

for *Osx*, no bone is observed, however *Runx2* expression is maintained (Nakashima et al., 2002). Conversely, *Osx* expression is absent in Runx2 mutant mice (Nakashima et al., 2002). This indicates that *Runx2* and *Osx* are involved in bone specification and differentiation, respectively.

Beside the specific transcription factors, major signaling pathways have also been demonstrated to be involved in skeletal development. Wnt pathway regulates skeleton differentiation through a cell-autonomous mechanism, which enhances osteoblast differentiation at the expense of chondrocytes (Day et al., 2005; Hill et al., 2005). Conditional inactivation of ß-catenin in mesenchyme blocks osteoblast differentiation and induces ectopic chondrocytes (Day et al., 2005). In addition, ß-catenin has been shown to control the expression of *Sox9* and *Runx2 in vitro* (Day et al., 2005). The role of FGF signaling in skeletal development comes from the observations that *FgfR3* and *FgfR1* inactivation in mouse leads to achondroplasia and hypochondroplasia (Deng et al., 1996; Jacob et al., 2006). In both mutant mice, an expansion of the hypertrophic chondrocyte zone is observed, suggesting that FGF signaling is a negative regulator of chondrocyte proliferation (Deng et al., 1996; Jacob et al., 2006). Inactivation of one member of the Hedgehog family, *Ihh* (Indian Hedgehog), leads to a decrease in chondrocyte proliferation and a defect in osteoblast formation (Vortkamp et al., 1996). This effect is mediated by the interaction between Ihh and Parathyroid hormone, which maintains the rate between cell proliferation and differentiation (Kronenberg, 2006). Interestingly, RUNX2 induces *Ihh* expression which inhibits *Runx2* expression by a feedback loop mechanism (Yoshida et al., 2004). Finally, BMPS (Bone Morphogenetic Proteins) are

important regulators of chondrocyte differentiation (Kobayashi et al., 2005, reviewed in Li and Cao, 2006) and have been shown to regulate *IHH* expression in chick embryos (Zhang et al., 2003).

Dense Regular Connective Tissue (Tendon)

Similarly to specialized CTs (bone and cartilage), tendons arise from distinct embryological origins depending on their position in the body (**Figure 1**). Tendons of the trunk originate from somites, more precisely from a subregion of the sclerotome named the syndetome (Brent et al., 2003), tendons of the craniofacial region derive from neural crest cells (Crane and Trainor, 2006; Grenier et al., 2009) and limb tendons derive from the lateral plate mesoderm (Kieny and Chevallier, 1979). Tendons attach muscles to bones by connecting muscle at the myotendinous junction and connecting bone at the enthesis, while ligaments connect bone to bone. The role of tendons is to transmit forces generated by muscle contractions to bones, in order to allow joint movements and maintain articular stability. The tendon extracellular matrix is rich in type I collagen fibers, which display a specific spatial organization parallel to the tendon axis. This specific organization lends mechanical properties to tendons (Benjamin and Ralphs, 2000). Ligaments are essential components of the skeletal joints. Their elasticity defines the range of motion of the joints, supports joint stability and protects joints and bones by their stretching capacities. Tendons and ligaments display similar structural collagen organization and molecular markers (Benjamin and Ralphs, 2000). However, genome-wide analysis identifies different levels of gene expression between adult tendons and ligaments (Pearse et al., 2009). However, tendon development has been more studied than ligament development (Tozer and Duprez, 2005).

In contrast to cartilage and bone, the master gene(s) involved in tendon specification during development is (are) still unknown. To date, *Scx* (Scleraxis) is the unique early tendon marker that has been described in vertebrates. *Scx* is specifically expressed in tendon progenitors and differentiated cells (Schweitzer et al., 2001). *Scx* mutant mice display severe tendon defects, leading to a severe impairment of limb and tail force-transmitting tendons, while anchoring tendons are less affected (Murchison et al., 2007). However, tendon progenitors are still present in $Scx^{-/-}$, indicating that *Scx* is not the master gene driving tenogenesis during development. Two additional transcription factors have been identified to be involved in tendon formation, the homeobox transcription factor *Mkx* (Mohawk), (Ito et al., 2010; Liu et al., 2010) and the zinc finger transcription factor *Egr1* (Early Growth Response 1), (Lejard et al., 2011, **Figure 2**). Both *Mkx* and *Egr1* mutant mice display tendon defects associated with a decrease in *Col1a1* expression levels and in type I collagen fiber number in tendons (Ito et al., 2010; Liu et al., 2010; Lejard et al., 2011; Guerquin et al., 2013). However, both *Mkx* and *Egr1* are expressed after *Scx* during development and are not specific to tendons, since they are expressed in many other lineages (Rackley et al., 1995; Anderson et al., 2006).

TGFß (Transforming growth factor) and FGF (Fibroblast growth factor) signaling pathways have been shown to regulate tendon specification and differentiation at different places of the body (recently reviewed in Gaut and Duprez, 2016). As mentioned above, **axial** tendon progenitors arise from a somitic subcompartment named the syndetome. The syndetome compartment, localized at the interface between the sclerotome and myotome, is formed by *Scx*-expressing cells. In chick embryos, axial tendons do not develop in the absence of axial muscles, as demonstrated by the absence of tendons after dermomyotome removal (Brent et al., 2003). Chick axial *SCX* expression is induced in response to FGF signaling arising from the myotome, which concomitantly downregulates *PAX1* expression in the sclerotome (Brent et al., 2003). In contrast to axial tendons, the initiation of **head and limb** tendons is independent of muscle. In the absence of limb or craniofacial muscles, *Scxa/SCX/Scx* expression is normally induced in limb and head of zebrafish, chick and mouse embryos (Schweitzer et al., 2001; Edom-Vovard et al., 2002; Grenier et al., 2009; Chen and Galloway, 2014). In chick limbs, *SCX* induction is known to be mediated via ectodermal signals, as shown by the absence of *SCX* expression after ectoderm removal (Schweitzer et al., 2001). BMP signaling from the limb mesenchyme represses *SCX* expression and overexpression of the BMP antagonist NOGGIN leads to ectopic *SCX* expression, indicating that tendon specification in chick limbs results from a balance between an unidentified factor coming from the ectoderm and BMP signaling from the mesenchyme (Schweitzer et al., 2001). TGFß is a key signaling molecule for tendon development. TGFß signaling is required and sufficient for *Scx/SCX* expression during development in chick and mouse embryos (Pryce et al., 2009; Havis et al., 2014, 2016), while FGF signaling is required and sufficient for *SCX* expression in undifferentiated chick limb cells but not in mouse limb cells (Pryce et al., 2009; Havis et al., 2014, 2016).

Although muscles are not necessary for head and limb tendon initiation, they are required for the maintenance of *Scxa/SCX/Scx* expression in tendons and for full tendon differentiation. In the absence of muscles, tendons degenerate in chick, mouse and zebrafish embryos (Kardon, 1998; Edom-Vovard et al., 2002; Grenier et al., 2009; Chen and Galloway, 2014). Moreover, overexpression of FGF4, which is normally expressed at the tips of muscles fibers, leads to ectopic expression of tendon-associated genes in chick limbs (Edom-Vovard et al., 2002; Eloy-trinquet et al., 2009). In addition, chick embryo immobilization decreases *SCX* expression in limb tendons and application of FGF4 or TGFβ ligands prevents *SCX* down-regulation consecutive to immobilization, demonstrating that FGF and TGFβ act downstream of mechanical forces to regulate tendon differentiation (Havis et al., 2016).

Dense Irregular Connective Tissue

The irregular CT constitutes a protective envelop for the different organs of the body, by embedding and scaffolding organs, with scattered cells embedded in high extracellular matrix content. Irregular CT is present all around organs, but also inside organs.

First studies on the differentiation of irregular CT have focused on the extracellular matrix composition. During development, Type I and type III collagen are both expressed in dense regular and irregular CTs, however type I collagen tends to replace type III collagen in adult tendons, while mature irregular CT is characterized by the expression of both type III and type VI collagen (Kieny and Mauger, 1984; Zhang et al., 2005; Gara et al., 2011; Stricker et al., 2012). Due to the lack of specific early molecular markers, the mechanisms driving irregular CT specification have been poorly investigated. However, the recent identification of transcription factors expressed in irregular CT has provided new insights into irregular CT formation and function (**Figure 2**).

The first marker identified in irregular CT fibroblasts is the transcription factor TCF4, belonging to the TCF/LEF family. In limbs of both mouse and chick embryos, *Tcf4*-expressing cells discriminate the lateral plate-derived mesodermal population from myogenic cells (Kardon et al., 2003; Mathew et al., 2011). When chick limb muscles differentiate, *TCF4* expression is restricted to muscle CT (Kardon et al., 2003) and colocalizes with type I collagen. Expression of *TCF4* in muscle CT persists at adult stages (Mathew et al., 2011). *TCF4* misexpression in chick limbs leads to muscle patterning defects, highlighting a non-cell autonomous effect of muscle CT on muscles, in which TCF4-expressing fibroblasts define a pre-pattern that ultimately drive muscle patterning (Kardon et al., 2003). However, low levels of *Tcf4* have been also observed genetically in myogenic cells (Murphy et al., 2011). BMP signaling has been shown to negatively regulate *TCF4* expression (Bonafede et al., 2006), while Wnt signaling positively regulates *TCF4* expression (Kardon et al., 2003) in developing chick limbs. *TCF4* is also expressed dynamically in avian jaw muscle CT and has been shown to be regulated by neural crest mesenchyme (Tokita and Schneider, 2009).

The T-box transcription factor *Tbx5* is another gene that has been characterized as expressed in fibroblasts constituting irregular CT. At early stage of mouse limb bud development (E11.5), *Tbx5* is broadly expressed in lateral plate mesodermal cells in domains overlapping with bone, tendon and muscle progenitors (Hasson et al., 2007). Disruption of *Tbx5* function in mice leads to disorganization of muscle CT during embryonic development (Hasson et al., 2010), which could be related to subtle alterations of muscle CT markers, as *Tcf4* and the *Osr* genes (see below), (Hasson et al., 2010). *Tbx5* positively regulates the expression of N-cadherin and ß-Catenin in muscle CT and as the expression levels of Wnt signaling targets are not affected in *Tbx5* mutant, it seems noteworthy that *Tbx5* mostly affects cell adhesion mechanisms independently of *Tcf4*.

Two orthologs of the Odd-Skipped genes, *Osr1* and *Osr2*, has been described as expressed in the irregular CT in chick and mouse embryos (Stricker et al., 2006, 2012). Both genes are expressed in a variety of organs such as kidney, eye, branchial arches, and dermis (So and Danielian, 1999; Lan et al., 2001; Stricker et al., 2006). In the developing limb of mouse and chick embryos, *Osr1* is expressed in all irregular CTs, displaying a partial overlap with *Tcf4* (Stricker et al., 2006). *Osr2*, although widely expressed in irregular CT, shows prevalence for muscle CT (Stricker et al., 2006, 2012). Both genes are also expressed in the mesenchyme of branchial arches in chick (Stricker et al., 2006) and mouse (Liu et al., 2013) embryos. Forced expression of OSR1 or OSR2 in chick mesenchymal progenitor limb cells induces the expression of irregular CT markers such as *COL3A1* and *COL6A1* and down-regulates the expression of markers of cartilage (specialized CT) and tendon (dense regular CT), (Stricker et al., 2012). Conversely, *OSR1* or *OSR2* inactivation down-regulates *COL3A1* and *COL6A1* expression, while increasing cartilage formation in chick limb cells (Stricker et al., 2012). Similarly, specific inactivation of *Osr1* in cranial neural crest cells result in the formation of an ectopic cartilage in the developing mouse tongue (Liu et al., 2013). OSR1 has been shown to bind *Sox9* promoter and repress *Sox9* expression, indicating that OSR1 prevents chondrogenesis in the mammalian tongue through repression of *Sox9* expression (Liu et al., 2013).

MUSCLE DEVELOPMENT

Embryonic Origins of Skeletal Muscles

In vertebrates, all skeletal muscles derive from paraxial mesodermal cells (**Figure 3**; reviewed in Stockdale et al., 2000; Noden and Francis-west, 2006), with the exception of a small population of neck muscles that have been shown to derive from the lateral plate mesoderm (Theis et al., 2010). Most of the knowledge about the paraxial mesodermal origin of skeletal muscles was established thanks to Di-I labeling (Selleck and Stern, 1991) and chick-quail graft experiments (Couly et al., 1992; Ordahl and Le Douarin, 1992). These lineage studies showed that although skeletal muscles share a common mesodermal origin, muscle organization significantly differs depending on their rostro-caudal position in the embryo.

Head muscles originate from cranial paraxial mesoderm. Cranial paraxial mesoderm lacks any initial signs of segmentation and mesodermal cells will only be segregated once they reach the branchial arches concomitantly with cranial neural crest cells (**Figure 3**; reviewed in Noden and Francis-west, 2006). Three distinct groups of cranial muscles can be distinguished: the extraocular muscles, originating from the prechordal mesoderm, the branchiomeric muscles including the muscles of the jaw, anterior neck and face, arising from the paraxial mesoderm and the tongue and posterior neck muscles, deriving from anterior somites (Noden, 1983; Couly et al., 1992; Trainor et al., 1994).

Truncal paraxial mesoderm caudal to the head emerges from already segmented embryonic structures, the somites, that will give rise to two main compartments all along the truncal axis of the embryo, the sclerotome and the dermomyotome (**Figure 3**, reviewed by Christ and Ordahl, 1995). Limb and axial skeletal muscles originate from the dermomyotome. The dorsomedial part of the dermomyotome gives rise to the epaxial musculature corresponding to the back and intercostal muscles, while the ventrolateral part of the dermomyotome gives rise to the hypaxial musculature corresponding to the diaphragm, abdominal and limb muscles (Ordahl and Le Douarin, 1992). Few muscles from the most posterior part of the head, including tongue muscles and muscles of the posterior pharyngeal arches also develop from the somites (Noden and Francis-west, 2006).

Molecular Cascades That Regulate Muscle Development

Lineage progression to establish skeletal muscle from a founder mesodermal cell in the embryo is common to all skeletal muscles. An undifferentiated mesodermal cell (fate is not acquired) will switch to a muscle progenitor state (fate being acquired) to finally end up as a differentiated muscle cell (functional entity). Such switches from an undifferentiated state to a fully differentiated state are regulated by the activation of different groups of transcription factors (**Figure 4**). Head, trunk and limb muscle progenitors are specified by different genetic programs, but once specified, myogenic cells use a common differentiation program.

In the body, myogenic specification requires *Pax3* and *Pax7* genes, belonging to the paired-box *Pax* family. PAX3 controls the delamination of epaxial myogenic progenitor cells (reviewed in Tajbakhsh and Buckingham, 2000). Moreover, the central domain of the dermomyotome gives rise to a PAX3/PAX7 progenitor population forming subsequent axial muscles. In *Pax3/Pax7* double-mutant mice, somitic cells do not enter the myogenic program, resulting in defective skeletal muscles (Kassar-Duchossoy et al., 2005; Relaix et al., 2005). The acquisition of a myogenic fate depends on a second group of transcription factors, named the basic Helix-Loop-Helix (bHLH) Myogenic Regulatory Factors (MRFs). MRFs have the ability to trigger skeletal muscle differentiation in non-muscle cells *in vitro* (Weintraub et al., 1991) and *in vivo* (Delfini and Duprez, 2004). *Myod1 (MyoD)*, *Myf5*, and *Myf6* (*Mrf4*) are considered as the muscle determination factors (Kassar-Duchossoy et al., 2004), while *MyoG* (Myogenin) is associated with muscle differentiation (Hasty et al., 1993). However, both *Myod1* and *Myf6 (Mrf4)* are also required for terminal muscle

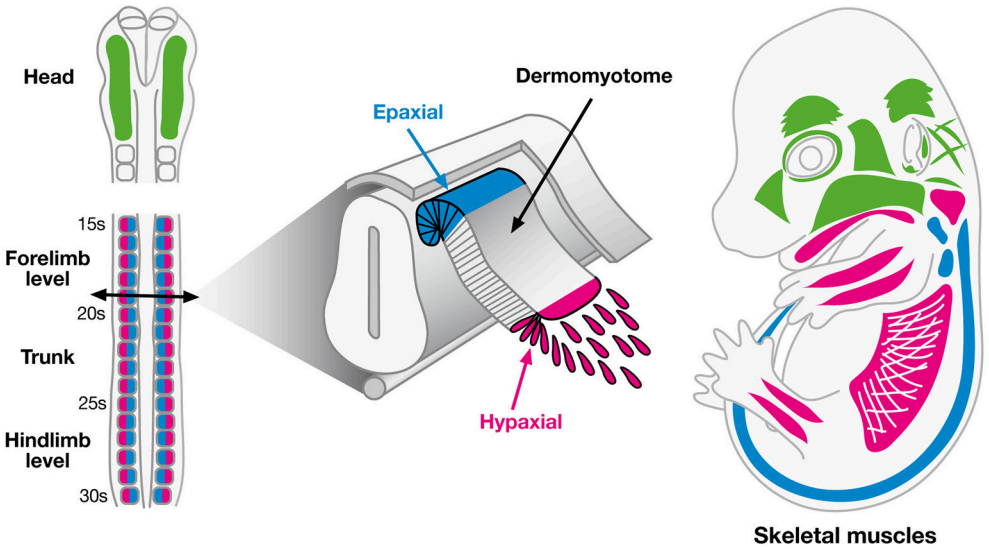

FIGURE 3 | Embryonic origins of skeletal muscles. Myogenic cells of skeletal muscles have two distinct embryonic origins. Myogenic cells of head muscles originate from the paraxial mesoderm (green), except the tongue and posterior neck muscles, which originate from the hypaxial lip of dermomyotome of cranial somites (pink). In the trunk, myogenic cells of back muscles derive from the epaxial lip of dermomyotome (blue), while myogenic cells of diaphragm and limb muscles derive from the hypaxial lip of dermomyotome (pink).

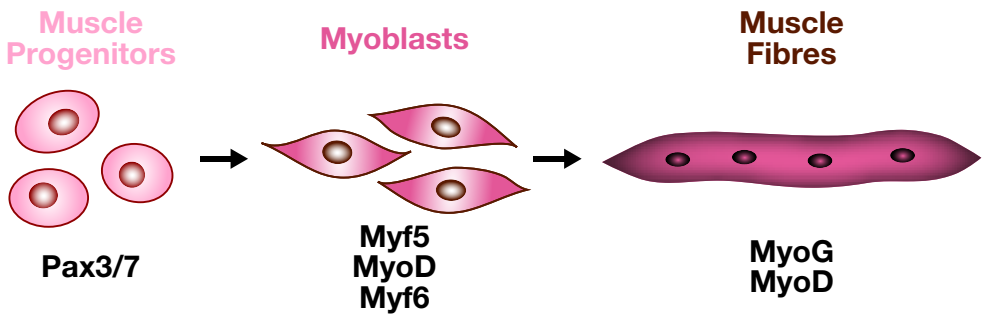

FIGURE 4 | From muscle progenitors to muscle fibers. The myogenic program is characterized by the successive and overlapping expression of specific transcription factors. PAX3 and PAX7 label the progenitor state. The MYF5, MYF6, MYOD myogenic factors label the entry into the myogenic program, MYOG is characteristic of differentiated multinucleated muscle cells.

differentiation (reviewed in Buckingham, 2006). *Myf5* and *Myf6* (*Mrf4*) regulate the entry of progenitor cells into the myogenic program when they delaminate from the lips of the dermomyotome to form the myotome, but subsequent hypaxial activation of *Myf5* is *Pax3*-dependent (Bajard et al., 2006). Early expression of *Myod1* depends on *Myf5*, *Myf6,* and *Pax3* as in the *Myf5*/*Myf6*/*Pax3* triple mutants, *Myod1* expression is altered and skeletal muscles do not form in the trunk and limbs (Tajbakhsh et al., 1997).

In vertebrates, the myogenic program of the head differs from the body musculature. While the expression of the myogenic regulatory factors *Myf5*, *Myod1*, and *Myog* in the craniofacial muscles is similar to what is observed in trunk/limb muscles (Hacker and Guthrie, 1998), the genetic hierarchies operating upstream of the myogenic genes are different for head muscles (branchiomeric and extraocular muscles). *Pax3* is not expressed in head muscles, and *Pax7* does not appear critical as head muscles form in the *Pax7* mutant mice (Relaix et al., 2004). While *Myf6* is not necessary for cranial myogenesis, other transcription factors among which *Tcf21* (*Capsulin*), *MyoR*, *Tbx1*, and *Pitx2* regulate the myogenic factors to form the different craniofacial muscles (Tzahor, 2009). *Tbx1* and *Pitx2* have been shown to activate *Myod1* and *Myf5* in the head and inactivation of *Tbx1* and *Pitx2* in mice causes severe reduction of specific groups of head muscles (Kelly et al., 2004; Zacharias et al., 2011). In mutant mice for *Tcf21* and *MyoR*, myogenic genes are not activated in branchiomeric muscles, and cells undergo cell death (Lu et al., 2002).

Myogenic factors are crucial intrinsic actors for correct development of muscle, however numerous studies have shown that their initiation and regulation also depends on secreted factors coming from the adjacent tissues. The influence of neural tube, neural crest cells, notochord and ectoderm on the formation of muscles has been previously extensively studied and showed that Shh, BMP, Wnt, FGF, and Notch signaling pathways participate to both axial and limb myogenesis (reviewed in Deries and Thorsteinsdóttir, 2016).

Connective Tissue-Mediated Muscle Morphogenesis

CTs and muscles are closely related during embryonic development and adult stages, suggesting that interactions between these tissues might be essential for their development. Classical experiments in avian embryos have demonstrated that signals involved in muscle differentiation and patterning partly derived from surrounding tissues (Lance-Jones, 1988; Ordahl and Le Douarin, 1992; Kardon, 1998). Over the last years, thanks to the identification of specific molecular markers for the different types of CT, progress has been made in the dissection of mechanisms underlying the interactions between CT and muscle development. These data have shown that depending on their embryological origin and their position throughout the body, mechanisms and signaling pathways coming from the diverse types of CT influence spatially and temporally muscle morphogenesis.

Specialized Connective Tissue-mediated Myogenesis (Bone and Cartilage)

Limb muscles and specialized CTs (bone and cartilage) do not exhibit direct physical interactions, as they are linked together via tendons. During limb development, processes regulating skeleton and muscle formation can be dissociated (Hasson et al., 2010; Li et al., 2010). Indeed, disruption of skeletogenesis, through the mutation in the LIM-homeodomain transcription factor *Lmx1b* in skeletal progenitors using the *Sox9-Cre*, has no effect on muscle development (Li et al., 2010). Similarly, inactivation of the BMP antagonist, *Noggin*, which is expressed in condensing cartilage and immature chondrocytes, leads to profound skeletal defects without affecting the early stages of myogenic differentiation (Tylzanowski et al., 2006). However, despite the fact that skeleton and muscle formation can be dissociated, it has been evidenced that skeleton-derived signals are required for proper myogenesis. Indeed, although no defect at the onset of myogenesis is observed in *Noggin* null-mutant mice, terminal muscle differentiation is impaired (Tylzanowski et al., 2006; Costamagna et al., 2016). The Indian hedgehog (Ihh) secreted factor which belongs to the Hedgehog family is secreted by developing chondrocytes (Vortkamp et al., 1996). In the absence of Ihh, muscles are affected (Bren-Mattison et al., 2011). As for Noggin null-mutant, muscle impairment is restricted to secondary myogenesis, resulting in a decrease in the muscle masses. Finally, *in vitro* experiments show that C2C12 myoblasts can be converted toward osteogenic lineage when exposed to BMPs (Lee et al., 2000).

Axial muscles develop from the myotomal compartment of the somite, which is formed by the delamination of cells deriving first from the dorsomedial lip of the dermomyotome and then from its caudal and rostral lips. This process is partly controlled by another somatic compartment, the sclerotome. During chick embryonic development, pioneer myoblasts, constituting the medial part of epithelial somites, express the receptor ROBO2, while its ligand SLIT1 is expressed in the caudal domain of the nascent sclerotome (Halperin-Barlev and Kalcheim, 2011). Loss-of-function assays targeting either ROBO2 or SLIT1 lead to similar results: disruption of the caudo/rostral migration of pioneer myoblasts and of myofibre formation, demonstrating that skeletal precursor-derived signals (sclerotome) regulate the myotome morphogenesis (Halperin-Barlev and Kalcheim, 2011). However, since the sclerotome give rise to both skeleton and tendon progenitors (syndetome), these experiments cannot discriminate between the effects of bone or tendon progenitors on muscle morphogenesis.

Skeletal elements in the **head** derive from the cranial neural crest cells (Couly et al., 1992). Using a *HoxA1/HoxB1* double-knockout mouse, it was shown that cranial neural crest cells fail to form and migrate into the second branchial arch. Despite the absence of neural crest cells (at the origin of skeletal progenitors), cranial myogenesis was initiated (Rinon et al., 2007). However, muscle patterning defects were observed, as evidenced by the expansion in *Tcf21* (Capsulin) and *Tbx1* expression (Rinon et al., 2007). Similarly, ablation of cranial neural crest cells in the chick embryo shows that early steps of head myogenesis are

not impaired by the removal of skeletal progenitors but that expression of myogenic genes is expanded to fill the entire arch mesenchyme, suggesting that the nature of the interactions between cranial skeleton and muscles are conserved in chick and mouse embryos (Tzahor et al., 2003; Rinon et al., 2007). Analysis of the molecular mechanisms demonstrate that BMP and Wnt signaling are important actors involved in these interactions (Tzahor et al., 2003; Rinon et al., 2007). However, cranial neural crest cells give rise to skeleton, tendon and CT progenitors. It is then difficult to determine in these experiments whether cranial myogenesis is controlled by interactions coming from prospective bone, tendon or muscle CT.

Dense Regular Connective Tissue (Tendon) As an Important Source of Signals during Muscle Development

Muscle and dense regular CT (tendon) displays interactions during their development. It is well established that tendon requires muscle to fully develop in chick, mouse and zebrafish embryos (reviewed in Gaut and Duprez, 2016). However, the influence of tendon on muscle development is less clear in vertebrates. During limb muscle development, muscle masses differentiate between tendon primordia. In experimentally tendon-depleted region in chick embryo, ectopic muscles form at the place where tendons normally develop, indicating the role of tendon in delimitating regions of muscle growth and differentiation (Kardon, 1998). The role of tendon cells on muscle development has been studied more in Drosophila. Drosophila tendon precursor cells are defined as a group of ectodermal cells, named the apodeme and characterized by the expression of the Early growth response (EGR)-like transcription factor *Stripe* (Frommer et al., 1996). Altering apodeme formation during the early steps of leg development affects the localization of myoblasts (Soler et al., 2016). Establishment of the myotendinous junctions also requires correct migration of myogenic cells toward tendon cells. This migration step is mediated through guidance cues delivered by tendon cells. In tendons, *Stripe* positively regulates the expression of the *Slit* gene (Volohonsky et al., 2007), coding for a secreted protein implicated in guidance cues during axonal migration (Wong et al., 2002). *Slit* is expressed by tendon cells, while its receptor *Robo* (Roundabout) is expressed in muscle (Kramer et al., 2001). Interestingly, *Slit* mutants present defects in muscle patterning (Ordan et al., 2015), revealing tendon-signaling requirement for proper muscle development. Tendon and muscle interactions via *Slit/Robo* is necessary for the migration arrest of muscle progenitors in Drosophila (Wayburn and Volk, 2009). The formation of the myotendinous junction in Drosophila also requires the transmembrane protein KON-TIKI, enriched at the tips of myotubes, and necessary to direct their migration and the subsequent recognition between muscle and tendon cells (Schnorrer et al., 2007). These data indicate that tendon cells are required for muscle morphogenesis through specific signals emanating from tendon cells and acting on myogenic cells. However, these signals remain to be elucidated during development of the vertebrate musculoskeletal system.

In zebrafish, *Tsp4b* (thrombospondin-4) appears critical to orchestrate tendon extracellular matrix assembly necessary for muscle attachment at the myotendinous junction (Subramanian and Schilling, 2014). Although it has been shown that the vertebrate orthologs of *Stripe*, *Egr1/2* are involved in vertebrate tendon differentiation (Lejard et al., 2011; Guerquin et al., 2013), there is no obvious defect in muscle formation in the absence of *Egr1*. Inactivation of *Tsp4* in mice shows that thrombospondin-4 controls the deposition of extracellular matrix in both tendon and muscle and is necessary for the correct organization of collagen fibrils in tendon (Frolova et al., 2014). However, the absence of *Tps4* also directly affects skeletal muscle structure, by controlling the expression of heparan-sulfate proteoglycans in muscle (Frolova et al., 2014). Finally, tendons have been shown to be required in late events of vertebrate muscle morphogenesis. Indeed, the translocation of myofibers to form the final position of the flexor digitorum superficialis muscle in the mouse forelimb is largely impaired in *Scx* mutant, showing that tendon is implicated in the final patterning and position of muscles (Huang et al., 2013).

Dense Irregular Connective Tissue Establish a Pre-pattern for Muscle Differentiation

Most of our knowledge concerning myogenesis regulation by signals produced by the irregular CT has been established in the limb. Each step of limb muscle development is tightly regulated by signals among which some are derived from the irregular CT. The different steps are the following. Somitic-PAX3-positive cells migrate toward the limb bud, invading the limb mesenchyme. Once they reached their target sites, PAX-3 positive cells proliferate and organize into dorsal and ventral muscle masses. Muscle differentiation is then initiated, followed by muscle mass growth and splitting (reviewed in Duprez, 2002; Deries and Thorsteinsdóttir, 2016).

Delamination and Migration of Muscle Progenitors

Delamination and migration of muscle progenitor cells from the ventrolateral lip of the dermomyotome are mediated via the tyrosine kinase receptor c-Met and its ligand, the Scatter Factor/Hepatocyte Growth Factor (SF/HGF), (Brand-Saberi et al., 1996; Heymann et al., 1996; Dietrich et al., 1999). Cells from the ventrolateral lip of the somite express *c-Met*, while SF/HGF is released by irregular CT progenitors in the limb mesenchyme. In *Hgf* or *c-Met* mutant mice, limb muscles are missing (Bladt et al., 1995). Dermomyotome development proceeds normally and migratory somatic precursors are correctly specified but they remain aggregated and fail to migrate toward limb buds (Dietrich et al., 1999). SF/HGF also regulates the migration of myogenic progenitors from occipital and cervical somites, giving rise to the tongue, diaphragm and shoulder muscles (Dietrich et al., 1999). These studies highlight the link between irregular CT and hypaxial muscle progenitors during the migration step of muscle morphogenesis. Other signaling pathways expressed in irregular CT are involved in the guidance of muscle progenitors to reach their target sites into the limb bud. The CXCL12 chemokine is expressed in restricted regions of limb bud irregular CT and

has been shown to attract muscle progenitors, which expressed the chemokine receptor CXCR4 (Vasyutina et al., 2005). Ectopic expression of CXCL12 in limb mesenchyme of chick embryos, or inactivation of *Cxcr4* in mouse embryos both give rise to aberrant localization of muscle progenitors in the limb (Vasyutina et al., 2005), demonstrating a chemoattractive role of CXCL12 positive-CT cells for *Cxcr4* expressing muscle precursors. During their migration toward the limb, muscle progenitors also express the receptor *EPHA4*, while its ligand *EPHRINA5* is expressed in specific areas of limb irregular CT (Swartz et al., 2001). Conversely to the chemoattractive role of CXCL12/CXCR4 signaling, EPHRINA5 acts as a repulsive signal for muscle cells expressing EPHA4 (Swartz et al., 2001), demonstrating that both chemoattractive and repulsive signals from irregular CT act simultaneously on muscle progenitors to restrict and define their pathway of migration. Finally, it is important for muscle progenitor cells to stay in an undifferentiated state during migration. It is likely that this step is regulated through secreted signals produced by the limb mesenchyme, however it is not clear yet which signaling exactly is involved in this process. Previous studies suggest that BMPs and FGFs secreted by limb irregular CT might be important to prevent differentiation in migrating cells by respectively inhibiting and promoting the expression of SF/HGF (Heymann et al., 1996; Pourquié et al., 1996; Scaal et al., 1999). In the chick embryo, FGF18 and retinoic acid, secreted by limb mesenchyme, control the timing of *Myod1* and *Myf5* expression in myogenic cells (Mok et al., 2014).

Muscle Differentiation and Patterning

During the whole processes of limb muscle morphogenesis, irregular CT and muscles (progenitors or differentiated cells) are in close association. Kardon et al. (2003) identified TCF4 as a putative actor in the process of irregular CT-mediated muscle morphogenesis. TCF4 is expressed in the lateral plate-derived mesoderm in close association with limb muscles during their differentiation and patterning. In the absence of limb muscles, TCF4 expression pattern is unchanged, suggesting that TCF4 expression may serve as a pre-pattern for limb musculature. To verify this hypothesis, *TCF4* gain- and loss-of-functions were performed in lateral plate-derived limb mesodermal cells. In all cases, muscle mispatterning was observed, demonstrating that TCF4 in irregular CT is important to establish the correct location of limb muscles (Kardon et al., 2003). *Tcf4* deletion in mice also lead to aponeurosis defects (Mathew et al., 2011). However, TCF4 is also expressed at low level in myogenic cells and is involved in the intrinsic regulation of muscle fiber type differentiation in mice (Mathew et al., 2011).

Recently, the role of irregular CT has also been involved in the context of a common and often lethal muscle diaphragm defect, called congenital diaphragmatic hernia (CDH). Merrell et al. (2015) have shown that the pleuroperitoneal folds, which are transient embryonic structures, give rise to the diaphragm irregular CT. Muscle progenitor cells arising from the ventrolateral dermomyotome of the cervical somites migrate into the *Tcf4*-positive pleuroperitoneal cells which guide muscle cells to organize the diaphragm morphogenesis. *Tcf4*-positive CT cells also express *Gata4*, known to be mutated in CDH, and Gata4 inactivation in diaphragm CT leads to hernias similar to those observed in CDH, demonstrating that this congenital muscular disease is related to a defect in muscle irregular CT (Merrell et al., 2015).

As previously mentioned, the human Holt-Oram syndrome is characterized by limb and heart musculoskeletal defects and irregular CT disorganization. This syndrome is due to a mutation in the *TBX5* gene, which is expressed in irregular CT during limb development (Hasson et al., 2010). *Tbx5* deletion leads to a defect in irregular CT organization during embryonic development (Hasson et al., 2010). In these conditions, while the early steps of limb myogenesis are not affected, ectopic splitting of nascent muscle bundles is observed. *Tbx5* inactivation leads to a disruption of muscle irregular CT, to an alteration of *Tcf4* expression, but also a marked decrease of ß-catenin and N-cadherin at the membranes of muscular irregular CT cells (Hasson et al., 2010). In addition, deletion of ß-catenin in the limb mesenchyme leads to ectopic muscle splitting consistent with a model in which the N-cadherin/ß-catenin complex in the muscle CT is critical for muscle patterning (Hasson et al., 2010). Finally, *Tbx5* deletion also alters the expression of mesenchymal secreted factors important in limb myogenesis, as CXCL12 and SF/HGF (Hasson et al., 2010). It is noteworthy that in synovial fibroblasts, *Cxcl12* is a target of *Tbx5* in human synovial fibroblasts (Karouzakis et al., 2014). Recently, it has been shown that the conditional deletion of another T-box gene, *Tbx3,* in the lateral plate mesoderm (using a Prx1-Cre transgene) leads to defects in myofiber formation in a subset of limb muscles in mice (Colasanto et al., 2016). These localized muscle defects are correlated with *Tbx3* expression in a subset of limb bones, tendons and muscle CT. Similar muscle defects are observed in patients with *TBX3* mutations that are responsible of the Ulnar-mammary syndrome (Colasanto et al., 2016). In addition to being expressed in limb skeletal elements, *Hoxa11* gene is also expressed in mouse muscle CT and *Hoxa11* inactivation disrupts limb muscle and tendon patterning in addition to the already known skeleton defect (Swinehart et al., 2013). Tendon and muscle phenotypes in heterozygous *Hoxa11* mutants are independent of skeletal patterning as abnormal tendon and muscle patterning are observed in *Hoxa11* mutants with normal skeleton (Swinehart et al., 2013). However, it cannot be excluded that, in this case, muscle mispatterning could be related to tendon abnormalities rather than to the muscle CT defect. Recently, Gu et al. (2016) have shown that in neonatal muscles, muscle interstitial cells activate NF-kB, which regulates EPHRINA5 to stimulate myoblast migration toward the end of growing fibers, where they subsequently fuse to contribute to muscle growth. These data show that muscle CT also contributes to the process of muscle maturation during neonatal development. However, these interstitial cells are characterized by the expression of NG2, a neural/glial antigen 2 expressed in pericytes and it cannot be excluded that these cells are of vascular origin (Gu et al., 2016).

Finally, differentiated muscle fibers also act on muscle CT formation. In mice deleted for *Lox* (Lysyl-oxidase), an enzyme regulating collagen organization and secreted from the myofibers, TGFβ signaling is decreased and promotes muscle CT

differentiation at the expense of muscle tissue (Kutchuk et al., 2015).

Connective Tissue Cell Involvement in Adult Muscle Homeostasis

In adult, skeletal muscle loss is observed in neuromuscular diseases, but also during aging, inactivity and chronic systemic disorders (i.e., diabetes, cancer, rheumatoid arthritis). The regenerative potential of skeletal muscle provides a compensatory response against such pathological muscle loss. The regenerative capacity of skeletal muscle relies on muscle stem cells (named satellite cells), which proliferate in response to exercise to facilitate muscle growth and remodeling, or following myotrauma to repair the injured muscle. Satellite cells are PAX3/7-positive progenitor cells located under the basal lamina that forms around muscle fibers of postnatal skeletal muscle. Satellite cells remain quiescent until the muscle is injured, when the lamina breaks down and activated satellite cells begin to proliferate before forming new muscle fibers (Relaix et al., 2005). *Myf5* is detected in the majority of quiescent satellite cells (Cornelison and Wold, 1997; Beauchamp et al., 2000) and activation of satellite cells is accompanied by expression of *Myod1* as well as higher levels of *Myf5*, leading to the downregulation of *Pax7*, activation of Myogenin, and new muscle fiber formation (Relaix et al., 2006, reviewed in Motohashi and Asakura, 2014). In the absence of *Pax7*-positive cells, the process of muscle regeneration failed and instead, fibrotic and fatty infiltration are observed, demonstrating the major contribution of muscle satellite cells in the formation of new muscle fibers (von Maltzahn et al., 2013). However, in response to muscle damage, non-myogenic cells can also participate to skeletal muscle regeneration, either by giving rise to myogenic stem cells or by stimulating the activation of resident muscle satellite cells.

A non-satellite cell population with myogenic capacity was first identified when it has been shown that bone-marrow-derived cells can participate directly to muscle regeneration (Ferrari et al., 1998). These cells, which normally reconstitute the hematopoietic lineage, can give rise to new satellite cells and myofibers during the muscle regeneration process (Asakura, 2012) and their transplantation into mdx mice (a model for Duchenne muscular dystrophy) improves muscle function (Sampaolesi et al., 2006). Similarly, a vascular progenitor population, which can be isolated from postnatal muscle, participate in muscle repair following arterial delivery in mice (Sampaolesi et al., 2003). Interestingly, pre-treatment of both mesenchymal bone-marrow stromal cells (Galvez et al., 2006) or vascular progenitors (Brzoska et al., 2012) with the CXCL12 chemokine improved the regeneration of injured muscle. CXCL12 is expressed in the adult muscle by the endomysium, i.e., the CT surrounding each muscle fiber (Hunger et al., 2012). Following muscle injury, CXCL12 secreted by muscle CT rapidly increases (Griffin et al., 2010) and chemoattracts both satellite cells and bone-marrow-derived cells to actively participate to the regeneration process (Ratajczak et al., 2003). In this context, CXCL12 would not only chemoattract stem cells toward the injury site, but would also increase their fusion with native muscle fibers (Griffin et al., 2010). These results demonstrate that signals provided by muscle irregular CT are not only crucial for muscle morphogenesis during development but also mediate the processes of muscle regeneration in the adult.

More recently, a population of interstitial muscle cells with myogenic potential has been identified (Mitchell et al., 2010). These cells, characterized by the expression of the PW1/*Peg3* gene and named PICs (PW1-positive interstitial cells) contribute to the satellite cell pool during muscle regeneration (Mitchell et al., 2010), although they do not express Pax3 or Pax7 (Pannérec et al., 2013). PICs can be subdivided into two distinct populations: PW1+ PDGFrα− and PW1+ PDGFrα+ cells. It has been establish that only PW1+ PDGFrα− PICs are associated with a myogenic potential while PW1+ PDGFrα+ cells give rise to adipocytes (Pannérec et al., 2013). Interestingly, PW1+ PDGFrα+ PICs express the pericyte marker NG2, indicating a possible overlap between these cells, and pericytes (Pannérec et al., 2013). Pericytes represent perivascular cells that are present in the muscle interstitium and associated with capillaries. They can be separated into two different populations: type-1 pericytes (NG2+ NESTIN− PDGFrα−) and type-2 pericytes (NG2+ NESTIN+ PDGFrα+), (Birbrair et al., 2013). Similarly to what has been described for PICs, the two different populations of pericytes have distinct cell fate potential: type-1 contribute to adipose tissue and type-2 to myogenesis (reviewed in Birbrair et al., 2014). Type-2 pericytes do not express Pax7, Myf5 and Myod1, but upregulate these markers before forming myotubes in regenerative conditions (Cappellari and Cossu, 2013).

Different studies also reported the participation of mesenchymal progenitors without myogenic capacity during muscle regeneration. These progenitors all arise from resident cells in the adult muscle interstitium (Joe et al., 2010; Uezumi et al., 2010). Based on the expression of PDGFRα, a cell population resident in the muscle interstitium has been isolated, which, under specific culture conditions, differentiate into fibroblasts, adipocytes or osteoblasts, but never give rise to muscle cells and has been named mesenchymal progenitors (Uezumi et al., 2010). Simultaneously, Rossi's group also identified a cell population with fibroblastic and adipogenic potential, but no myogenic potential (Joe et al., 2010). These progenitors were isolated on the basis of SCA1 and CD34 expression, and termed Fibro/Adipogenic progenitors (FAPs), (Joe et al., 2010). Interestingly, mesenchymal PDGFRα+ progenitors express SCA1 (Joe et al., 2010, Uezumi et al., 2010) and FAPs express PDGFRα, highlighting the possibility that mesenchymal progenitors and FAPs actually represent a unique progenitor population. FAPs/mesenchymal progenitors are activated upon muscle injury and promote myoblast differentiation in co-cultures (Joe et al., 2010), but also exhibit a strong adipogenic and fibrogenic potential *in vitro*, indicating a potential contribution of FAPs to fibrotic and adipose accumulation in diseased muscles (Uezumi et al., 2010). It is then proposed that a balance between satellite cell-dependent myogenesis and FAPs-dependent adipogenesis/fibrogenesis regulates muscle homeostasis and regeneration. After muscle injury, FAPs/mesenchymal progenitors start to proliferate before satellite cells and invade the space between regenerating muscle fibers, where they

generate factors promoting myogenesis. When regeneration proceeds efficiently, FAPs/mesenchymal progenitors are discarded from the tissue through apoptotic signals emanating from satellite cells. If regeneration fails, FAPs/mesenchymal progenitors persist and differentiate into adipocytes and fibroblasts, leading to fatty and fibrotic degeneration (reviewed in Natarajan et al., 2010; Judson et al., 2013). Depending on the surrounding environment, FAPs/mesenchymal progenitors will preferentially give rise to fibroblasts or adipocytes. Addition of TGFß to FAPs/mesenchymal progenitors *in vitro* induces the expression of fibrosis markers leading to fibroblastic differentiation at the expense of adipocyte differentiation (Uezumi et al., 2011). Interestingly, PDGFRα+ expressing FAPs/mesenchymal progenitors accumulate preferentially into fibrotic regions, suggesting a specific role for PDGFRα in muscle fibrosis (Uezumi et al., 2014). This hypothesis is supported by the observation that, in adult and embryonic mouse, an elevated level of PDGFRα leads to an abnormal increase in CT differentiation (Olson and Soriano, 2009).

The participation of irregular CT to muscle regeneration has been also highlighted by a recent set of experiments. CT fibroblasts identified by *Tcf4* expression have been shown to proliferate close to muscle satellite cells following injury and conditional ablation of *Tcf4*-positive cells prior to muscle lesion leads to premature satellite cell differentiation, depletion of the early pool of satellite cells, and small regenerated fibers, indicating that *Tcf4*-positive fibroblasts participate in muscle regeneration (Murphy et al., 2011). It remains unclear whether a direct relationship exists between FAPs/mesenchymal progenitors and TCF4-positive cells. However, *Tcf4*-positive cells express PDGFRα (Murphy et al., 2011) and accumulating evidence suggests that FAPs/mesenchymal and irregular CT progenitors share common features (Sudo et al., 2007; Haniffa et al., 2009). Extracellular matrix components also contribute directly to the regenerative potential of muscle. Indeed, it has been shown that a fibronectin-rich fibrosis is essential during the initial step of regeneration to activate the proliferation of muscle satellite cells (Bentzinger et al., 2013). Irregular CT progenitors, FAPs and PICs could be potential sources of fibronectin and might contribute to the transient fibronectin-rich promyogenic fibrosis during muscle regeneration. However, activated satellite myogenic cells themselves release fibronectin into their microenvironment and inactivation of fibronectin using a *Myf5*-Cre reporter impairs the regenerative potential of muscle, suggesting that this effect could be also related to a cell-autonomous role of satellite cell derived-fibronectin (Bentzinger et al., 2013).

The importance of muscle CT has been also evidenced in muscle disorders. Indeed, mutations in *COL6A1, COL6A2*, and *COL6A3* genes, which give rise to the main collagens expressed in muscle CT, have been observed in congenital Ullrich muscular dystrophy and in Behlem myopathy. Mutant mice for *Col6a1* display alterations of muscle sarcoplasmic reticulum and mitochondria (Pan et al., 2014) and *Col6a3* mutant mice display myopathic and connective tissue phenotypes similar to the *Col6a1* null mice (Pan et al., 2013), demonstrating that collagen VI mutations result in disorders with combined muscle and connective tissue involvement. In addition, *Col6a1* mutant mice showed delayed muscle regeneration and reduced satellite cell self-renewal. Transplantation of wild-type fibroblasts in muscles of *Col6a1* mutant mice rescues muscle satellite cells, indicating that COL6A1 in the muscle environment can modulate satellite cell behavior (Urciuolo et al., 2013).

Finally, during muscle hypertrophic activity, satellite cells can regulate fibrogenic cell collagen expression via exosome secretion, showing that muscle cell progenitors can also act with their surrounding environment to facilitate tissue plasticity (Fry et al., 2016). Similarly, Abou-Khalil et al. (2015) have shown that *Pax7*-positive muscle satellite cells are involved in bone repair, providing a direct evidence of a muscle contribution to specialized CT (bone and cartilage) formation.

Taken together, these data evidence interactions between different cell populations promoting muscle progenitor activation during regeneration, with a central role of muscle irregular CT in this process. Changes in CT local environment may contribute to muscle pathologies and age-related loss of muscle stem cell competence by implicating pivotal signaling pathways and genes similar to those described to mediate the CT-dependent muscle morphogenesis during development.

CONCLUSIONS

The development of skeletal muscle has been extensively studied for decades and most of the studies have first concentrated on the elucidation of the intrinsic mechanisms underlying the conversion of muscle progenitors toward a functional skeletal muscle organ. The identification of specific myogenic transcription factors has allowed us to decipher the importance of these intrinsic gene networks in the specification and differentiation of muscles during embryonic development. In parallel, the role of neighboring tissues on muscle morphogenesis has been investigated and highlighted the influence of the neural tube, notochord and ectoderm on the early steps of axial muscle morphogenesis, mostly via the effect of the secreted factors Wnts, BMPs, and SHH. More recently, the role of CTs in muscle morphogenesis has been investigated, thanks to the identification of transcription factors specifically expressed in the different types of CT surrounding (tendon) or composing (muscle CT) the developing muscle (previously reviewed in Hasson, 2011). These studies demonstrate that mesenchymal cells at the origin of the different CT types deliver information necessary for a correct muscle morphogenesis, from the early steps of myoblast migration and fusion to the late stages of muscle maturation. Secreted factors as BMPs, FGFs, and chemokines as CXCL12 are important in this dialogue between CTs and muscles, which also implicate a reverse interaction between both tissues, as muscle cells are necessary for the tendons to develop correctly and for the organization of irregular CT and bone in adult. A right balance between myogenic and CT cells is particular necessary during the muscle regeneration process. Indeed, impairment of the regenerative potential after injury or in neuromuscular diseases results in the progressive replacement of the muscle mass by fibrotic tissue (Farup et al., 2015). Thus, bi-directional communication between muscle and CT is critical for a correct assembly of the musculoskeletal system during development as well as to maintain its homeostasis in the adult.

AUTHOR CONTRIBUTIONS

SN, DD, and CF have equally contributed to making the original plan and writing the manuscript.

FUNDING

This work was supported by the Fondation pour la Recherche Médicale (FRM) DEQ20140329500, Agence Nationale de la Recherche (ANR) ANR-12-BSV1-0038, Association Française contre les Myopathies (AFM) N°16826. SN was part of the MyoGrad International Research Training Group for Myology and received financial support from the AFM (AFM 201505 32272).

ACKNOWLEDGMENTS

We thank S. Gournet for illustrations.

REFERENCES

Abou-Khalil, R., Yang, F., Lieu, S., Julien, A., Perry, J., Pereira, C., et al. (2015). Role of muscle stem cells during skeletal regeneration. *Stem Cells* 33, 1501–1511. doi: 10.1002/stem.1945

Adhami, M., Rashid, H., Chen, H., and Javed, A. (2014). Runx2 activity in committed osteoblasts is not essential for embryonic skeletogenesis. *Connect. Tissue Res.* 55, 102–106. doi: 10.3109/03008207.2014.923873

Akiyama, H., Chaboissier, M., Martin, J., and Crombrugghe, B. D. S. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* 16, 2813–2828. doi: 10.1101/gad.1017802

Anderson, D. M., Arredondo, J., Hahn, K., Valente, G., Martin, J. F., Wilson-Rawls, J., et al. (2006). Mohawk is a novel homeobox gene expressed in the developing mouse embryo. *Dev. Dyn.* 235, 792–801. doi: 10.1002/dvdy.20671

Asakura, A. (2012). Skeletal muscle-derived hematopoietic stem cells: muscular dystrophy therapy by bone marrow transplantation. *J. Stem Cell Res. Ther.* 3(Suppl. 11), S11–S15. doi: 10.4172/2157-7633.s11-005

Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P., and Buckingham, M. E. (2006). A novel genetic hierarchy functions during hypaxial myogenesis : Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev.* 20, 2450–2464. doi: 10.1101/gad.382806

Beauchamp, J., Heslop, L., Yu, D., Tajbakhsh, S., Kelly, R., Wernig, A., et al. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J. Cell Biol.* 151, 1221–1233. doi: 10.1083/jcb.151.6.1221

Benjamin, M., and Ralphs, J. R. (2000). The cell and developmental biology of tendons and ligaments. *Int. Rev. Cytol.* 196, 85–130. doi: 10.1016/S0074-7696(00)96003-0

Bentzinger, C. F., Wang, Y. X., Von Maltzahn, J., Soleimani, V. D., Yin, H., and Rudnicki, M. A. (2013). Fibronectin regulates Wnt7a signaling and satellite cell expansion. *Cell Stem Cell* 12, 75–87. doi: 10.1016/j.stem.2012.09.015

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and De Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nature* 22, 85–89.

Birbrair, A., Zhang, T., Wang, Z.-M., Messi, M. L., Enikolopov, G. N., Mintz, A., et al. (2013). Role of pericytes in skeletal muscle regeneration and fat accumulation. *Stem Cells Dev.* 22, 2298–2314. doi: 10.1089/scd.2012.0647

Birbrair, A., Zhang, T., Wang, Z. M., Messi, M. L., Mintz, A., and Delbono, O. (2014). Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle. *Front. Aging Neurosci.* 6:245. doi: 10.3389/fnagi.2014.00245

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* 376, 768–771. doi: 10.1038/376768a0

Bonafede, A., Kohler, T., Rodriguez-Niedenfuhr, M., and Brand-Saberi, B. (2006). BMPs restrict the position of premuscle masses in the limb buds by influencing Tcf4 expression. *Dev. Biol.* 299, 330–344. doi: 10.1016/j.ydbio.2006.02.054

Brand-Saberi, B., Müller, T. S., Wilting, J., Christ, B., and Birchmeier, C. (1996). Scatter factor/hepatocyte growth factor (SF/HGF) induces emigration of myogenic cells at interlimb level *in vivo*. *Dev. Biol.* 179, 303–308. doi: 10.1006/dbio.1996.0260

Bren-Mattison, Y., Hausburg, M., and Olwin, B. B. (2011). Growth of limb muscle is dependent on skeletal-derived Indian hedgehog. *Dev. Biol.* 356, 486–495. doi: 10.1016/j.ydbio.2011.06.002

Brent, A. E., Schweitzer, R., and Tabin, C. J. (2003). A somitic compartment of tendon progenitors. *Cell* 113, 235–248. doi: 10.1016/S0092-8674(03)00268-X

Brzoska, E., Kowalewska, M., Markowska-Zagrajek, A., Kowalski, K., Archacka, K., Zimowska, M., et al. (2012). Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells. *Biol. Cell* 104, 722–737. doi: 10.1111/boc.201200022

Buckingham, M. (2006). Myogenic progenitor cells and skeletal myogenesis in vertebrates. *Curr. Opin. Genet. Dev.* 16, 525–532. doi: 10.1016/j.gde.2006.08.008

Cappellari, O., and Cossu, G. (2013). Pericytes in development and pathology of skeletal muscle. *Circ. Res.* 113, 341–347. doi: 10.1161/CIRCRESAHA.113.300203

Chen, J. W., and Galloway, J. L. (2014). The development of zebrafish tendon and ligament progenitors. *Development* 141, 2035–2045. doi: 10.1242/dev.104067

Christ, B., and Ordahl, C. (1995). Early stages of chick somite development. *Anat. Embryol.* 191, 381–396. doi: 10.1007/BF00304424

Christ, B., and Wilting, J. (1992). From somites to vertebral column. *Ann. Anat.* 174, 23–32. doi: 10.1016/S0940-9602(11)80337-7

Colasanto, M. P., Eyal, S., Mohassel, P., Bamshad, M., Bonnemann, C., Zelzer, E., et al. (2016). Development of a subset of forelimb muscles and their attachment sites requires the ulnar-mammary syndrome gene Tbx3. *Development* 9, 1257–1269. doi: 10.1242/dev.147645

Cornelison, D. D. W., and Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* 191, 270–283. doi: 10.1006/dbio.1997.8721

Costamagna, D., Mommaerts, H., Sampaolesi, M., and Tylzanowski, P. (2016). Noggin inactivation affects the number and differentiation potential of muscle progenitor cells *in vivo*. *Sci. Rep.* 6:31949. doi: 10.1038/srep31949

Couly, G. F., Coltey, P. M., and Le Douarin, N. M. (1992). The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development* 114, 1–15.

Crane, J. F., and Trainor, P. A. (2006). Neural crest stem and progenitor cells. *Annu. Rev. Cell Dev. Biol.* 22, 267–286. doi: 10.1146/annurev.cellbio.22.010305.103814

Day, T. F., Guo, X., Garrett-beal, L., and Yang, Y. (2005). Wnt / ß -catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev. Cell* 8, 739–750. doi: 10.1016/j.devcel.2005.03.016

Delfini, M.-C., and Duprez, D. (2004). Ectopic Myf5 or MyoD prevents the neuronal differentiation program in addition to inducing skeletal muscle differentiation, in the chick neural tube. *Development* 131, 713–723. doi: 10.1242/dev.00967

Deng, C., Wynshaw-boris, A., Zhou, F., Kuo, A., and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* 84, 911–921. doi: 10.1016/S0092-8674(00)81069-7

Deries, M., and Thorsteinsdóttir, S. (2016). Axial and limb muscle development: dialogue with the neighbourhood. *Cell. Mol. Life Sci.* 73, 4415–4431. doi: 10.1007/s00018-016-2298-7

Dietrich, S., Abou-rebyeh, F., Brohmann, H., Bladt, F., and Sonnenberg-Riethmacher, E. (1999). The role of SF / HGF and c-Met in the development of skeletal muscle. *Development* 1629, 1621–1629.

Ducy, P., Starbuck, M., Priemel, M., Shen, J., Pinero, G., Geoffroy, V., et al. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* 13, 1025–1036. doi: 10.1101/gad.13.8.1025

Duprez, D. (2002). Signals regulating muscle formation in the limb during embryonic development. *Int. J. Dev. Biol.* 46, 915–925. doi: 10.1387/IJDB.12455629

Edom-Vovard, F., Schuler, B., Bonnin, M.-A., Teillet, M.-A., and Duprez, D. (2002). Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons. *Dev. Biol.* 247, 351–366. doi: 10.1006/dbio.2002.0707

Eloy-trinquet, S., Wang, H., and Duprez, D. (2009). Fgf signaling components are associated with muscles and tendons during limb development. *Dev. Dyn.* 238, 1195–1206. doi: 10.1002/dvdy.21946

Farup, J., Madaro, L., Puri, P. L., and Mikkelsen, U. R. (2015). Interactions between muscle stem cells, mesenchymal- derived cells and immune cells in muscle homeostasis, regeneration and disease. *Cell Death Dis.* 6, e1830–e1813. doi: 10.1038/cddis.2015.198

Ferrari, G., Angelis, G. C., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., et al. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors published by: American Association for the advancement of Science linked references are available on JSTOR for this article: muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 279, 1528–1530. doi: 10.1126/science.279.5356.1528

Frolova, E. G., Drazba, J., Krukovets, I., Kostenko, V., Blech, L., Harry, C., et al. (2014). Control of organization and function of muscle and tendon by thrombospondin-4. *Matrix Biol.* 37, 35–48. doi: 10.1016/j.matbio.2014.02.003

Frommer, G., Vorbruggen, G., Pasca, G., Jackle, H., and Volk, T. (1996). Epidermal egr-like zinc finger protein of Drosophila participates in myotube guidance. *EMBO J.* 15, 1642–1649.

Fry, C. S., Kirby, T. J., Kosmac, K., McCarthy, J. J., and Peterson, C. A. (2016). Myogenic progenitor cells control extracellular matrix production by fibroblasts during skeletal muscle hypertrophy. *Cell Stem Cell* 20, 1–14. doi: 10.1016/j.stem.2016.09.010

Galvez, B. G., Sampaolesi, M., Brunelli, S., Covarello, D., Gavina, M., Rossi, B., et al. (2006). Complete repair of dystrophic skeletal muscle by mesoangioblasts with enhanced migration ability. *J. Cell Biol.* 174, 231–243. doi: 10.1083/jcb.200512085

Gara, S. K., Grumati, P., Squarzoni, S., Sabatelli, P., Urciuolo, A., Bonaldo, P., et al. (2011). Differential and restricted expression of novel collagen VI chains in mouse. *Matrix Biol.* 30, 248–257. doi: 10.1016/j.matbio.2011.03.006

Gaut, L., and Duprez, D. (2016). Tendon development and diseases. *Wiley Interdiscip. Rev. Dev. Biol.* 5, 5–23. doi: 10.1002/wdev.201

Grenier, J., Teillet, M.-A., Grifone, R., Kelly, R. G., and Duprez, D. (2009). Relationship between neural crest cells and cranial mesoderm during head muscle development. *PLoS ONE* 4:e4381. doi: 10.1371/journal.pone.0004381

Griffin, C. A, Apponi, L. H., Long, K. K., and Pavlath, G. K. (2010). Chemokine expression and control of muscle cell migration during myogenesis. *J. Cell Sci.* 123, 3052–3060. doi: 10.1242/jcs.066241

Gu, J. M., Wang, D. J., Peterson, J. M., Shintaku, J., Liyanarachchi, S., Coppola, V., et al. (2016). An NF-κB - EphrinA5-dependent communication between NG2+ interstitial cells and myoblasts promotes muscle growth in neonates. *Dev. Cell* 36, 215–224. doi: 10.1016/j.devcel.2015.12.018

Guerquin, M., Charvet, B., Nourissat, G., Havis, E., Ronsin, O., Bonnin, M., et al. (2013). Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. *J. Clin. Invest.* 123, 3564–3576. doi: 10.1172/JCI67521

Hacker, A., and Guthrie, S. (1998). A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. *Development* 125, 3461–3472.

Halperin-Barlev, O., and Kalcheim, C. (2011). Sclerotome-derived Slit1 drives directional migration and differentiation of Robo2-expressing pioneer myoblasts. *Development* 138, 2935–2945. doi: 10.1242/dev.065714

Haniffa, M. A., Collin, M. P., Buckley, C. D., and Dazzi, F. (2009). Mesenchymal stem cells: the fibroblasts' new clothes? *Haematologica* 94, 258–263. doi: 10.3324/haematol.13699

Hasson, P. (2011). "Soft" tissue patterning: muscles and tendons of the limb take their form. *Dev. Dyn.* 240, 1100–1107. doi: 10.1002/dvdy.22608

Hasson, P., Del Buono, J., and Logan, M. P. (2007). Tbx5 is dispensable for forelimb outgrowth. *Development* 134, 85–92. doi: 10.1242/dev.02622

Hasson, P., DeLaurier, A., Bennett, M., Grigorieva, E., Naiche, L. A., Papaioannou, V. E., et al. (2010). Tbx4 and Tbx5 acting in connective tissue are required for limb muscle and tendon patterning. *Dev. Cell* 18, 148–156. doi: 10.1016/j.devcel.2009.11.013

Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., et al. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* 364, 501–506. doi: 10.1038/364501a0

Havis, E., Bonnin, M.-A., Olivera-Martinez, I., Nazaret, N., Ruggiu, M., Weibel, J., et al. (2014). Transcriptomic analysis of mouse limb tendon cells during development. *Development* 141, 3683–3696. doi: 10.1242/dev.108654

Havis, E., Bonnin, M., de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFβ and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Heymann, S., Koudrova, M., Arnold, H.-H., Köster, M., and Braun, T. (1996). Regulation and function of SF/HGF during migration of limb muscle precursor cells in chicken. *Dev. Biol.* 180, 566–578. doi: 10.1006/dbio.1996.0329

Hill, T. P., Später, D., Taketo, M. M., Birchmeier, W., and Hartmann, C. (2005). Canonical Wnt / ß-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev. Cell* 8, 727–738. doi: 10.1016/j.devcel.2005.02.013

Huang, A. H., Riordan, T. J., Wang, L., Eyal, S., Zelzer, E., Brigande, J. V., et al. (2013). Repositioning forelimb superficialis muscles: tendon attachment and muscle activity enable active relocation of functional myofibers. *Dev. Cell* 26, 544–551. doi: 10.1016/j.devcel.2013.08.007

Hunger, C., Ödemis, V., and Engele, J. (2012). Expression and function of the SDF-1 chemokine receptors CXCR4 and CXCR7 during mouse limb muscle development and regeneration. *Exp. Cell Res.* 318, 2178–2190. doi: 10.1016/j.yexcr.2012.06.020

Ito, Y., Toriuchi, N., Yoshitaka, T., Ueno-kudoh, H., Sato, T., and Yokoyama, S. (2010). The Mohawk homeobox gene is a critical regulator of tendon differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 10538–10542. doi: 10.1073/pnas.1000525107

Jacob, A. L., Smith, C., Partanen, J., and Ornitz, D. M. (2006). Fibroblast growth factor receptor 1 signaling in the osteo-chondrogenic cell lineage regulates sequential steps of osteoblast maturation. *Dev. Biol.* 296, 315–328. doi: 10.1016/j.ydbio.2006.05.031

Joe, A. W. B., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., et al. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. *Nat. Cell Biol.* 12, 153–163. doi: 10.1038/ncb2015

Judson, R. N., Zhang, R., and Rossi, F. M. A. (2013). Tissue-resident mesenchymal stem/progenitor cells in skeletal muscle: collaborators or saboteurs? *FEBS J.* 280, 4100–4108. doi: 10.1111/febs.12370

Kardon, G. (1998). Muscle and tendon morphogenesis in the avian hind limb. *Development* 125, 4019–4032.

Kardon, G., Harfe, B. D., and Tabin, C. J. (2003). A Tcf4-positive mesodermal population provides a prepattern for vertebrate limb muscle patterning. *Dev. Cell* 5, 937–944. doi: 10.1016/S1534-5807(03)00360-5

Karouzakis, E., Trenkmann, M., Gay, R. E., Michel, B. A., Gay, S., and Neidhart, M. (2014). Epigenome analysis reveals TBX5 as a novel transcription factor involved in the activation of rheumatoid arthritis synovial fibroblasts. *J. Immunol.* 193, 4945–4951. doi: 10.4049/jimmunol.1400066

Karsenty, G., and Wagner, E. F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* 2, 389–406. doi: 10.1016/S1534-5807(02)00157-0

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., et al. (2004). Mrf4 determines skeletal muscle identity in Myf5:myod double-mutant mice. *Nature* 431, 466–471. doi: 10.1038/nature02876

Kassar-Duchossoy, L., Giacone, E., Gayraud-morel, B., Jory, A., Gomès, D., and Tajbakhsh, S. (2005). Pax3 / Pax7 mark a novel population of primitive myogenic cells during development. *Genes (Basel).* 19, 1426–1431. doi: 10.1101/gad.345505

Kelly, R. G., Jerome-majewska, L. A., and Papaioannou, V. E. (2004). The del22q11. 2 candidate gene Tbx1 regulates branchiomeric myogenesis. *Hum. Mol. Genet.* 13, 2829–2840. doi: 10.1093/hmg/ddh304

Kieny, M., and Chevallier, A. (1979). Autonomy of tendon development in the embryonic chick wing. *J. Embryol. Exp. Morph.* 49, 153–165.

Kieny, M., and Mauger, A. (1984). Immunofluorescent localization of extracellular matrix components during muscle morphogenesis. 1. in normal chick embryos. *J. Exp. Zool.* 232, 327–341. doi: 10.1002/jez.1402320220

Kobayashi, T., Lyons, K. M., McMahon, A. P., and Kronenberg, H. M. (2005). BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18023–18027. doi: 10.1073/pnas.0503617102

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., et al. (1997). Targeted DISruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89, 755–764. doi: 10.1016/S0092-8674(00)80258-5

Kramer, S. G., Kidd, T., Simpson, J. H., Goodman, C. S., Kramer, S. G., Kidd, T., et al. (2001). Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. *Science* 292, 737–740. doi: 10.1126/science.1058766

Kronenberg, H. M. (2006). PTHrP and Skeletal Development. *Ann. N. Y. Acad. Sci.* 1068, 1–13. doi: 10.1196/annals.1346.002

Kutchuk, L., Laitala, A., Soueid-Bomgarten, S., Shentzer, P., Rosendahl, A. H., Eilot, S., et al. (2015). Muscle composition is regulated by a Lox-TGFbeta feedback loop. *Development* 142, 983–993. doi: 10.1242/dev.113449

Lan, Y., Kingsley, P. D., Cho, E., and Jiang, R. (2001). Osr2, a new mouse gene related to Drosophila odd-skipped, exhibits dynamic expression patterns during craniofacial, limb, and kidney development. *Mech. Dev.* 107, 175–179. doi: 10.1016/S0925-4773(01)00457-9

Lance-Jones, C. (1988). The effect of somite manipulation on the development of motoneuron projection patterns in the embryonic chick hindlimb. *Dev. Biol.* 126, 408–419. doi: 10.1016/0012-1606(88)90150-9

Lee, K., Kim, H., Li, Q., Chi, X., Ueta, C., Komori, T., et al. (2000). Runx2 is a common target of transforming growth factor β 1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent Runx2 is a common target of transforming growth factor $_{NL}$ 1. *Mol. Cell. Biol.* 20, 8783–8792. doi: 10.1128/MCB.20.23.8783-8792.2000

Lefebvre, V., Behringer, R. R., and De Crombrugghe, B. (2001). L-Sox5, Sox6 and SOx9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis. Cartilage.* 9, 69–75. doi: 10.1053/joca.2001.0447

Lefebvre, V., Li, P., and De Crombrugghe, B. (1998). A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* 17, 5718–5733. doi: 10.1093/emboj/17.19.5718

Lejard, V., Blais, F., Guerquin, M. J., Bonnet, A., Bonnin, M. A., Havis, E., et al. (2011). EGR1 and EGR2 involvement in vertebrate tendon differentiation. *J. Biol. Chem.* 286, 5855–5867. doi: 10.1074/jbc.M110.153106

Li, X., and Cao, X. U. (2006). BMP Signaling and Skeletogenesis. *Ann. N. Y. Acad. Sci.* 40, 26–40. doi: 10.1196/annals.1346.006

Li, Y., Qiu, Q., Watson, S. S., Schweitzer, R., and Johnson, R. L. (2010). Uncoupling skeletal and connective tissue patterning: conditional deletion in cartilage progenitors reveals cell- autonomous requirements for Lmx1b in dorsal-ventral limb patterning. *Development* 137, 1181–1188. doi: 10.1242/dev.045237

Liu, H., Lan, Y., Xu, J., Chang, C., Brugmann, S. A., and Jiang, R. (2013). Odd-skipped related-1 controls neural crest chondrogenesis during tongue development. *Proc. Natl. Acad. Sci. U.S.A.* 110, 18555–18560. doi: 10.1073/pnas.1306495110

Liu, W., Watson, S. S., Lan, Y., Keene, D. R., Ovitt, C. E., Liu, H., et al. (2010). The Atypical homeodomain transcription factor mohawk controls tendon morphogenesis. *Mol. Cell. Biol.* 30, 4797–4807. doi: 10.1128/MCB.00207-10

Lu, J., Bassel-duby, R., Hawkins, A., Chang, P., Valdez, R., Wu, H., et al. (2002). Control of facial muscle development by MyoR and capsulin. *Science* 298, 2378–2381. doi: 10.1126/science.1078273

Mathew, S. J., Hansen, J. M., Merrell, A. J., Murphy, M. M., Lawson, J. A., Hutcheson, D. A., et al. (2011). Connective tissue fibroblasts and Tcf4 regulate myogenesis. *Development* 138, 371–384. doi: 10.1242/dev.057463

Merrell, A. J., Ellis, B. J., Fox, Z. D., Lawson, J. A., Jeffrey, A., and Kardon, G. (2015). Muscle connective tissue controls development of the diaphragm and is a source of congenital diaphragmatic hernias. *Nat. Genet.* 47, 496–504. doi: 10.1038/ng.3250

Mitchell, K. J., Pannerec, A., Cadot, B., Parlakian, A., Besson, V., Gomes, E. R., et al. (2010). Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. *Nat. Cell Biol.* 12, 257–266. doi: 10.1038/ncb2025

Mok, G. F., Cardenas, R., Anderton, H., Campbell, K. H. S., and Sweetman, D. (2014). Interactions between FGF18 and retinoic acid regulate differentiation of chick embryo limb myoblasts. *Dev. Biol.* 396, 214–223. doi: 10.1016/j.ydbio.2014.10.004

Motohashi, N., and Asakura, A. (2014). Muscle satellite cell heterogeneity and self-renewal. *Front. Cell Dev. Biol.* 2:1. doi: 10.3389/fcell.2014.00001

Murchison, N. D., Price, B. A., Conner, D. A., Keene, D. R., Olson, E. N., Tabin, C. J., et al. (2007). Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 134, 2697–2708. doi: 10.1242/dev.001933

Murphy, M. M., Lawson, J. A., Mathew, S. J., Hutcheson, D. A., and Kardon, G. (2011). Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development* 138, 3625–3637. doi: 10.1242/dev.064162

Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., et al. (2002). The novel zinc finger-containing transcription factor Osterix is required for Osteoblast differentiation and bone formation. *Cell* 108, 17–29. doi: 10.1016/S0092-8674(01)00622-5

Natarajan, A., Lemos, D. R., and Rossi, F. M. (2010). A double-edged sword in skeletal muscle regeneration Fibro / adipogenic progenitors. *Cell Cycle* 9, 2045–2046. doi: 10.4161/cc.9.11.11854

Noden, D. (1983). The embryonic origins of avian cephalic and cervical muscles associated connective tissues. *Am. J. Anat.* 168, 257–276. doi: 10.1002/aja.1001680302

Noden, D. M., and Francis-west, P. (2006). The differentiation and morphogenesis of craniofacial muscles. *Dev. Dyn.* 235, 1194–1218. doi: 10.1002/dvdy.20697

Noden, D. M., and Trainor, P. A. (2005). Relations and interactions between cranial mesoderm and neural crest populations. *J. Anat.* 207, 575–601. doi: 10.1111/j.1469-7580.2005.00473.x

Olson, L. E., and Soriano, P. (2009). Increased PDGFRa activation disrupts connective tissue development and drives systemic fibrosis. *Dev. Cell* 16, 303–313. doi: 10.1016/j.devcel.2008.12.003

Omelyanenko, N. P., and Slutsky, L. I. (2013). *Chapter 1: Peculiarities of Connective Tissue Histophysiology, Biochemistry and Molecular Biology,* ed S. P. Mironov (CRC Press; Taylor and Francis Group), 1–15.

Ordahl, C. P., and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. *Development* 114, 339–353.

Ordan, E., Brankatschk, M., Dickson, B., Schnorrer, F., and Volk, T. (2015). Slit cleavage is essential for producing an active, stable, non-diffusible short-range signal that guides muscle migration. *Development* 142, 1431–1436. doi: 10.1242/dev.119131

Pan, T. C., Zhang, R. Z., Arita, M., Bogdanovich, S., Adams, S. M., Gara, S. K., et al. (2014). A mouse model for dominant collagen VI disorders heterozygous deletion of Col6a3 EXON 16. *J. Biol. Chem.* 289, 10293–10307. doi: 10.1074/jbc.M114.549311

Pan, T. C., Zhang, R. Z., Markova, D., Arita, M., Zhang, Y., Bogdanovich, S., et al. (2013). COL6A3 protein deficiency in mice leads to muscle and tendon defects similar to human collagen VI congenital muscular dystrophy. *J. Biol. Chem.* 288, 14320–14331. doi: 10.1074/jbc.M112.433078

Pannérec, A., Formicola, L., Besson, V., Marazzi, G., and Sassoon, D. A. (2013). Defining skeletal muscle resident progenitors and their cell fate potentials. *Development* 140, 2879–2891. doi: 10.1242/dev.089326

Pearse, R., Esshaki, D., Tabin, C. J., and Murray, M. M. (2009). Genome-wide expression analysis of intra- and extraarticular connective tissue. *J. Orthop. Res.* 27, 427–434. doi: 10.1002/jor.20774

Pourquié, O., Fan, C., Coltey, M., Hirsinger, E., Watanabe, Y., Bre, C., et al. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* 84, 461–471. doi: 10.1016/S0092-8674(00)81291-X

Pryce, B. A., Watson, S. S., Murchison, N. D., Staverosky, J. A., Dünker, N., and Schweitzer, R. (2009). Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 136, 1351–1361. doi: 10.1242/dev.027342

Rackley, R. R., Kessler, P. M., Campbell, C., and Williams, B. R. G. (1995). *In-Situ* expression of the early growth-response Gene-1 during murine nephrogenesis. *J. Urol.* 154, 700–705. doi: 10.1016/S0022-5347(01)67136-2

Ratajczak, M., Majka, M., Kucia, M., Drukala, J., Pietrzkowski, Z., Peiper, S., et al. (2003). Expression of functional CXCR4 by muscle satellite cells and secretion of sdf-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic STEm/Progenitor cells in muscles. *Stem Cells* 21, 363–371. doi: 10.1634/stemcells.21-3-363

Relaix, F., Montarras, D., Zaffran, S., Gayraud-morel, B., Rocancourt, D., Tajbakhsh, S., et al. (2006). Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J. Cell Biol.* 172, 91–102. doi: 10.1083/jcb.200508044

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev.* 18, 1088–1105. doi: 10.1101/gad.301004

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* 435, 948–953. doi: 10.1038/nature03594

Rinon, A., Lazar, S., Marshall, H., Büchmann-Møller, S., Neufeld, A., Elhanany-Tamir, H., et al. (2007). Cranial neural crest cells regulate head muscle patterning and differentiation during vertebrate embryogenesis. *Development* 134, 3065–3075. doi: 10.1242/dev.002501

Sampaolesi, M., Blot, S., Antona, G. D., Granger, N., Tonlorenzi, R., Perani, L., et al. (2006). Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* 444, 574–579. doi: 10.1038/nature05282

Sampaolesi, M., Torrente, Y., Innocenzi, A., Tonlorenzi, R., D'antona, G., Pellegrino, M., et al. (2003). Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesangioblasts. *Science* 301, 487–492. doi: 10.1126/science.1082254

Scaal, M., Bonafede, A., Dathe, V., Sachs, M., Cann, G., Christ, B., et al. (1999). SF/HGF is a mediator between limb patterning and muscle development. *Development* 126, 4885–4893.

Schnorrer, F., Kalchhauser, I., and Dickson, B. J. (2007). The transmembrane protein Kon-tiki couples to Dgrip to mediate myotube targeting in Drosophila. *Dev. Cell* 12, 751–766. doi: 10.1016/j.devcel.2007.02.017

Schweitzer, R., Chyung, J. H., Murtaugh, L. C., Brent, A. E., Rosen, V., Olson, E. N., et al. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 128, 3855–3866.

Selleck, M. A. J., and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen ' s node in the chick embryo. *Development* 112, 615–626.

Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J. M., Behringer, R. R., et al. (2001). The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev. Cell* 1, 277–290. doi: 10.1016/S1534-5807(01)00003-X

So, P. L., and Danielian, P. S. (1999). Cloning and expression analysis of a mouse gene related to Drosophila odd-skipped. *Mech. Dev.* 84, 157–160. doi: 10.1016/S0925-4773(99)00058-1

Soler, C., Laddada, L., and Jagla, K. (2016). Coordinated development of muscles and tendon-like structures: early interactions in the Drosophila leg. *Front. Physiol.* 7:22. doi: 10.3389/fphys.2016.00022

Stockdale, F. (1992). Myogenic cell lineages. *Dev. Biol.* 154, 284–298. doi: 10.1016/0012-1606(92)90068-R

Stockdale, F., Nikovits, W., and Christ, B. (2000). Molecular and cellular biology of avian somite. *Dev. Dyn.* 321, 304–321. doi: 10.1002/1097-0177(2000)9999:9999<::AID-DVDY1057>3.0.CO;2-5

Stricker, S., Brieske, N., Haupt, J., and Mundlos, S. (2006). Comparative expression pattern of odd-skipped related genes Osr1 and Osr2 in chick embryonic development. *Gene Expr. Patterns* 6, 826–834. doi: 10.1016/j.modgep.2006.02.003

Stricker, S., Mathia, S., Haupt, J., Seemann, P., Meier, J., and Mundlos, S. (2012). Odd-skipped related genes regulate differentiation of embryonic limb mesenchyme and bone marrow mesenchymal stromal cells. *Stem Cells Dev.* 21, 623–633. doi: 10.1089/scd.2011.0154

Subramanian, A., and Schilling, T. F. (2014). Thrombospondin-4 controls matrix assembly during development and repair of myotendinous junctions. *Elife* 3:e02372. doi: 10.7554/eLife.02372

Sudo, K., Kanno, M., Miharada, K., Ogawa, S., Hiroyama, T., Saijo, K., et al. (2007). Mesenchymal progenitors able to differentiate into Osteogenic, Chondrogenic, and / or Adipogenic cells *in vitro* are present in most primary fibroblast-like cell populations. *Stem Cells* 1610–1617. doi: 10.1634/stemcells.2006-0504

Swartz, M. E., Eberhart, J., Pasquale, E. B., and Krull, C. E. (2001). EphA4/ephrin-A5 interactions in muscle precursor cell migration in the avian forelimb. *Development* 128, 4669–4680.

Swinehart, I. T., Schlientz, A. J., Quintanilla, C. A., Mortlock, D. P., and Wellik, D. M. (2013). Hox11 genes are required for regional patterning and integration of muscle, tendon and bone. *Development* 140, 4574–4582. doi: 10.1242/dev.096693

Tajbakhsh, S. (2009). Skeletal muscle stem cells in developmental versus regenerative myogenesis. *J. Intern. Med.* 266, 372–389. doi: 10.1111/j.1365-2796.2009.02158.x

Tajbakhsh, S., and Buckingham, M. (2000). The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr. Top. Dev. Biol.* 48, 225–268. doi: 10.1016/S0070-2153(08)60758-9

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis : Pax-3 and Myf-5 Act Upstream of MyoD. *Cell* 89, 127–138. doi: 10.1016/S0092-8674(00)80189-0

Takarada, T., Hinoi, E., Nakazato, R., Ochi, H., Xu, C., Tsuchikane, A., et al. (2013). An Analysis of skeletal development in osteoblast–transcription factor–2 (Runx2) knockout mice. *J. Bone Miner. Res.* 28, 2064–2069. doi: 10.1002/jbmr.1945

Tedesco, F. S., Dellavalle, A., Diaz-manera, J., Messina, G., and Cossu, G. (2010). Repairing skeletal muscle : regenerative potential of skeletal muscle stem cells. *J. Clin. Invest.* 120, 11–19. doi: 10.1172/JCI40373

Theis, S., Patel, K., Valasek, P., Otto, A., Pu, Q., Harel, I., et al. (2010). The occipital lateral plate mesoderm is a novel source for vertebrate neck musculature. *Development* 137, 2961–2971. doi: 10.1242/dev.049726

Tokita, M., and Schneider, R. (2009). Developmental origins of species-specific muscle pattern. *Dev. Biol.* 331, 311–325. doi: 10.1016/j.ydbio.2009.05.548

Tozer, S., and Duprez, D. (2005). Tendon and ligament: development, repair and disease. *Birth Defects Res. C Embryo Today Rev.* 75, 226–236. doi: 10.1002/bdrc.20049

Trainor, P. A., Tan, S., and Tam, P. P. L. (1994). Cranial paraxial mesoderm : regionalisation of cell fate and impact on craniofacial development in mouse embryos. *Development* 120, 2397–2408.

Tylzanowski, P., Mebis, L., and Luyten, F. P. (2006). The noggin null mouse phenotype is strain dependent and haploinsufficiency leads to skeletal defects. *Dev. Dyn.* 235, 1599–1607. doi: 10.1002/dvdy.20782

Tzahor, E. (2009). Heart and craniofacial muscle development: a new developmental theme of distinct myogenic fields. *Dev. Biol.* 327, 273–279. doi: 10.1016/j.ydbio.2008.12.035

Tzahor, E., Mootoosamy, R. C., Poon, A. C., Abzhanov, A., Tabin, C. J., Dietrich, S., et al. (2003). Antagonists of Wnt and BMP signaling promote the formation of vertebrate head muscle. *Genes Dev.* 17, 3087–3099. doi: 10.1101/gad.1154103

Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S., and Tsuchida, K. (2010). Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat. Cell Biol.* 12, 143–152. doi: 10.1038/ncb2014

Uezumi, A., Ikemoto-Uezumi, M., and Tsuchida, K. (2014). Roles of nonmyogenic mesenchymal progenitors in pathogenesis and regeneration of skeletal muscle. *Front. Physiol.* 5:68. doi: 10.3389/fphys.2014.00068

Uezumi, A., Ito, T., Morikawa, D., Shimizu, N., and Yoneda, T. (2011). Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J. Cell Sci.* 124, 3654–3664. doi: 10.1242/jcs.086629

Urciuolo, A., Quarta, M., Morbidoni, V., Gattazzo, F., Molon, S., Grumati, P., et al. (2013). Collagen VI regulates satellite cell self-renewal and muscle regeneration. *Nat. Commun.* 4:1964. doi: 10.1038/ncomms2964

Vasyutina, E., Stebler, J., Brand-Saberi, B., Schulz, S., Raz, E., and Birchmeier, C. (2005). CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. *Genes Dev.* 19, 2187–2198. doi: 10.1101/gad.346205

Volohonsky, G., Edenfeld, G., Klämbt, C., and Volk, T. (2007). Muscle-dependent maturation of tendon cells is induced by post-transcriptional regulation of stripeA. *Development* 134, 347–356. doi: 10.1242/dev.02735

von Maltzahn, J., Jones, A. E., Parks, R. J., and Rudnicki, M. A. (2013). Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16474–16479. doi: 10.1073/pnas.1307680110

Vortkamp, A., Lee, K., Lanske, B., Segre, G., Kronenberg, H., and Tabin, C. (1996). Regulation of rate of cartilage by Indian hedgehog and PTH-related protein. *Science* 273, 613–622. doi: 10.1126/science.273.5275.613

Wachtler, F., Christ, B., and Jacob, H. J. (1981). On the determination of mesodermal tissues in the avian embryonic wing bud. *Anat. Embryol.* 161, 283–289. doi: 10.1007/BF00301826

Wayburn, B., and Volk, T. (2009). LRT, a tendon-specific leucine-rich repeat protein, promotes muscle-tendon targeting through its interaction with Robo. *Development* 136, 3607–3615. doi: 10.1242/dev.040329

Weintraub, H., Dwarki, V. J., Verma, I., Davis, R., Hollenberg, S., Snider, L., et al. (1991). Muscle-specific transcriptional activation by MyoD. *Genes Dev.* 5, 1377–1386. doi: 10.1101/gad.5.8.1377

Wong, K., Park, H. T., Wu, J. Y., and Rao, Y. (2002). Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr. Opin. Genet. Dev.* 12, 583–591. doi: 10.1016/S0959-437X(02)00343-X

Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., et al. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* 18, 952–963. doi: 10.1101/gad.1174704

Zacharias, A. L., Lewandoski, M., Rudnicki, M. A., and Gage, P. J. (2011). Pitx2 is an upstream activator of extraocular myogenesis and survival. *Dev. Biol.* 349, 395–405. doi: 10.1016/j.ydbio.2010.10.028

Zhang, D., Schwarz, E. M., Rosier, R. N., Zuscik, M. J., Puzas, J. E., and Keefe, R. J. O. (2003). ALK2 functions as a BMP type I receptor and induces Indian hedgehog. *J. Bone Miner. Res.* 18, 1593–1604. doi: 10.1359/jbmr.2003.18.9.1593

Zhang, G., Young, B. B., Ezura, Y., Favata, M., Soslowsky, L. J., Chakravarti, S., et al. (2005). Development of tendon structure and function: regulation of collagen fibrillogenesis. *J. Musculoskelet. Neuronal Interact.* 5, 5–21.

Zhao, Q., Eberspaecher, H., Lefebvre, V., and De Crombrugghe, B. (1997). Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev. Dyn.* 209, 377–386. doi: 10.1002/(SICI)1097-0177(199708)209:4<377::AID-AJA5>3.0.CO;2-F

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 01 May 2017
doi: 10.3389/fcell.2017.00046

Pitx2 in Embryonic and Adult Myogenesis

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Skeletal muscle is a heterogeneous tissue that represents between 30 and 38% of the human body mass and has important functions in the organism, such as maintaining posture, locomotor impulse, or pulmonary ventilation. The genesis of skeletal muscle during embryonic development is a process controlled by an elaborate regulatory network combining the interplay of extrinsic and intrinsic regulatory mechanisms that transform myogenic precursor cells into functional muscle fibers through a finely tuned differentiation program. However, the capacity of generating muscle still remains once these fibers have matured. Adult myogenesis resembles many of the embryonic morphogenetic episodes and depends on the activation of satellite cells that have the potential to differentiate into new muscle fibers. *Pitx2* is a member of the *bicoid* family of homeodomain transcription factors that play an important role in morphogenesis. In the last decade, *Pitx2* has emerged as a key element involved in the fine-tuning mechanism that regulates skeletal-muscle development as well as the differentiation and cell fate of satellite cells in adult muscle. Here we present an integrative view of all aspects of embryonic and adult myogenesis in which *Pitx2* is involved, from embryonic development to satellite-cell proliferation, fate specification, and differentiation. Those new *Pitx2* functions on satellite-cell biology might open new perspectives to develop therapeutic strategies for muscular disorders.

Keywords: *Pitx2*, myogenic precursor cells, embryonic myogenesis, adult myogenesis, satellite cell and regeneration

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Consejo Superior de Investigaciones Científicas, Spain

Reviewed by:
Solveig Thorsteinsdottir, Universidade de Lisboa, Portugal
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Thimios Mitsiadis, University of Zurich, Switzerland

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *31 January 2017*
Accepted: *12 April 2017*
Published: *01 May 2017*

Citation:
Hernandez-Torres F, Rodríguez-Outeiriño L, Franco D and Aranega AE (2017) Pitx2 in Embryonic and Adult Myogenesis. Front. Cell Dev. Biol. 5:46. doi: 10.3389/fcell.2017.00046

INTRODUCTION

Skeletal muscle is a heterogeneous tissue that represents between 30 and 38% of the human body mass (Janssen et al., 2000). It is composed of individual muscle fibers, diversified in size, shape, and contractile protein content, to fulfill the different functional needs of the vertebrate body such as maintaining body posture, locomotor impulse, or pulmonary ventilation. The genesis of skeletal muscle during embryonic development and postnatal life is a process controlled by an extremely elaborate regulatory network that combines the interplay of extrinsic (e.g., morphogens, neurohormonal input, muscle damage, etc.) and intrinsic elements (gene regulatory elements). The intrinsic elements form hierarchical interactions between transcriptional regulators, regulatory RNAs, and chromatin-remodeling factors. In this sense, during embryogenesis, muscle progenitors are specified by the sequential expression of a network of transcription factors composed of PAX3 and PAX7, and the basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs) MYOD,

MYF5, MYF6 (also called MFR4), and MYOG (Bentzinger et al., 2012; Moncaut et al., 2013). In addition, during adult life the skeletal muscle has the ability to resume developmental mechanisms that compensate for the physiological turnover and damage in order to maintain tissue homeostasis (Schmalbruch and Lewis, 2000; Pellettieri and Alvarado, 2007). This adult myogenesis depends on the activation of satellite cells (SCs), that have the potential to proliferate, differentiate, and generate new fibers, or repair existing ones (Chargé and Rudnicki, 2004). It has been well-established that SCs are closely related to progenitors of embryonic origin (Gros et al., 2005; Relaix et al., 2005; Schienda et al., 2006; Hutcheson et al., 2009; Lepper and Fan, 2010). Thus, many similarities have been discovered between prenatal myogenesis and regeneration in the mature skeletal musculature, such as common transcription factors and signaling molecules (Tajbakhsh, 2009).

During the last two decades the homeobox transcription factor *Pitx2* has emerged as a key element in the fine-tuning mechanism that regulates skeletal-muscle development. Concurrently, several recent experimental pieces of evidence point to the role of *Pitx2* in SC biology. Here, we present an integrative view of the role of *Pitx2* in prenatal and adult myogenesis (from embryonic development to SC proliferation), fate specification, and differentiation. Finally we discuss the potential therapeutic use of *Pitx2* in the future.

PRENATAL AND ADULT MYOGENESIS

In vertebrates, skeletal-muscle development is a biphasic process. A primary (embryonic) myogenesis takes place to generate primary muscle fibers, between embryonic day (E) 9.5 and E14.5 in the mouse. This is followed during fetal stages by a secondary myogenesis which gives rise to the bulk of skeletal-muscle fibers present at birth (Kelly and Zacks, 1969; Biressi et al., 2007; Tajbakhsh, 2009; Deries and Thorsteinsdóttir, 2016). All skeletal-muscle cells have the same underlying functions, although their progenitors within the paraxial mesoderm are spread throughout the embryo at the onset of myogenesis. This bears emphasizing since the genetic networks that control myogenesis present differences depending on the location of those myogenic precursors in the embryo.

Embryonic Myogenesis: The Trunk and Limb Muscles

The muscles of the trunk and limbs derive from somites (**Figure 1A**), which are transient paraxial mesodermal structures that form pairwise on either side of the neural tube, following an anterior-posterior developmental gradient. The somite is initially a spherical unit of polarized epitheloid cells that soon after subdivides into two compartments, the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome. Shortly afterwards, myogenic precursor cells from the epaxial and hypaxial lips of the dermomyotome undergo an epithelial-mesenchymal transition (EMT) and accumulate underneath, where they differentiate and elongate to form the myocytes of the myotome, the first myogenic structure to develop in the body (Buckingham and Relaix, 2015; Deries and Thorsteinsdóttir, 2016). The epaxial region of the myotome gives rise to the deep back muscles, whereas the hypaxial myotome is the source of body wall muscles and most other trunk muscles (Buckingham and Relaix, 2015; Deries and Thorsteinsdóttir, 2016). In segments adjacent to the limb-region cells of the hypaxial dermomyotome undergo an EMT, leave the epithelial structure, and migrate toward the fore and hind limbs to form dorsal and ventral muscle masses in the limb-bud mesenchyme, where they begin to differentiate and express muscle-specific genes (Biressi et al., 2007; Deries and Thorsteinsdóttir, 2016).

Cell commitment in the somite is highly dependent on a number of transcription factors which act in a hierarchical molecular cascade to orchestrate the specification, determination, and differentiation of myogenic precursors. In the genetic hierarchy that regulates the onset of trunk myogenesis, *Pax3* and *Myf5* play a dominant role (Buckingham and Relaix, 2015). *Pax3* is already transcribed in pre-somitic mesoderm adjacent to the first somite (Schubert et al., 2001) and then throughout the newly formed somites. As somites mature *Pax3* expression becomes confined to the dermomyotome (Goulding et al., 1991) and persists in myogenic progenitor cells that delaminate and migrate from the somite to more distant sites of myogenesis such as the limb (Buckingham and Relaix, 2015). Myogenic cells that have activated the myogenic determination genes *Myf5*/*Myf6* and *MyoD* downregulate *Pax3* and delaminate from the edges of the dermomyotome (Buckingham and Relaix, 2015). The epaxial myotome then start to form. This depends on the early epaxial activation of *Myf5*, which is driven by *Wnt* and *Shh* signaling, without any *Pax3* and/or *Pax7* requirement (Borello et al., 2006; Buckingham and Relaix, 2015). These cells do not activate *MyoD* but rather *Myog* and differentiate (Kablar et al., 2003). On the other hand, the activation of *Myf5* in the hypaxial somite as well as in the limb depends on PAX3 (Bajard et al., 2006; Buckingham and Relaix, 2015). At this stage MYF6 also acts as a myogenic determination factor (Kassar-Duchossoy et al., 2004). The *Myod* gene is activated after the onset of *Myf5* expression in the rest of the dermomyotome and limbs (Hu et al., 2008). Finally, the transcription factor MYOG is required for the onset of the expression of terminal differentiation genes needed for the fusion of myocytes and the formation of myotubes (Bentzinger et al., 2012).

Embryonic Myogenesis: The Head Muscles

Although, all skeletal muscle throughout the body originates within paraxial mesoderm, in the head, identifiable compartments such as the somites in the trunk are not evident histologically or by most molecular criteria. This unsegmented head mesoderm is remodeled at the early stages of embryonic development (**Figures 1B,C**). The unsegmented head mesoderm gives rise to all craniofacial skeletal muscles, which can be cataloged as four distinct populations: extra-ocular (EOMs), branchial, laryngoglossal, and axial neck muscles (Noden and Francis-West, 2006; Tzahor, 2015). EOMs are formed by cells from the cranial paraxial mesoderm that migrate through the first branchial arch (FBA) as well as from the prechordal mesoderm (Jacob et al., 1984; Evans and Noden, 2006; Tzahor,

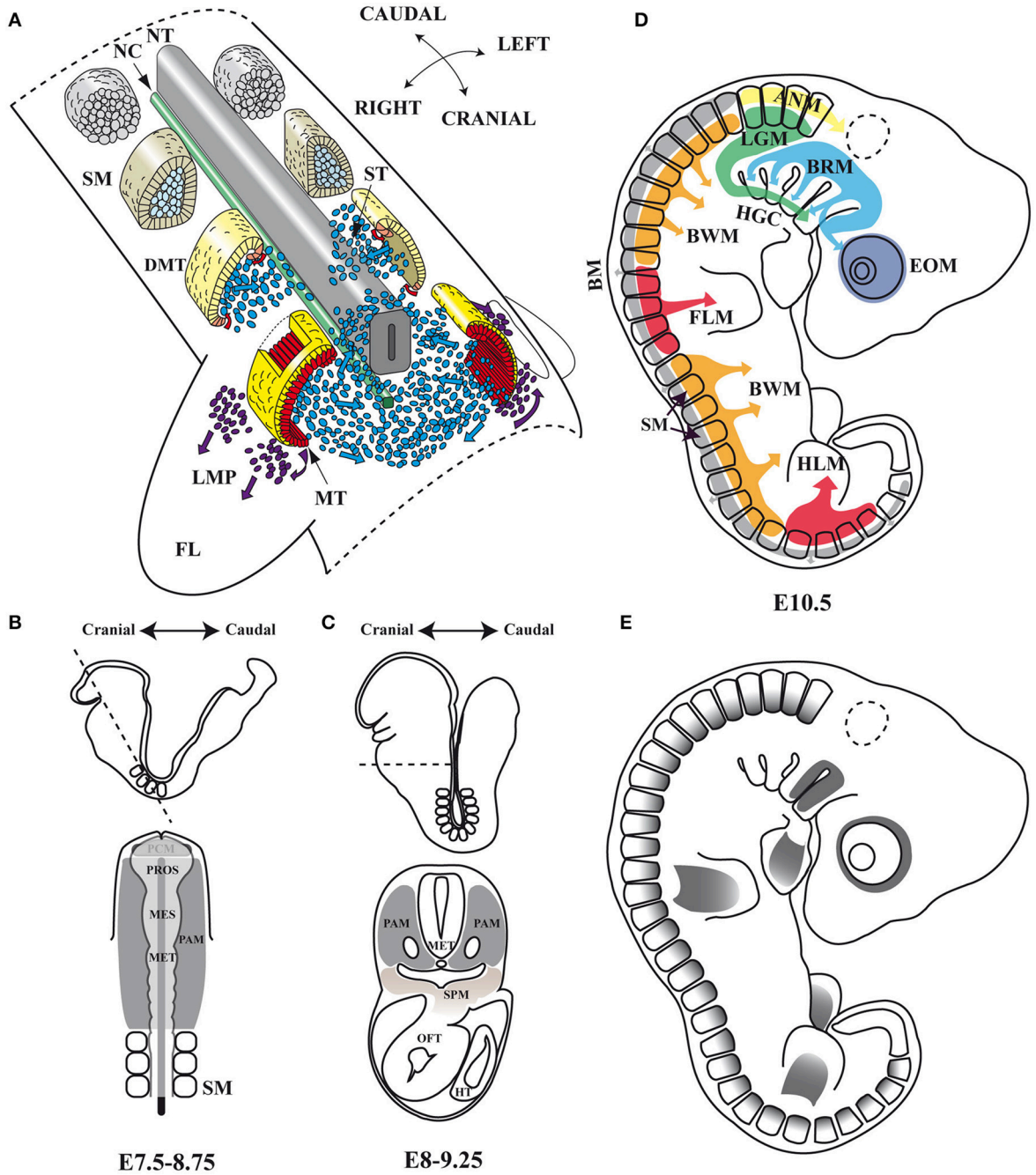

FIGURE 1 | Embryonic myogenesis (A) Schematic representation of somite maturation. Somites mature following an anterior to posterior developmental gradient (Modified from *Gray's Anatomy*. The Anatomical Basis of Clinical Practice, 40th Edition Standring, 2008): myogenic precursor cells arise from the epaxial and hypaxial lips of the dermomyotome after archive epithelial-mesenchymal transition (EMT) and migrate toward the limbs to form dorsal and ventral muscle masses where they begin to differentiate. **(B,C)** Head frontal and transverse planes of a mouse embryo between stages of development E7.5–8.75 and E8–9.25 in mouse. At an open neural plate stage, head mesoderm in a frontal plane includes the prechordal mesoderm and the paraxial mesoderm. When the neural tube closes dorsally and the endoderm ventrally, the prechordal mesoderm is integrated within the remaining paraxial mesoderm, which is located anterior to the somites. Dashed line illustrates the cutting plane. **(D)** Origins of skeletal muscles: Myogenic precursors arise from different paraxial mesoderm compartments. **(E)** *Pitx2* expression domains at the E10.5 stage of development in mouse. NT, neural tube; NC, notochord; SM, somites; DMT, dermomyotome; ST, sclerotome; MT, myotome; LMP, limb muscle precursors; FL, forelimb; PAM, head paraxial mesoderm; PCM, prechordal mesoderm; PROS, prosencephalon; MES, mesencephalon; MET, metencephalon; SPM, splanchnic mesoderm; OFT, outflow tract of heart; HT, heart tube; EOM, extra-ocular muscles; BRM, branchial muscles; LGM, laryngoglossal muscles; HGC, hypoglossal cord; ANM, axial neck muscles; BM, back muscles; BWM, body wall muscles; FLM, forelimbs muscles; HLM, hind limbs muscles.

2015; **Figure 1D**). Branchial arch muscles are formed mainly by migrating cells from the cranial paraxial mesoderm and the lateral splanchnic mesoderm (Harel et al., 2009; Sambasivan et al., 2009; Tzahor, 2015). Laryngoglossal muscles develop from migratory myoblasts arising from occipital somites that form a condensed mesenchymal band, the hypoglossal cord, which elongates and similarly brings myoblasts ventral to pharynx (Hammond, 1965; Hazelton, 1970; Tzahor, 2015). Finally, in the transition zone between the head and the trunk are the axial neck muscles. They arise from medio-dorsal and latero-ventral domains of occipital and cervical somites (Noden, 1983; Couly et al., 1992; Matsuoka et al., 2005).

The genetic hierarchy governing primary myogenesis in the trunk does not appear to operate for head-muscle formation. Activation of the myogenic program in the head therefore depends on different upstream factors, responds differently to signaling pathways and also displays site-dependent regulation. Branchial-arch-derived muscles depend on *Myf5/Myf6/Myod*, whereas extra-ocular muscle formation is initiated by *Myf5/Myf6* and in their absence cannot be restored by *Myod* (Tajbakhsh and Buckingham, 1999).

Fetal Myogenesis

During fetal myogenesis, secondary fibers in trunk, limbs, and head are generated by the fusion of fetal myoblasts. Secondary fibers form initially at the site of innervation of the primary fiber and are surrounded by the same basal lamina as the primary fiber on which they lie (Duxson et al., 1989). The secondary myotubes remain attached for a short period to primary fibers and subsequently elongate and become independent fibers, which can be distinguished from primary fibers by their relative small size (Kelly and Zacks, 1969). Although, the genetic networks that rule this second stage of prenatal myogenesis is less understood, it is known that the MRFs MYF5, MYOD, and MYOG are also crucial, since in $Myog^{-/-}$ as well as $Myf5^{-/-}$:$MyoD^{-/-}$ double-mutant secondary myogenesis is completely inhibited (Venuti et al., 1995; Kassar-Duchossoy et al., 2004).

Adult Myogenesis

The regulatory inputs that orchestrate myogenesis during prenatal myogenesis are partially reactivated in adult muscle repair. In adulthood, the maintenance as well as the repair of muscle tissue are both directed mainly by SCs. These cells, originally identified via electron microscopy in 1961 by Alexander Mauro, are located underneath the basal lamina and adjacent to the plasma membrane of the skeletal-muscle myofiber (Mauro, 1961; **Figure 2**). In their quiescent state, SC express the transcription factor *Pax7* and represent a genuine stem-cell population indispensable for skeletal-muscle repair (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011; Miersch et al.,

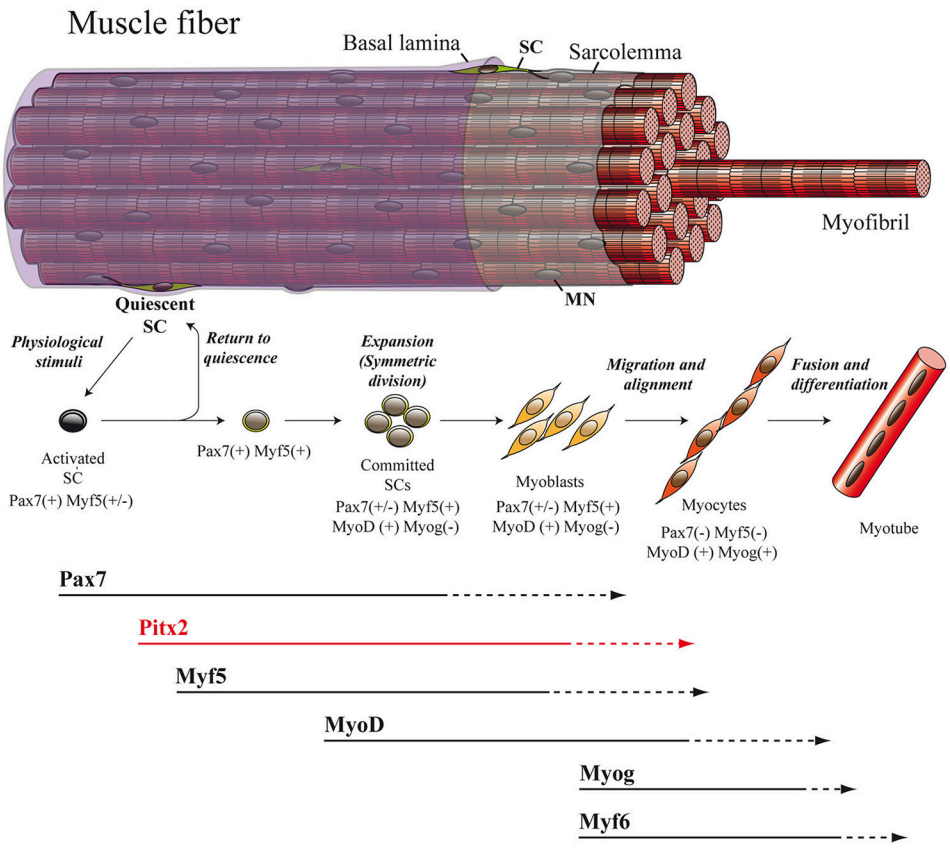

FIGURE 2 | Adult myogenesis. The overall myogenic differentiation pathway includes the activation of quiescent SCs, commitment to differentiation, proliferation, fusion to form myotubes and ultimately maturation into myofibers. SC, satellite cell; MN, myonucleus.

2017; Stuelsatz et al., 2017). It has been established that SCs in adult muscle represent a lineage continuum of the embryonic myogenic progenitor cells. Thus, while SCs of the body and limbs arise from somites, in common with the muscle that they are associated with (Armand et al., 1983; Gros et al., 2005; Relaix et al., 2005; Schienda et al., 2006), the SCs located in head muscles also originate from the cranial mesoderm (Harel et al., 2009). Within a context of physiological stimuli (physical exercise or pathological conditions) SCs become activated, proliferate, differentiate and fuse to form multinucleated myofibers in order to undergo proper myogenesis (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011; Miersch et al., 2017; Stuelsatz et al., 2017; **Figure 2**). In this regard, numerous studies have revealed a striking similarity between adult and embryonic myogenesis, where the core regulatory network composed of the MRFs MYF5, MYOD, MYOG, and MYF6 is mainly required (Bentzinger et al., 2012; Segalés et al., 2016; **Figure 2**).

THE *PITX2* GENE

The *Pitx* gene family includes three vertebrate paralogues, *Pitx1, Pitx2,* and *Pitx3,* which have been cloned in multiple organisms (Gage et al., 1999b; Knopp et al., 2013). These three genes encode transcription factors that belong to the *bicoid*-related subclass of homeodomain proteins (Gage et al., 1999b) The members of this family share an almost identical protein sequence within their homeodomains, varying mainly in the N-terminal region (Gage et al., 1999b; Knopp et al., 2013). Mutations or misregulation of *Pitx1, Pitx2,* and *Pitx3* result in developmental disorders in humans, such as Facioscapulohumeral Muscular Dystrophy (FSHD; Dixit et al., 2007), Axenfeld-Rieger syndrome (Semina et al., 1996), and Anterior Segment Mesenchymal Dysgenesis (ASMD; Semina et al., 1998), respectively. Muscle expression of these genes during development has been systematically studied. Thus, *Pitx1* is highly expressed in developing hind-limb-bud mesenchyme and is shown to determine hind-limb identity in mice (Lanctôt et al., 1999; Szeto et al., 1999), chicks (Logan and Tabin, 1999), and fish (Shapiro et al., 2004). On the other hand, *Pitx3* is widely expressed in all skeletal muscles of the head, trunk and limbs (Semina et al., 1998; L'honoré et al., 2007). Curiously, despite its apparent importance in muscle development, the investigation of $Pitx3^{-/-}$ mice indicates that *Pitx3* on its own is not required for myogenesis (L'honoré et al., 2007). In this scenario *Pitx2,* the third *Pitx* family member is strongly upregulated and appears to fully compensate for the loss of *Pitx3* during muscle formation (L'honoré et al., 2007). *Pitx2* is also able to control the growth ability of hind-limb mesenchyme together with *Pitx1* (Marcil et al., 2003), indicating the importance of *Pitx2* in the control of skeletal myogenesis during development.

In mice, the *Pitx2* (*Pituitary homeobox 2 or Paired-like homeodomain transcription factor 2*) gene is mapped on chromosome 3 (3G3; 3 57.84 cM) (Gage and Camper, 1997) and is transcribed into three distinct isoforms: *Pitx2a, Pitx2b*, and *Pitx2c*. *Pitx2a* and *Pitx2b* share the same promoter while *Pitx2c* uses an alternative one upstream of exon 4 (Schweickert et al., 2000). In human, *PITX2* is mapped on chromosome 4 (4q25) and maintains a similar genetic structure, but presents a fourth isoform (Arakawa et al., 1998; Cox et al., 2002). This fourth isoform is generated by the *PITX2C* alternative promoter and differential splicing, being able to suppress the transcriptional activity of the other *PITX2* isoforms (Cox et al., 2002). All *Pitx2* isoforms share a K50 DNA-binding homeodomain which binds to the consensus sequence TAATCC (Amendt et al., 1998; Chaney et al., 2005), thus being able to induce a transcriptional activation of *Prl* (Amendt et al., 1998) or *Anf* (Ganga et al., 2003) promoters. The *Pitx2* gene was isolated independently by several research groups and designated as *Otlx2* (Muccielli et al., 1996), *Rieg* (Semina et al., 1996), *Ptx2* (Gage and Camper, 1997), *Brx1* (Kitamura et al., 1997), and *Arp1* (Arakawa et al., 1998). Although, most of these works focused on the role of this gene in the development of brain structures, the authors reported the expression of *Pitx2* in the mesenchyme of the eye, the first and second branchial arches, the fore and hind limbs as well as the dermomyotome at somite stages E8.5 and 10.5 in mouse, and its equivalent stages in chicken (**Figure 1E**). Soon afterwards, a role for *Pitx2* was also described in left-right asymmetry, being proposed as the molecular transducer of embryonic left-right signaling during early developmental stages at the level of organs such as heart, gut, and/or stomach (Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999).

PITX2 WITHIN THE GENETIC HIERARCHIES THAT CONTROL MUSCLE DEVELOPMENT

Pitx2 Function during Embryonic Myogenesis

Pitx2 in Trunk and Limb Muscle Development

The first evidence involving *Pitx2* in the molecular process controlling myogenesis was provided by Kitamura et al. (1999). These authors reported *Pitx2* expression co-localizing in dermomyotomes with *Pax3*, a muscle specification marker playing a key role in delamination and migration of the somitic muscle progenitor cells to the limb buds (Goulding et al., 1994; Tajbakhsh et al., 1997). Later, Marcil et al. demonstrated the presence of PITX2 protein in the myoblasts of the limb bud, displaying an expression pattern similar to that of PAX3 and MYOD (Marcil et al., 2003). All these data suggested that PITX2 was involved in muscle patterning. A more detailed temporal and spatial analysis during initial muscle-cell-cluster formation, by using *lacZ* expression from a *Pitx2* gene insertion, revealed the presence of a *Pitx2*-expressing cell cluster lateral to the dermomyotome (Shih et al., 2007b). This cluster first appeared at the forelimb level at E10.25. After E10.5, Pitx2(+/LacZ)-expressing cells were then also detected on sections of the limbs. Curiously, *Pax3* and the muscle-regulatory factors (MRFs) stained only subsets of $Pitx2^{+}$ cells within these areas, and virtually all $Pitx2^{+}$ cells in these areas express at least one of these known myogenic markers (Shih et al., 2007b). These observations led the authors to conclude that *Pitx2* marks the muscle

lineage more completely than any of the known markers does. In agreement with the interpretation that muscle progenitors express *Pitx2*, L'Honoré et al. (2007) found extensive co-labeling of myotome- and dermomyotome-proliferating cells with PITX2, PAX3, and with PAX7. Notably, they also observed PAX3-positive cells that have completed migration at the proximal limb bud also express PITX2 while not all PITX2-positive cells expressed PAX3. All these data suggest that *Pitx2* might be a player within the molecular pathways controlling muscle-progenitor fate.

Sometime afterwards, additional information regarding the hierarchical position occupied by *Pitx2* within the genetic cascade that control somite-derived myogenesis was inferred by using $Pitx2^{-/-}$, $Myf5^{nlacZ/nlacZ}$ and $Pitx2^{-/-};Myf5^{nlacZ/nlacZ}$ double-mutant mice (L'honoré et al., 2010). In this work, the authors showed that PITX2 protein directly regulates *Myod* expression through binding to its core enhancer in wild-type limbs. In agreement, the authors described a delayed myogenic differentiation and a *Myod* down regulation in $Pitx2^{-/-}$ limb buds and proposed that this phenotype appears to be due to the failure to activate the *Myod* core enhancer. However, although the inactivation of *Myf5* and *Myf6 in* $Myf5^{nlacZ/nlacZ}$ mutant embryos (*Myf6* is inactivated in *cis* in this mutant; Tajbakhsh et al., 1997) did not affect *Myod* expression in limb buds, this inactivation in a $Pitx2^{-/-}$ background ($Pitx2^{-/-};Myf5^{nlacZ/nlacZ}$) induced a synergic effect that resulted not in a downregulation but in almost a complete loss of *Myod* expression compared with $Pitx2^{-/-};Myf5^{nlacZ/+}$ embryos, where the presence of one active *Myf5* allele prevented *Myod* loss in about 60% of myogenic precursors cells. These results imply that *Myf5* and/or *Myf6* cooperate with *Pitx2* to control *Myod* expression during early limb-bud myogenesis (**Figure 3A2**). In contrast to limb-muscle cells, myotome expression of *Myod* was not delayed in $Pitx2^{-/-}$ embryos. Nevertheless, in $Myf5^{nlacZ/nlacZ}$ mutant embryos, *Myod* expression was delayed by ∼2 days. Therefore, the onset of *Myod* expression in the myotome does not appear to depend on PITX2 but mostly on MYF5/MYF6. Nonetheless the inactivation of *Myf5* and/or *Myf6* in a $Pitx2^{-/-}$ background ($Pitx2^{-/-};Myf5^{nlacZ/nlacZ}$) led to an almost complete loss of *Myod* expression in myotome, as happened in limbs (L'honoré et al., 2010). These results indicate that MYF5 and/or MYF6 also cooperate with PITX2 to control *Myod* expression during myotome development (**Figure 3A1**).

In addition, it should be stressed that the analysis of *Pax3* mutant *Splotch* mice revealed a deficit of *Pitx2* expression restricted to the myotome (L'honoré et al., 2010). This deficit is not observed in neighboring mesenchyme, indicating that *Pitx2* is downstream of *Pax3* during myotome myogenesis. This is also supported by transcriptome analyses of $Pax3^{GFP/+}$ *and* $Pax3^{GFP/PAX3-FKHR}$ transgenic mice carried out by Lagha et al. (2010) since, in gain-of-function screens for PAX3 targets, they found an up-regulation of *Pitx2* in somites but not in limb buds. Although, all these seminal works suggest that *Pitx2* could be acting downstream of *Pax3* and in parallel with *Myf5*, at least in the myotome, as noted above, not all PITX2-expressing cells were positive for PAX3, and limb expression of *Pitx2* precedes *Myf5* (L'honoré et al., 2010). Therefore, additional studies using conditional *Pitx2* inactivation in specific myogenic cell populations would help to elucidate the function of Pitx2 in embryonic myogenesis.

Scientific evidence also relates *Pitx2* to cell proliferation in myogenic cells and somite derivates. Notably, *Pitx2* has been reported to be a target gene in the *Wnt/Dvl2/beta-catenin* pathway and operates in specific cell types to control proliferation by regulating expression of the growth-control genes *Ccnd1*, *Ccnd2*, and *c-Myc* (Kioussi et al., 2002; Baek et al., 2003). These authors established that the PITX2 N-terminal domain is required for its effects on proliferation in a myoblast cell line. We have previously demonstrated that *Pitx2c* is the main *Pitx2*-isoform expressed in Sol8 myoblasts and that overexpression of *Pitx2c* in Sol8 cells led to an increase in proliferative capacity and completely blocked terminal differentiation, mainly because high levels of *Pax3* expression were maintained (Martínez-Fernández et al., 2006). Additional data *in vivo* have supported the role of *Pitx2* in cell proliferation during myogenesis. In this sense, Abu-Elmagd et al. (2010) showed that *Pitx2* loss of function in chicken embryos decreased the number of differentiated myocytes/myofibers in the somites, whereas *Pitx2* overexpression increased myocyte/myofiber numbers, particularly in the epaxial region of the myotome. In agreement with Abu-Elmagd et al. and by using $Pitx2c^{-/-}$ mutant embryos, we have reported that *Pitx2c* plays a pivotal role in the control of the subtle equilibrium between proliferation and differentiation during trunk and limb myogenesis. This control is exercised by balancing *Pax3+/Pax7+* myogenic population *in vivo* as well as regulating key myogenic transcription factors such as *Pax3* through the repression of *miR-27* (Lozano-Velasco et al., 2011; **Figures 3A1,A2**). This new function of *Pitx2c* mediated by miRNAs introduces a new level of complexity in the intricate regulatory network that governs myogenesis in the embryo.

Pitx2 during Head-Muscle Development

As mentioned above, *Pax3* controls the myogenic specification of muscle embryonic progenitors in trunk and limbs (Tajbakhsh et al., 1997). However, it has been proposed that, instead of *Pax3, Pitx2* plays a major role as an upstream regulator of craniofacial myogenesis (Zacharias et al., 2011; Buckingham and Rigby, 2014). This is supported by the fact that EOM development is impaired in *Pitx2* null mice (Gage et al., 1999a; Kitamura et al., 1999). However, in the early studies it was not evident whether this muscle dysgenesis in *Pitx2* mutant mice resulted from an intrinsic defect in the developing myoblasts or was secondary to the loss of *Pitx2* expression in the periocular mesenchyme. Other authors have subsequently suggested that this phenotype could be due to the *Pitx2* effect on proliferation rate of myogenic precursors (Noden and Francis-West, 2006), in agreement with previously reported data (Kioussi et al., 2002; Martínez-Fernández et al., 2006). The hypothesis that *Pitx2* plays a part in controlling cell proliferation in myogenic cells in this context is also supported by the fact that conditional inactivation of *Pitx2* in neural-crest-derived cells does not affect the early differentiation of eye muscles (Evans and Gage, 2005), while conditional *Pitx2* deletion in the mesoderm induces a down-regulation of *Myf5*, *Myf6*, *Myod1*, and *Myog* expression and, therefore, blocks the onset of myogenesis of EOM (Zacharias

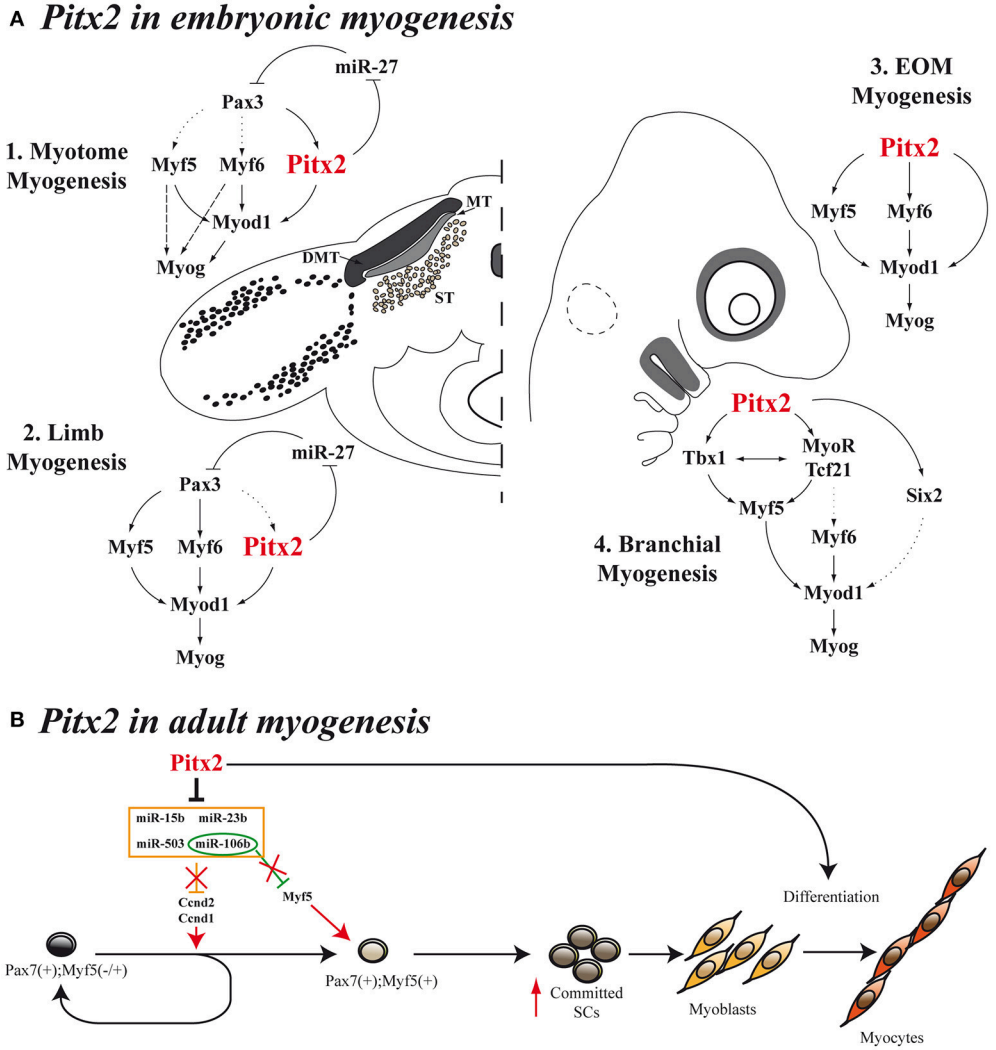

FIGURE 3 | Models for *Pitx2* functions in myogenesis. (A) During embryonic stages, *Pitx2* contribution is different depending on the initial muscle-cell clusters [myotome myogenesis **(A1)**, limb myogenesis **(A2)**, EOM myogenesis **(A3)**, or branchial myogenesis **(A4)**]. First myocytes of the myotome differentiate through *Myf5* and/or *Myf6* directly to *Myog* without turning on MyoD. This is represented by dashed arrows. Dotted arrows represent direct molecular relationships that still remain elusive **(B)** Proposed model for *Pitx2* in adult myogenesis promoting activation and commitment of SCs.

et al., 2011). In this regard, in 2009, Sambasivan et al. by analyzing double defective mutant mouse embryos *Myf5*(*Myf6*) (*Myf5*$^{nlacZ/+}$, *Myf5*$^{nlacZ/nlacZ}$) and *Myf4*$^{-/-}$ mutants, showed that *Pitx2* cannot ensure survival and activation of *Myod* expression in EOM in the absence of both *Myf5* and *Myf6* (Sambasivan et al., 2009). Shortly afterwards, Zacharias et al. were able to inactivate the expression of *Pitx2* in mesodermal EOM precursors by using a tamoxifen inducible *UBC- CreER*^{T2} promoter (Zacharias et al., 2011). This inactivation clearly showed that *Pitx2* is required for EOM precursor specification and survival, acting as an anti-apoptotic factor in the pre-myogenic mesoderm and subsequently activating the myogenic program in these cells through direct binding to *Myf5* and *Myod* promoters (Zacharias et al., 2011). Taken together, all these data clearly suggest that *Pitx2* is an upstream regulator of *Myf5*, *Myf6*, and *Myod* in EOM embryonic myogenesis (**Figure 3A3**).

Pitx2 is also expressed in the myogenic precursors of the FBA. *Tbx1* expression on FBA premyoblast is required for specification leading to *Myf5* and *Myod1* activation in those cells (Kelly et al., 2004). Notably, systemic *Pitx2* mutants, whether *Pitx2*$^{-/-}$ (Dong et al., 2006) or *Pitx2*$^{LacZ/LacZ}$ (Shih et al., 2007a), display a down regulation of *Tbx1* expression in this structure, although *Pitx2* expression is unaffected in *Tbx1* null mutants (Dong et al., 2006). These data, together with the fact that *Pitx2* directly interacts with *Tbx1* regulatory elements (Shih et al., 2007a) suggest that *Pitx2* is an upstream activator of *Tbx1* in FBA. A fuller analysis of both systemic *Pitx2* mutants reveals that the inactivation of *Pitx2* in FBA results in increased cell death in the mesodermal core and loss of early premyoblast specification markers such as *Six2*,

Tcf21, and *MyoR* (Dong et al., 2006; Shih et al., 2007a). Although, the role that *Six2* could play in the myogenesis of the FBA remains elusive, *Tcf21* and *MyoR* are known to be upstream effectors of *Myf5*, *Myod*, and *Myog* in these initial muscle-cell clusters (Lu et al., 2002). Jointly, these results indicate that *Pitx2* controls the expression of *Myod1* and the onset of myogenesis in FBA through *Tbx1, Tcf21*, and *MyoR* (**Figure 3A4**).

Pitx2 during Fetal Myogenesis

Most of what is known about *Pitx2* concerns early (embryonic) myogenesis. However, a new role for *Pitx2* has recently been unraveled during fetal myogenesis. L'Honoré et al. by using *Pitx2:Pitx3* double conditional mutants, have shown that *Pitx2/3* control the expression of the antioxidant system through the regulation of *Nrf1* and antioxidant enzymes during muscle differentiation (L'honoré et al., 2014a). Thus, *Pitx2/3 depletion* at the onset of differentiation induces an abnormal increase of reactive oxygen species (ROS) levels in differentiating myoblasts and leads to impaired myogenesis due to apoptosis of these cells. These results emphasize the role of *Pitx2* controlling redox conditions during fetal myogenesis.

Pitx2 Is Emerging as a Key Transcription Factor That Modulates Adult Myogenesis

During adult life the maintenance and repair of skeletal-muscle tissue is directed by SCs. The regulation of SC function in adults requires the redeployment of many of the regulatory networks fundamental for developmental myogenesis. Although, several efforts have been made during the last few years to disentangle the role of *Pitx2* in embryonic and fetal stages of myogenesis, studies linking *Pitx2* to adult myogenesis have only recently emerged and are still controversial.

The first evidence regarding *Pitx2* expression in SCs was reported by Ono et al. (2010). These authors showed that all *Pitx2* isoforms are expressed in proliferating SC-derived myoblasts. They analyzed SCs with a different ontology, comparing those of the extensor digitorum longus (EDL) of the limb with SCs from the masseter of the head (MAS). They found that *Pitx2b* and *Pitx2c* levels were higher in cells from the EDL than from the MAS, with *Pitx2c* being the main *Pitx2* isoform expressed in proliferating limb SCs (Ono et al., 2010). Based on these distinct gene-expression profiles, the authors suggest that, even after activation and entry into the cell cycle, SCs retain an identity consistent with their ontogeny underlying their distinct properties. Subsequent studies have pointed out that *Pitx2a*, *Pitx2b,* and *Pitx2c* were expressed at very low levels in proliferating SCs, but increased during the early stages of myogenic differentiation. Meanwhile the constitutive expression of any *Pitx2* isoform suppressed SC proliferation, with the cells undergoing greater myogenic differentiation (Knopp et al., 2013). However, additional evidence underlying the functional relevance of *Pitx2* on SC proliferation has been reported. For example, Herbet et al. demonstrated that *Pitx2* is crucial in maintaining the phenotype of myogenic precursor cells in the extraocular muscles (EOM; Hebert et al., 2013). In this analysis, the authors found that the higher levels of *Pitx2* expression in EOM in comparison with limb muscles were concomitant with longer proliferative state in EOM-derived SCs as compared with limb cells. In addition, the knockdown of *Pitx2* in SCs isolated from EOM slowed their proliferation rate, and a similar trend was seen for SCs isolated from tibialis anterioris muscle. These data led to the conclusion that *Pitx2* helps maintain a proliferating pool of myogenic precursor cells. Finally, the authors highlight that this greater proliferative capacity may facilitate the repair of damaged EOM tissue, thereby contributing to the sparing of EOM in muscular dystrophies (Hebert et al., 2013).

More recently, a study conducted in our laboratory has provided additional information about the molecular mechanisms by which the *Pitx2* transcription factor regulates cell proliferation in SCs (Lozano-Velasco et al., 2015). We have reported that *Pitx2c* expression is higher in early-activated SCs than in long-term activated ones, and our *in vitro Pitx2c* gain-of-function experiments have revealed that *Pitx2c* stimulates *Ccnd1* and *Ccnd2* expression, accelerating cell proliferation during early satellite-cell activation. Moreover, we have demonstrated that such *Pitx2c* effect on SCs proliferation is due to *Pitx2c*-mediated downregulation of the *miRNAs miR-15b*, *miR-106b*, *miR-23b*, and *miR-503* (**Figure 3B**). The existence of the *Pitx2-miRNA* pathway controlling the expression of key regulatory cell-cycle genes in early-activated SCs revealed a role of *Pitx2* in satellite-cell activation. Although, muscle SCs are promising targets for cell therapies, the paucity of SCs that can be isolated or expanded from adult muscle tissue is limiting; thus these findings provide new molecular tools to overcome such a bottleneck. It bears noting that our analyses also showed that *Pitx2c* can increase *Myf5* expression by down-regulating *miR-106b* (**Figure 3B**), thus expanding the $Myf5^{+}$ satellite-cell population and revealing a role for *Pitx2c* in promoting satellite-cell populations more primed for myogenic commitment (Lozano-Velasco et al., 2015). In this context it should be highlighted that in several muscular disorders such as muscular dystrophies, the progressive muscle wasting and weakness is often associated with exhaustion of muscle-regeneration potential. Therefore, the progressive loss of muscle mass has been attributed, at least partly, to the inability of muscle stem cells to efficiently regenerate tissue loss as the result of the disease (Berardi et al., 2014). Thus, critical for the development of effective strategies to treat muscle disorders is the optimization of approaches targeting muscle stem cells and capable of regenerating tissue loss as the result of the disease or as the result of normal muscle turnover (Bertoni, 2014). Notably, very recent reports have been pointed out that muscle stem cells should be considered as a therapeutic target for restoring muscle function in individuals with DMD (Chal et al., 2015; Dumont et al., 2015). Therefore, identification of new *Pitx2* functions in the context of SC biology may significantly contribute to the clarification of the molecular and cellular mechanisms of skeletal-muscle regeneration and may help to develop therapeutic strategies for muscular disorders.

Notably, the analysis of adult single and double *Pitx2:Pitx3* conditional mutant mouse lines targeted to the muscle stem-cell compartment revealed that double mutant SCs undergo senescence with impaired regeneration after injury, suggesting

that Pitx2-mediated changes in ROS levels are required for differentiation of SCs (L'honoré et al., 2014b).

All these data provide new insight into the function of *Pitx2* in the molecular mechanisms that control SC behavior and might thus have future application to enhance the regenerative capacity of these myogenic precursor cells. Further analysis using *in vivo* models could aid in understanding how the *Pitx2*-mediated effects on SCs can influence the kinetics of muscle regeneration.

CONCLUSIONS AND FUTURE CHALLENGES

The data reviewed above show that *Pitx2* is a comprehensive marker for cells undergoing myogenic progression, more so than any of the MRFs. This supports models that include a *Pitx2*-dependent pathway in virtually all skeletal muscles. Many pieces of experimental evidence have pointed out that *Pitx2* is the first molecular signal specifying all myogenic precursors in the head muscles. However, although several works have characterized *Pitx2* as a key transcription factor in the molecular cascade regulating trunk- and limb-muscle progenitors, additional work is needed to elucidate the function of *Pitx2* in specification vs. determination during trunk and limb myogenesis. In addition, since seminal works have revealed that *Pitx2* functions on myogenic cells may be due to *Pitx2*-mediated regulation of miRNAs, the role of *Pitx2* in the post-transcriptional control of myogenesis should be further explored.

In parallel, the role of *Pitx2* during adult myogenesis is beginning to be explored. Skeletal muscle has the ability to repair and regenerate due to the presence of resident SCs. SC function in adults requires redeployment of many of the regulatory networks fundamental to developmental myogenesis. Currently, SCs are considered potential therapeutic targets for restoring muscle function in muscle degenerative disorders such as muscular dystrophies. Recent works indicate that *Pitx2* is expressed in proliferating SCs and can promote differentiation of satellite-cell-derived myoblasts. Moreover, the identification of *Pitx2-miRNA* pathways that regulate satellite-cell behavior as well as the impact of Pitx2 on redox condition during satellite-cell differentiation may open insights toward future applications to modulate satellite-cell fate during muscle regeneration. Therefore, these findings propose *Pitx2* as a new player on skeletal-muscle satellite-cell biology and may help to develop therapeutic strategies for muscular disorder.

AUTHOR CONTRIBUTIONS

FH, LR, and AA conceived of the structure and content. FH wrote the first draft document. FH and LR designed and produced the figures. LR and DF critically revised the manuscript for intellectual content. AA corrected, edited, and approved the final version of the document to be published.

ACKNOWLEDGMENTS

This work was partially supported by grants BFU2012-38111, BFU2015-67131 (Ministerio de Economía y Competitividad, Gobierno de España), CTS-1614, P08-CTS-03878, BIO-302 (Junta de Andalucia), and AFM2012-16074 (AFM).

REFERENCES

Abu-Elmagd, M., Robson, L., Sweetman, D., Hadley, J., Francis-West, P., and Münsterberg, A. (2010). Wnt/Lef1 signaling acts via Pitx2 to regulate somite myogenesis. *Dev. Biol.* 337, 211–219. doi: 10.1016/j.ydbio.2009.10.023

Amendt, B. A., Sutherland, L. B., Semina, E. V., and Russo, A. F. (1998). The molecular basis of Rieger syndrome. Analysis of Pitx2 homeodomain protein activities. *J. Biol. Chem.* 273, 20066–20072. doi: 10.1074/jbc.273.32.20066

Arakawa, H., Nakamura, T., Zhadanov, A. B., Fidanza, V., Yano, T., Bullrich, F., et al. (1998). Identification and characterization of the ARP1 gene, a target for the human acute leukemia ALL1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 95, 4573–4578. doi: 10.1073/pnas.95.8.4573

Armand, O., Boutineau, A. M., Mauger, A., Pautou, M. P., and Kieny, M. (1983). Origin of satellite cells in avian skeletal muscles. *Arch. Anat. Microsc. Morphol. Exp.* 72, 163–181.

Baek, S. H., Kioussi, C., Briata, P., Wang, D., Nguyen, H. D., Ohgi, K. A., et al. (2003). Regulated subset of G1 growth-control genes in response to derepression by the Wnt pathway. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3245–3250. doi: 10.1073/pnas.0330217100

Bajard, L., Relaix, F., and Lagha, M. (2006). A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev.* 20, 2450–2464. doi: 10.1101/gad.382806

Bentzinger, C. F., Wang, Y. X., and Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harb. Perspect. Biol.* 4:a008342. doi: 10.1101/cshperspect.a008342

Berardi, E., Annibali, D., Cassano, M., Crippa, S., and Sampaolesi, M. (2014). Molecular and cell-based therapies for muscle degenerations: a road under construction. *Front. Physiol.* 5:119. doi: 10.3389/fphys.2014.00119

Bertoni, C. (2014). Emerging gene editing strategies for Duchenne muscular dystrophy targeting stem cells. *Front. Physiol.* 5:148. doi: 10.3389/fphys.2014.00148

Biressi, S., Molinaro, M., and Cossu, G. (2007). Cellular heterogeneity during vertebrate skeletal muscle development. *Dev. Biol.* 308, 281–293. doi: 10.1016/j.ydbio.2007.06.006

Borello, U., Berarducci, B., Murphy, P., Bajard, L., Buffa, V., Piccolo, S., et al. (2006). The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. *Development* 133, 3723–3732. doi: 10.1242/dev.02517

Buckingham, M., and Relaix, F. (2015). PAX3 and PAX7 as upstream regulators of myogenesis. *Semin. Cell Dev. Biol.* 44, 115–125. doi: 10.1016/j.semcdb.2015.09.017

Buckingham, M., and Rigby, P. W. J. (2014). Gene regulatory networks and transcriptional mechanisms that control myogenesis. *Dev. Cell* 28, 225–238. doi: 10.1016/j.devcel.2013.12.020

Campione, M., Steinbeisser, H., Schweickert, A., Deissler, K., van Bebber, F., Lowe, L. A., et al. (1999). The homeobox gene Pitx2: mediator of asymmetric left-right signaling in vertebrate heart and gut looping. *Development* 126, 1225–1234.

Chal, J., Oginuma, M., Al Tanoury, Z., Gobert, B., Sumara, O., Hick, A., et al. (2015). Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. *Nat. Biotechnol.* 33, 962–969. doi: 10.1038/nbt.3297

Chaney, B. A., Clark-Baldwin, K., Dave, V., Ma, J., and Rance, M. (2005). Solution structure of the K50 class homeodomain PITX2 bound to DNA and implications for mutations that cause Rieger syndrome. *Biochemistry* 44, 7497–7511. doi: 10.1021/bi0473253

Chargé, S. B. P., and Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* 84, 209–238. doi: 10.1152/physrev.00019.2003

Couly, G. F., Coltey, P. M., and Le Douarin, N. M. (1992). The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development* 114, 1–15.

Cox, C. J., Espinoza, H. M., McWilliams, B., Chappell, K., Morton, L., Hjalt, T. A., et al. (2002). Differential regulation of gene expression by PITX2 isoforms. *J. Biol. Chem.* 277, 25001–25010. doi: 10.1074/jbc.M201737200

Deries, M., and Thorsteinsdóttir, S. (2016). Axial and limb muscle development: dialogue with the neighbourhood. *Cell. Mol. Life Sci.* 73, 4415–4431. doi: 10.1007/s00018-016-2298-7

Dixit, M., Ansseau, E., Tassin, A., Winokur, S., Shi, R., Qian, H., et al. (2007). DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18157–18162. doi: 10.1073/pnas.0708659104

Dong, F., Sun, X., Liu, W., Ai, D., Klysik, E., Lu, M. F., et al. (2006). Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle. *Development* 133, 4891–4899. doi: 10.1242/dev.02693

Dumont, N. A., Wang, Y. X., von Maltzahn, J., Pasut, A., Bentzinger, C. F., Brun, C. E., et al. (2015). Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. *Nat. Med.* 21, 1455–1463. doi: 10.1038/nm.3990

Duxson, M. J., Usson, Y., and Harris, A. J. (1989). The origin of secondary myotubes in mammalian skeletal muscles: ultrastructural studies. *Development* 107, 743–750.

Evans, A. L., and Gage, P. J. (2005). Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development. *Hum. Mol. Genet.* 14, 3347–3359. doi: 10.1093/hmg/ddi365

Evans, D. J. R., and Noden, D. M. (2006). Spatial relations between avian craniofacial neural crest and paraxial mesoderm cells. *Dev. Dyn.* 235, 1310–1325. doi: 10.1002/dvdy.20663

Gage, P. J., and Camper, S. A. (1997). Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum. Mol. Genet.* 6, 457–464. doi: 10.1093/hmg/6.3.457

Gage, P. J., Suh, H., and Camper, S. A. (1999a). Dosage requirement of Pitx2 for development of multiple organs. *Development* 126, 4643–4651.

Gage, P. J., Suh, H., and Camper, S. A. (1999b). The bicoid-related Pitx gene family in development. *Mamm. Genome* 10, 197–200. doi: 10.1007/s003359900970

Ganga, M., Espinoza, H. M., Cox, C. J., Morton, L., Hjalt, T. A., Lee, Y., et al. (2003). PITX2 isoform-specific regulation of atrial natriuretic factor expression: synergism and repression with Nkx2.5. *J. Biol. Chem.* 278, 22437–22445. doi: 10.1074/jbc.M210163200

Goulding, M., Lumsden, A., and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* 120, 957–971.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R., and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *EMBO J.* 10, 1135–1147.

Gros, J., Manceau, M., Thomé, V., and Marcelle, C. (2005). A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature* 435, 954–958. doi: 10.1038/nature03572

Hammond, W. S. (1965). Origin of hypoglossal muscles in the chick embryo. *Anat. Rec.* 151, 547–557. doi: 10.1002/ar.1091510405

Harel, I., Nathan, E., Tirosh-Finkel, L., Zigdon, H., Guimarães-Camboa, N., Evans, S. M., et al. (2009). Distinct origins and genetic programs of head muscle satellite cells. *Dev. Cell* 16, 822–832. doi: 10.1016/j.devcel.2009.05.007

Hazelton, R. D. (1970). A radioautographic analysis of the migration and fate of cells derived from the occipital somites in the chick embryo with specific reference to the development of the hypoglossal musculature. *J. Embryol. Exp. Morphol.* 24, 455–466.

Hebert, S. L., Daniel, M. L., and McLoon, L. K. (2013). The role of Pitx2 in maintaining the phenotype of myogenic precursor cells in the extraocular muscles. *PLoS ONE* 8:e58405. doi: 10.1371/journal.pone.0058405

Hu, P., Geles, K. G., Paik, J.-H., DePinho, R. A., and Tjian, R. (2008). Codependent activators direct myoblast-specific MyoD transcription. *Dev. Cell* 15, 534–546. doi: 10.1016/j.devcel.2008.08.018

Hutcheson, D. A., Zhao, J., Merrell, A., Haldar, M., and Kardon, G. (2009). Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for beta-catenin. *Genes Dev.* 23, 997–1013. doi: 10.1101/gad.1769009

Jacob, M., Jacob, H. J., Wachtler, F., and Christ, B. (1984). Ontogeny of avian extrinsic ocular muscles. I. A light- and electron-microscopic study. *Cell Tissue Res.* 237, 549–557. doi: 10.1007/BF00228439

Janssen, I., Heymsfield, S. B., Wang, Z. M., and Ross, R. (2000). Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. *J. Appl. Physiol.* 89, 81–88.

Kablar, B., Krastel, K., Tajbakhsh, S., and Rudnicki, M. A. (2003). Myf5 and MyoD activation define independent myogenic compartments during embryonic development. *Dev. Biol.* 258, 307–318. doi: 10.1016/S0012-1606(03)00139-8

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomès, D., Rocancourt, D., Buckingham, M., Shinin, V., et al. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* 431, 466–471. doi: 10.1038/nature02876

Kelly, A. M., and Zacks, S. I. (1969). The histogenesis of rat intercostal muscle. *J. Cell Biol.* 42, 135–153. doi: 10.1083/jcb.42.1.135

Kelly, R. G., Jerome-Majewska, L. A., and Papaioannou, V. E. (2004). The del22q11.2 candidate gene Tbx1 regulates branchiomeric myogenesis. *Hum. Mol. Genet.* 13, 2829–2840. doi: 10.1093/hmg/ddh304

Kioussi, C., Briata, P., Baek, S. H., Rose, D. W., Hamblet, N. S., Herman, T., et al. (2002). Identification of a Wnt/Dvl/beta-Catenin –> Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell* 111, 673–685. doi: 10.1016/S0092-8674(02)01084-X

Kitamura, K., Miura, H., Miyagawa-Tomita, S., Yanazawa, M., Katoh-Fukui, Y., Suzuki, R., et al. (1999). Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. *Development* 126, 5749–5758.

Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T., and Kato, K. (1997). Expression patterns of Brx1 (Rieg gene), Sonic hedgehog, Nkx2.2, Dlx1 and Arx during zona limitans intrathalamica and embryonic ventral lateral geniculate nuclear formation. *Mech. Dev.* 67, 83–96. doi: 10.1016/S0925-4773(97)00110-X

Knopp, P., Figeac, N., Fortier, M., Moyle, L., and Zammit, P. S. (2013). Pitx genes are redeployed in adult myogenesis where they can act to promote myogenic differentiation in muscle satellite cells. *Dev. Biol.* 377, 293–304. doi: 10.1016/j.ydbio.2013.02.011

Lagha, M., Sato, T., Regnault, B., Cumano, A., Zuniga, A., Licht, J., et al. (2010). Transcriptome analyses based on genetic screens for Pax3 myogenic targets in the mouse embryo. *BMC Genomics* 11:696. doi: 10.1186/1471-2164-11-696

Lanctôt, C., Moreau, A., Chamberland, M., Tremblay, M. L., and Drouin, J. (1999). Hindlimb patterning and mandible development require the Ptx1 gene. *Development* 126, 1805–1810.

Lepper, C., and Fan, C.-M. (2010). Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. *Genesis* 48, 424–436. doi: 10.1002/dvg.20630

Lepper, C., Partridge, T. A., and Fan, C.-M. (2011). An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development* 138, 3639–3646. doi: 10.1242/dev.067595

L'honoré, A., Commère, P.-H., Ouimette, J.-F., Montarras, D., Drouin, J., and Buckingham, M. (2014a). Redox regulation by Pitx2 and Pitx3 is critical for fetal myogenesis. *Dev. Cell* 29, 392–405. doi: 10.1016/j.devcel.2014.04.006

L'honoré, A., Coulon, V., Marcil, A., Lebel, M., Lafrance-Vanasse, J., Gage, P., et al. (2007). Sequential expression and redundancy of Pitx2 and Pitx3 genes during muscle development. *Dev. Biol.* 307, 421–433. doi: 10.1016/j.ydbio.2007.04.034

L'honoré, A., Drouin, J., Buckingham, M., and Montarras, D. (2014b). Pitx2 and Pitx3 transcription factors: two key regulators of the redox state in adult skeletal muscle stem cells and muscle regeneration. *Free Radic. Biol. Med.* 75, S37. doi: 10.1016/j.freeradbiomed.2014.10.781

L'honoré, A., Ouimette, J.-F., Lavertu-Jolin, M., and Drouin, J. (2010). Pitx2 defines alternate pathways acting through MyoD during limb and somitic myogenesis. *Development* 137, 3847–3856. doi: 10.1242/dev.053421

Logan, M., Pagán-Westphal, S. M., Smith, D. M., Paganessi, L., and Tabin, C. J. (1998). The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* 94, 307–317. doi: 10.1016/S0092-8674(00)81474-9

Logan, M., and Tabin, C. J. (1999). Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. *Science* 283, 1736–1739. doi: 10.1126/science.283.5408.1736

Lozano-Velasco, E., Contreras, A., Crist, C., Hernandez-Torres, F., Franco, D., and Aránega, A. E. (2011). Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis. *Dev. Biol.* 357, 165–178. doi: 10.1016/j.ydbio.2011.06.039

Lozano-Velasco, E., Vallejo, D., Esteban, F. J., Doherty, C., Hernandez-Torres, F., Franco, D., et al. (2015). A Pitx2-miRNA pathway modulates cell proliferation in myoblasts and skeletal-muscle satellite cells and promotes

their commitment to a myogenic cell fate. *Mol. Cell. Biol.* 35, 2892–2909. doi: 10.1128/MCB.00536-15

Lu, J.-R., Bassel-Duby, R., Hawkins, A., Chang, P., Valdez, R., Wu, H., et al. (2002). Control of facial muscle development by MyoR and capsulin. *Science* 298, 2378–2381. doi: 10.1126/science.1078273

Marcil, A., Dumontier, E., Chamberland, M., Camper, S. A., and Drouin, J. (2003). Pitx1 and Pitx2 are required for development of hindlimb buds. *Development* 130, 45–55. doi: 10.1242/dev.00192

Martínez-Fernández, S., Hernandez-Torres, F., Franco, D., Lyons, G. E., Navarro, F., and Aránega, A. E. (2006). Pitx2c overexpression promotes cell proliferation and arrests differentiation in myoblasts. *Dev. Dyn.* 235, 2930–2939. doi: 10.1002/dvdy.20924

Matsuoka, T., Ahlberg, P. E., Kessaris, N., Iannarelli, P., Dennehy, U., Richardson, W. D., et al. (2005). Neural crest origins of the neck and shoulder. *Nature* 436, 347–355. doi: 10.1038/nature03837

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9, 493–495. doi: 10.1083/jcb.9.2.493

Miersch, C., Stange, K., Hering, S., Kolisek, M., Viergutz, T., and Röntgen, M. (2017). Molecular and functional heterogeneity of early postnatal porcine satellite cell populations is associated with bioenergetic profile. *Sci. Rep.* 7:45052. doi: 10.1038/srep45052

Moncaut, N., Rigby, P. W. J., and Carvajal, J. J. (2013). Dial M(RF) for myogenesis. *FEBS J.* 280, 3980–3990. doi: 10.1111/febs.12379

Muccielli, M. L., Martinez, S., Pattyn, A., Goridis, C., and Brunet, J. F. (1996). Otlx2, an Otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. *Mol. Cell. Neurosci.* 8, 258–271. doi: 10.1006/mcne.1996.0062

Murphy, M. M., Lawson, J. A., Mathew, S. J., Hutcheson, D. A., and Kardon, G. (2011). Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development* 138, 3625–3637. doi: 10.1242/dev.064162

Noden, D. M. (1983). The embryonic origins of avian cephalic and cervical muscles and associated connective tissues. *Am. J. Anat.* 168, 257–276. doi: 10.1002/aja.1001680302

Noden, D. M., and Francis-West, P. (2006). The differentiation and morphogenesis of craniofacial muscles. *Dev. Dyn.* 235, 1194–1218. doi: 10.1002/dvdy.20697

Ono, Y., Boldrin, L., Knopp, P., Morgan, J. E., and Zammit, P. S. (2010). Muscle satellite cells are a functionally heterogeneous population in both somite-derived and branchiomeric muscles. *Dev. Biol.* 337, 29–41. doi: 10.1016/j.ydbio.2009.10.005

Pellettieri, J., and Alvarado, A. S. (2007). Cell turnover and adult tissue homeostasis: from humans to planarians. *Annu. Rev. Genet.* 41, 83–105. doi: 10.1146/annurev.genet.41.110306.130244

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* 435, 948–953. doi: 10.1038/nature03594

Ryan, A. K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., et al. (1998). Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* 394, 545–551. doi: 10.1038/29004

Sambasivan, R., Gayraud-Morel, B., Dumas, G., Cimper, C., Paisant, S., Kelly, R. G., et al. (2009). Distinct regulatory cascades govern extraocular and pharyngeal arch muscle progenitor cell fates. *Dev. Cell* 16, 810–821. doi: 10.1016/j.devcel.2009.05.008

Sambasivan, R., Yao, R., Kissenpfennig, A., Van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., et al. (2011). Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development* 138, 3647–3656. doi: 10.1242/dev.067587

Schienda, J., Engleka, K. A., Jun, S., Hansen, M. S., Epstein, J. A., Tabin, C. J., et al. (2006). Somitic origin of limb muscle satellite and side population cells. *Proc. Natl. Acad. Sci. U.S.A.* 103, 945–950. doi: 10.1073/pnas.0510164103

Schmalbruch, H., and Lewis, D. M. (2000). Dynamics of nuclei of muscle fibers and connective tissue cells in normal and denervated rat muscles. *Muscle Nerve* 23, 617–626. doi: 10.1002/(SICI)1097-4598(200004)23:4<617::AID-MUS22>3.0.CO;2-Y

Schubert, F. R., Tremblay, P., Mansouri, A., Faisst, A. M., Kammandel, B., Lumsden, A., et al. (2001). Early mesodermal phenotypes in splotch suggest a role for Pax3 in the formation of epithelial somites. *Dev. Dyn.* 222, 506–521. doi: 10.1002/dvdy.1211

Schweickert, A., Campione, M., Steinbeisser, H., and Blum, M. (2000). Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left-right asymmetry. *Mech. Dev.* 90, 41–51. doi: 10.1016/S0925-4773(99)00227-0

Segalés, J., Perdiguero, E., and Muñoz-Cánoves, P. (2016). Regulation of muscle stem cell functions: a focus on the p38 MAPK signaling pathway. *Front. Cell Dev. Biol.* 4:91. doi: 10.3389/fcell.2016.00091

Semina, E. V., Ferrell, R. E., Mintz-Hittner, H. A., Bitoun, P., Alward, W. L., Reiter, R. S., et al. (1998). A novel homeobox gene PITX3 is mutated in families with autosomal-dominant cataracts and ASMD. *Nat. Genet.* 19, 167–170. doi: 10.1038/527

Semina, E. V., Reiter, R., Leysens, N. J., Alward, W. L., Small, K. W., Datson, N. A., et al. (1996). Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat. Genet.* 14, 392–399. doi: 10.1038/ng1296-392

Shapiro, M. D., Marks, M. E., Peichel, C. L., Blackman, B. K., Nereng, K. S., Jónsson, B., et al. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* 428, 717–723. doi: 10.1038/nature02415

Shih, H. P., Gross, M. K., and Kioussi, C. (2007a). Cranial muscle defects of Pitx2 mutants result from specification defects in the first branchial arch. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5907–5912. doi: 10.1073/pnas.0701122104

Shih, H. P., Gross, M. K., and Kioussi, C. (2007b). Expression pattern of the homeodomain transcription factor Pitx2 during muscle development. *Gene Expr. Patterns* 7, 441–451. doi: 10.1016/j.modgep.2006.11.004

Standring, S. (2008). *Gray's Anatomy. The Anatomical Basis of Clinical Practice*. London: Churchill Livingstone Elsevier.

Stuelsatz, P., Keire, P., and Yablonka-Reuveni, Z. (2017). Isolation, culture, and immunostaining of skeletal muscle myofibers from wildtype and nestin-GFP mice as a means to analyze satellite cell. *Methods Mol. Biol.* 1556, 51–102. doi: 10.1007/978-1-4939-6771-1_4

Szeto, D. P., Rodriguez-Esteban, C., Ryan, A. K., O'Connell, S. M., Liu, F., Kioussi, C., et al. (1999). Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. *Genes Dev.* 13, 484–494. doi: 10.1101/gad.13.4.484

Tajbakhsh, S. (2009). Skeletal muscle stem cells in developmental versus regenerative myogenesis. *J. Intern. Med.* 266, 372–389. doi: 10.1111/j.1365-2796.2009.02158.x

Tajbakhsh, S., and Buckingham, M. (1999). The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr. Top. Dev. Biol.* 48, 225–268.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell* 89, 127–138. doi: 10.1016/S0092-8674(00)80189-0

Tzahor, E. (2015). Head muscle development. *Results Probl. Cell Differ.* 56, 123–142. doi: 10.1007/978-3-662-44608-9_6

Venuti, J. M., Morris, J. H., Vivian, J. L., Olson, E. N., and Klein, W. H. (1995). Myogenin is required for late but not early aspects of myogenesis during mouse development. *J. Cell Biol.* 128, 563–576. doi: 10.1083/jcb.128.4.563

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., et al. (1998). Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* 94, 299–305. doi: 10.1016/S0092-8674(00)81473-7

Zacharias, A. L., Lewandoski, M., Rudnicki, M. A., and Gage, P. J. (2011). Pitx2 is an upstream activator of extraocular myogenesis and survival. *Dev. Biol.* 349, 395–405. doi: 10.1016/j.ydbio.2010.10.028

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 28 February 2017
doi: 10.3389/fcell.2017.00014

Sonic Hedgehog Signaling in Limb Development

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The gene encoding the secreted protein Sonic hedgehog (Shh) is expressed in the polarizing region (or zone of polarizing activity), a small group of mesenchyme cells at the posterior margin of the vertebrate limb bud. Detailed analyses have revealed that Shh has the properties of the long sought after polarizing region morphogen that specifies positional values across the antero-posterior axis (e.g., thumb to little finger axis) of the limb. Shh has also been shown to control the width of the limb bud by stimulating mesenchyme cell proliferation and by regulating the antero-posterior length of the apical ectodermal ridge, the signaling region required for limb bud outgrowth and the laying down of structures along the proximo-distal axis (e.g., shoulder to digits axis) of the limb. It has been shown that Shh signaling can specify antero-posterior positional values in limb buds in both a concentration- (paracrine) and time-dependent (autocrine) fashion. Currently there are several models for how Shh specifies positional values over time in the limb buds of chick and mouse embryos and how this is integrated with growth. Extensive work has elucidated downstream transcriptional targets of Shh signaling. Nevertheless, it remains unclear how antero-posterior positional values are encoded and then interpreted to give the particular structure appropriate to that position, for example, the type of digit. A distant cis-regulatory enhancer controls limb-bud-specific expression of *Shh* and the discovery of increasing numbers of interacting transcription factors indicate complex spatiotemporal regulation. Altered Shh signaling is implicated in clinical conditions with congenital limb defects and in the evolution of the morphological diversity of vertebrate limbs.

Keywords: Sonic hedgehog, limb, digits, mouse, chick, positional information

OPEN ACCESS

Edited by:
Andrea Erika Münsterberg,
University of East Anglia, UK

Reviewed by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *07 December 2016*
Accepted: *08 February 2017*
Published: *28 February 2017*

Citation:
Tickle C and Towers M (2017) Sonic Hedgehog Signaling in Limb Development. Front. Cell Dev. Biol. 5:14. doi: 10.3389/fcell.2017.00014

INTRODUCTION

Over 20 years ago the first evidence was presented that *Sonic hedgehog* (*Shh*), an orthologue of the *Drosophila Hedgehog* (*Hh)* gene, encodes the long sought after morphogen that specifies antero-posterior pattern in developing vertebrate limbs (Riddle et al., 1993). Grafting experiments in chick wing buds in the 1960s revealed that a group of morphologically indistinguishable mesenchyme cells at the posterior margin of the wing bud (the margin nearest the tail), later known as the polarizing region (or zone of polarizing activity), is an important cell-cell signaling center that controls development across the antero-posterior axis (Saunders and Gasseling, 1968). Tissue transplanted from the posterior margin of one chick wing bud to the anterior margin of another was shown to have the striking ability to duplicate the pattern of three digits, so that another set develop in mirror-image symmetry to the normal set. Based on these observations it was proposed that the

polarizing region produces a diffusible morphogen that specifies antero-posterior positional values (Wolpert, 1969). These positional values are interpreted so that a structure, such as a digit with an appropriate identity, develops in the correct position.

The key pieces of evidence that Shh is the polarizing morphogen are that *Shh* transcripts were found to be localized to the polarizing region of the chick wing bud (**Figures 1a–f**) and that *Shh*-expressing cells grafted to the anterior margin of chick wing buds can produce the same effects as grafts of the polarizing region (Riddle et al., 1993). Earlier experiments revealed that tissue from the posterior margin of mammalian limb buds grafted to the anterior margin of chick wing buds could duplicate the pattern of chick wing digits (Tickle et al., 1976; Fallon and Crosby, 1977). This is explained by the finding that *Shh* is expressed at the posterior margin of mammalian limb buds (Echelard et al., 1993; Odent et al., 1999). *Shh* has now been shown to be expressed at the posterior margin of the limb buds of all vertebrates studied to date, including the fin buds of the most primitive chondrichthyan fishes such as the shark (Dahn et al., 2007).

Experiments in which the polarizing region was grafted to the anterior margin of another chick wing bud showed that polarizing region signaling also plays a role in controlling the width of the limb bud and that widening of the bud is required to specify a complete set of new antero-posterior positional values (Tickle et al., 1975; Smith and Wolpert, 1981). The earliest detected effect of a polarizing region graft was an increase in cell proliferation in adjacent mesenchyme in the host wing bud (Cooke and Summerbell, 1980). In addition, it was proposed that the polarizing region controls the production of a factor by the mesenchyme that maintains the apical ectodermal ridge over the region of the wing bud that will give rise to distal structures including the digits (Zwilling and Hansborough, 1956). The apical ectodermal ridge is a signaling region that rims the bud and is required for proximal-distal patterning and outgrowth and the laying down of structures along this axis; the extent of the apical ectodermal ridge across the antero-posterior axis controls the width of the wing bud and determines the number of digits that can form. The effects of the polarizing region on the apical ectodermal ridge also link antero-posterior and proximo-distal pattern formation. This explains the observation that polarizing region grafts made at later stages of development affect the antero-posterior pattern of more-distal structures (Summerbell, 1974).

Early experiments highlighted the complex relationship between the polarizing region and apical ectodermal ridge. In order for a polarizing region to signal, it has to contact the apical ectodermal ridge (Tickle et al., 1975) and this interaction is required in order for the polarizing region to maintain production of the apical ridge maintenance factor by the mesenchyme that will form distal structures. In addition, in the chick wing bud, the polarizing region itself demarcates the posterior limit of the apical ectodermal ridge and grafts of the polarizing region placed under the apical ectodermal ridge flatten it (Saunders and Gasseling, 1968). Interestingly, it has also been shown that the dorsal ectoderm of the wing bud, which produces a signal controlling the development of the dorsal pattern of structures (e.g., extensor muscles), is also required for the polarizing region to signal (Yang and Niswander, 1995). Thus, signaling along all three axes of the developing limb bud is integrated.

It has now been shown that Shh affects cell proliferation in the chick wing bud by controlling expression of genes encoding cell cycle regulators including D cyclins independently of the apical ectodermal ridge (Towers et al., 2008). Work on mouse limb development has shown that Shh controls expression of the *Gremlin1* gene, which encodes the BMP antagonist that acts as the apical ridge maintenance factor (Zuniga et al., 1999). In addition, it has also been demonstrated that short-range Shh signaling can flatten the apical ridge above the polarizing region (Bouldin et al., 2010).

Experiments on chick wing buds have identified FGFs as the apical ectodermal ridge signals that promote outgrowth and also maintain *Shh* expression in the polarizing region (Laufer et al., 1994; Niswander et al., 1994). Genetic experiments in mouse have identified Wnt7a as the dorsalizing signal that also contributes to regulating *Shh* expression (Parr and McMahon, 1995). Loss of Wnt7a function in the mouse limb results in the transformation of dorsal to ventral fates and loss of posterior digits (Parr and McMahon, 1995). This second phenotype is consistent with a function for Wnt7a in controlling *Shh* expression since no digits form in the fore-limbs of $Shh^{-/-}$ mouse embryos and only a single digit—considered to be an anterior digit 1—is present in hind-limbs (Chiang et al., 1996).

In this review, we will emphasize the parallel contributions that experimental chick embryology and mouse genetics have played in providing the current picture of Shh function in the limb. We will provide an in-depth picture of how Shh specifies antero-posterior positional values in the limb buds of these two main vertebrate models and how this is integrated with its role in growth. We will consider how *Shh* expression in the limb is initiated, maintained and eventually extinguished and how cells respond to the Shh signal. We will finally review clinical conditions affecting the limb and examples of evolutionary diversification of limb morphology that are associated with changes in Shh signaling.

SPECIFICATION OF ANTERO-POSTERIOR PATTERN

Chick Wing

Detailed embryological experiments on the chick wing bud have been crucial in establishing the signaling parameters of the polarizing region morphogen. The polarizing region was first discovered in the chick wing bud, where it overlaps with a region of programmed cell death, known as the posterior necrotic zone (Saunders and Gasseling, 1962). Indeed, the original grafting experiments were designed to investigate how this region of cell death is controlled (Saunders and Gasseling, 1968). Tissue from the posterior margin of a chick wing bud was grafted to the anterior margin of a second wing bud and this resulted in a mirror-image pattern of digits across the antero-posterior axis. The normal chick wing has three digits (designated at this time as 2, 3, and 4) but following a polarizing region graft to the

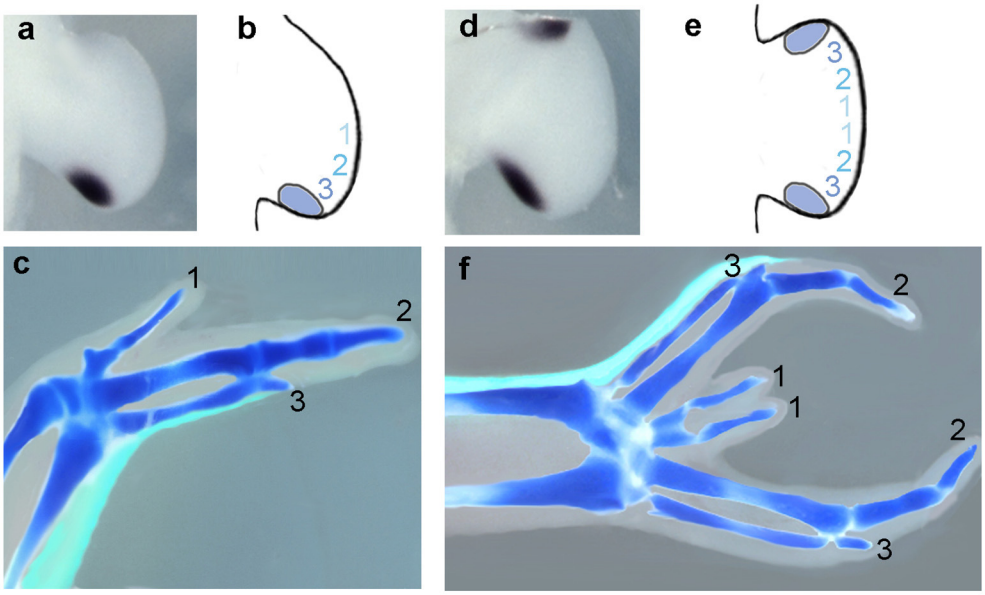

FIGURE 1 | Shh as a morphogen in the chick wing bud. (a) *Sonic hedgehog* (*Shh*) expression in the polarizing region at the posterior margin of the early chick wing bud (Riddle et al., 1993). **(b)** A gradient of Shh in the chick wing bud (blue shaded numbers) specifies antero-posterior positional values for three digits (1,2, and 3) in cells adjacent to polarizing region over 12 h. **(c)** Chick wing digit skeleton with polarizing region descendants fate-mapped by GFP-expression (green) (Towers et al., 2011). Digits form in tissue adjacent to descendants of the polarizing region that form narrow strip of cells along posterior wing margin. **(d)** Chick wing bud with anterior polarizing region graft expresses *Shh* at both anterior and posterior margins (Towers et al., 2011). **(e)** Mirror-image symmetrical positional values specified as in **(b)** as a result of Shh being produced by both graft and host. **(f)** Chick wing digit skeleton pattern with grafted polarizing region **(d)** and progeny fate mapped by GFP expression (Towers et al., 2011). Six digits form in an anterior to posterior pattern 3-2-1-1-2-3 and grafted polarizing region descendants form narrow strip of cells along anterior wing margin. In all cases, data shown is representative of data in the original cited papers.

anterior margin, six digits can develop in the pattern 4-3-2-2-3-4. Note that recent evidence supports numbering of the digits as 1, 2, and 3 (Towers et al., 2011), and this numbering system is now generally accepted and will be used in this review. This grafting experiment provided an assay for polarizing activity and antero-posterior pattern that could readily be scored by the distinct skeletal morphology of each of the three digits of the chick wing. It should be noted that grafts of the polarizing region also affect the antero-posterior pattern of the wing forearm skeleton and soft tissues (Shellswell and Wolpert, 1977; Robson et al., 1994). Thus, following a polarizing region graft, two ulnae develop and the pattern of muscles is also duplicated. The myogenic cells of the muscle originate in the somites and migrate into the limb bud but the pattern of the wing muscles is dictated by the connective tissue, which is derived from the lateral plate mesoderm (Chevalier and Mauger, 1977). Therefore, the duplicated pattern of muscles following a polarizing region graft will be based on the response of the cells that give rise to the muscle connective tissue.

The experimental parameters determined for polarizing region signaling in the chick wing (reviewed in Towers and Tickle, 2009) are consistent with the suggestion that the polarizing region produces a long-range morphogen that sets up a concentration gradient across the antero-posterior axis of the wing bud and specifies positional values (Wolpert, 1969). According to this model, the positional values at particular threshold concentrations govern digit identity, with the highest threshold concentration in tissue closest to the polarizing region specifying the most-posterior digit, digit 3, and the lowest threshold concentration in tissue further away specifying the most-anterior digit, digit 1. Thus, any candidate molecule for the polarizing region morphogen must act in a concentration-dependent manner (Tickle, 1981) and provide a long-range signal (Honig, 1981).

The first defined molecule found to mimic the duplicating activity of polarizing region grafts was the vitamin A derivative, retinoic acid (Tickle et al., 1982, 1985) but it was subsequently shown that retinoic acid acts indirectly (Noji et al., 1991; Wanek et al., 1991) by inducing *Shh* expression (Riddle et al., 1993). There is now good evidence that Shh acts in a concentration-dependent fashion to induce digit duplications. When *Shh*–expressing cells, or beads soaked in bacterially produced ShhN protein (the active N-terminal fragment produced by autocatalytic cleavage of the large precursor Shh protein), are placed at the anterior margin of a chick wing bud, the extent of digit duplication depends on the number of *Shh*–expressing cells grafted or the concentration of ShhN protein in which the beads are soaked (Yang et al., 1997). Fewer *Shh*-expressing cells or lower concentrations of Shh elicit duplication of only the anterior digit 1 (Yang et al., 1997). Grafts of *Shh*-expressing cells that induce full digit duplications were also shown to result in two ulnae developing in the forearm

together with a duplicated pattern of muscles (Duprez et al., 1999).

The original model for how antero-posterior values are specified in the chick wing bud did not consider the dynamic nature of the process, although experiments showed that the extent of duplication following a polarizing graft depended on the length of time that the graft was left in place (Smith, 1980). A similar time dependency was subsequently seen with Shh–soaked beads (Yang et al., 1997). Furthermore, fate mapping experiments showed that cells near a Shh-soaked-bead give rise to an anterior digit 1 when the bead is removed after a short time, but give rise to a more posterior digit (2) if the bead is left in place for longer (Yang et al., 1997). This process by which positional values of cells change over time in response to an increasing concentration of morphogen is known as promotion (see also (Gurdon et al., 1995). An alternative process in which wing bud cells acquire a stable positional value depending on the duration of Shh signaling and then are displaced by growth can be ruled out because an anterior digit 1 has been shown to arise in tissue which was not originally adjacent to a polarizing region graft (see Tickle, 1995).

The parameters of polarizing region discussed above were determined in experiments in which additional digits were induced following polarizing region grafts to the anterior margin. But what is the evidence that Shh acts long range and how does Shh signaling specify antero-posterior positional values during normal development of the chick wing? Measurements of Shh activity in slices taken from different positions across the bud using an *in vitro* cell-differentiation assay are consistent with there being a concentration gradient of Shh across the bud, with Shh activity of a posterior slice being 5–6 times higher than that of a middle slice (Zeng et al., 2001). Another indication that Shh spreads across the wing bud and provides a long range signal is that high levels of the transcripts of known direct gene targets of Shh signaling, including *Ptch1* (encoding the main receptor for Shh), and *Gli1* (encoding a transcriptional effector of Shh signaling) encompass the posterior two-thirds of the wing bud, including adjacent tissue in addition to the polarizing region (Marigo et al., 1996). It should also be noted that following a polarizing region graft or implantation of an Shh bead to the anterior margin of the chick wing, there is a burst of high level *Ptch1* expression in the anterior part of the wing bud, which then subsides and is later followed by the establishment of a stable domain of high level *Ptch1* expression (Drossopoulou et al., 2000). This suggests that cells could respond to and interpret two waves of Shh signaling; the first defining the size of the domain that can give rise to digits, and the second, promoting the growth of this domain and specifying positional values.

The temporal specification of positional values specified by Shh in normal wing development has been directly addressed by applying cyclopamine, a small molecule inhibitor of Hh signaling at the level of Smoothened to chick embryos, at a series of short time intervals after the onset of *Shh* expression in wing buds (Towers et al., 2011). Smoothened, a member of the G-protein coupled receptor superfamily, is normally activated upon Shh binding to Ptch1, and this triggers of activation of the Gli family of transcription factors (see section on Mechanisms of Shh signaling). Application of cyclopamine about 4 h after the onset of *Shh* expression results in the development of just the anterior digit 1, the anterior and middle digits (1 and 2) develop when cyclopamine is added at 8 h while a complete set of digits (1, 2, and 3) develop when cyclopamine is added at 12 h (Towers et al., 2011). Furthermore, fate mapping experiments show that promotion is occurring with cells next to the polarizing region first being specified to form the anterior digit 1, then being promoted to form the middle digit 2 and finally the posterior digit 3 (**Figure 2A**).

The effects of Shh signaling on antero-posterior growth must be included in any comprehensive model for specification of antero-posterior pattern in the chick wing. Application of cyclopamine in the experiments described above demonstrated that Shh signaling has effects on both specification of antero-posterior positional values and growth because this treatment not only prevented promotion but also expansion of the region of the wing bud that will give rise to distal structures leading to the development of fewer digits (Towers et al., 2008, 2011). When growth alone is targeted by adding trichostatin A or colchicine, and following over-expression of the cyclin-dependent kinase inhibitor—$p21^{Cip1}$—at a similar series of time points, fewer digits also develop, but because specification of positional values and promotion by Shh signaling are unaffected, the digits that develop are posterior digits (Towers et al., 2008). These experiments show that specification of antero-posterior positional values in the early chick wing bud is coupled with growth that determines the width of the wing bud.

The cyclopamine experiments also show that antero-posterior values are specified over a relatively short time period during early wing bud development. However, these values will not be interpreted in terms of digit identity until much later in development when the digit condensations develop (**Figure 2A**). When the *Shh*-expressing region is completely removed from the early wing bud at the time when the positional values that specify two digits are specified, truncated wings develop with posterior structures being preferentially lost (Pagan et al., 1996), showing the crucial importance of Shh signaling in stimulating antero-posterior expansion and maintaining the apical ectodermal ridge. Resulting skeletons bear resemblance to those of the wings of the chicken mutant *Oligozeugodactyly* (*Ozd*) that develop devoid of Shh (Ros et al., 2003). It is unclear why *Shh* continues to be expressed at the posterior margin of the chick wing bud long after the antero-posterior values have been specified (**Figure 2A** see section Termination of *Shh* expression).

Chick Leg

The chick leg has four morphologically distinct digits (numbered 1, 2, 3, and 4 in antero-posterior sequence). Early grafting experiments demonstrated that chick leg buds also have a polarizing region but it was noted that when the leg polarizing region was grafted to a chick wing bud, a toe frequently developed in the duplicated wings (Summerbell and Tickle, 1977). It has since been demonstrated using grafts from the Green Fluorescent Protein-expressing transgenic chicken to make fate maps of the polarizing region that the chick leg polarizing region gives rise to the most posterior digit 4, whereas in the chick wing all the digits

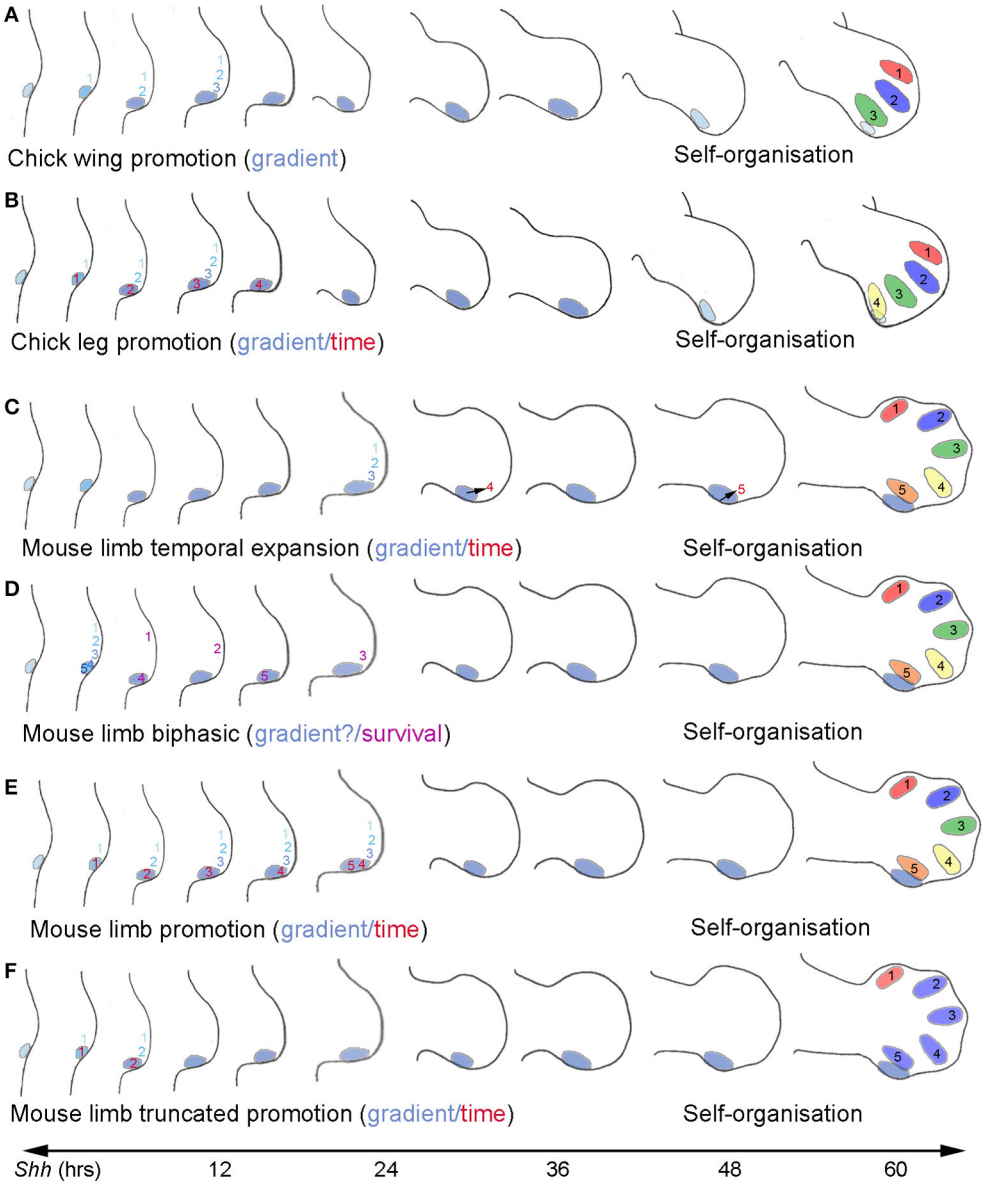

FIGURE 2 | Comparison of models of Shh function in chick and mouse limbs. (A) Chick wing promotion model. Positional values of digits 1, 2, and 3 specified adjacent to polarizing region (blue shading) and promoted over 12 h through a series of increasingly posterior positional values by a concentration gradient of paracrine Shh signaling (graded blue shading—note coloring of polarizing region also shows strength of *Shh* expression. *Shh* terminated at around 60 h as digit condensations form by self-organization (black numbers). Colors of developing digits indicate a different positional value that cells were specified with. **(B)** Chick leg promotion model. Positional values of digits 1, 2, and 3 specified as **(A)** but polarizing region cells promoted through progressively anterior positional values over 16 h in response to time of autocrine Shh signaling (red numbers) and form digit 4. *Shh* terminated at around 60 h. **(C)** Mouse limb temporal expansion. Positional values of digits 1, 2, and 3 specified adjacent to the polarizing region by a gradient of paracrine Shh signaling over approximately 24 h– it is unclear whether promotion is involved (see **A**). Positional values of digits 4 and 5 specified in polarizing region sometime before *Shh* terminates at 60 h according to duration of autocrine Shh signaling. *Shh* terminates at around 60 h. **(D)** Mouse limb biphasic model. Positional values of digits 1, 2, 3, 4, and 5 specified by Shh, possibly by a gradient of paracrine signaling from the polarizing region in approximately 6 h. It is unclear whether promotion is involved and is possible in this time (see **A**), or if Shh levels can reach concentrations predicted required to specify posterior positional values. Shh signaling over the next 16 h required for specified digit progenitor cells to proliferate and form condensations in the order digit 1, 4, 2, 5, and 3 (purple numbers). **(E)** Mouse limb promotion model. Positional values of digits 1, 2, 3, and 4 specified as **(B)** and polarizing region enlarges sufficiently to give rise to digits 4 and 5 by self-organization. Note promotion model does not easily explain digit 5 patterning that requires a shorter exposure to form than digit 3 (see **D**). **(F)** Mouse limb truncated promotion model. Anterior positional values specified (1 and 2) specified by autocrine and paracrine signaling and then cells become refractory to further posterior promotion. Digits form by self-organization: 1, 2, and 3 from cells adjacent to polarizing region, digits 4 and 5 from the polarizing region.

come from tissue anterior to the polarizing region (Towers et al., 2011; see **Figure 1c**).

Shh is expressed at the posterior margin of chick leg buds for a similar duration to its expression in chick wing buds. Furthermore, it has been demonstrated by treating leg buds with cyclopamine that the positional values that specify the three anterior digits of the chick leg are promoted in response to paracrine Shh signaling in an identical fashion to those that specify the three digits of the chick wing (Towers et al., 2011). However, the positional value for the most posterior digit 4 is promoted in response to autocrine Shh signaling (**Figure 2B**). Thus, when Shh signaling was attenuated in the chick leg bud by cyclopamine 4 h after onset of *Shh* expression, two toes with digit 1 identities arose—one from the polarizing region, the other from adjacent anterior tissue, while when Shh signaling was attenuated after 8 h, three digits develop, toes with digit 2 identities from the polarizing region and adjacent cells and a toe with a digit 1 identity from cells further away, and so on, until by 16 h, all the antero-posterior positional values in the leg bud have been specified (Towers et al., 2011). These observations show that although it takes slightly longer to specify antero-posterior positional values in the leg compared to the wing, this process is nevertheless accomplished in the early leg bud, and, as in the wing bud, some considerable time elapses before these positional values are interpreted (**Figure 2B**). It should be noted that, in the *Ozd* chicken mutant, a single digit 1 forms in the leg (Ros et al., 2003).

Mouse Limb

The mouse limb has five digits (1, 2, 3, 4 and 5 in antero-posterior sequence) and digits 2–5 all have three phalanges making them morphologically very similar. Fate maps of the mouse limb polarizing region made by tracing genetically labeled cells that have expressed *Shh* show that the two posterior digits of the mouse limb are entirely derived from the polarizing region, and while there is some contribution to digit 3, the two anterior digits come from cells outside of the polarizing region (Harfe et al., 2004).

Shh is expressed at the posterior margin of limb buds of mouse embryos between E9.5–E12.0 (60 h; Zhu et al., 2008, **Figure 2C**, note expression is between E10-E12.5 in hind-limbs). At E10.5, a graded distribution of Shh across the posterior third of the mouse hind limb bud has been detected by immunohistochemical analysis (Gritli-Linde et al., 2001) in keeping with paracrine Shh signaling specifying antero-posterior positional values as in the chick wing. *Shh* is expressed not only at the posterior margin but also at the anterior of the limbs of several polydactylous mouse mutants (Masuya et al., 1995) consistent with Shh functioning as a polarizing signal in mouse limbs. In contrast, in mouse embryos lacking *Shh* function, the limbs taper toward the tip, and only one digit-like structure (interpreted as digit 1) develops in the hind-limb, while no digits develop in the fore-limb (Chiang et al., 1996). This indicates that Shh is required for the outgrowth of the limb and for the development of structures distal to the elbow/knee in the mouse limb. It should also be noted that in mouse embryos lacking *Shh* function the development of muscles in this distal region of the limb is severely compromised (Kruger et al., 2001) Experiments in which Smoothened activity is deleted specifically in the prospective myogenic cells show that Shh signaling has direct effects on these cells; timing myogenic differentiation, promoting slow muscle differentiation and controlling their migration into the distal part of the limb (Anderson et al., 2012; Hu et al., 2012).

In chick limbs, antero-posterior positional values clearly relate to the identity of a digit that develops in an appropriate position. However, this is not readily observable in the mouse limb due to the difficulties in determining which digits are present in mouse limbs conditionally lacking Shh function. Therefore, there is currently no general consensus about the model which best reflects how positional values are specified in the mouse limb bud. The various models are now discussed below (also see **Figures 2C–F**).

The first formal model to be proposed for the mouse limb was the temporal expansion model (Harfe et al., 2004). In this model, anterior positional values for digit 2 (and in part for digit 3) are specified in a concentration-dependent fashion by paracrine Shh signaling and then, posterior positional values (for digits 4 and 5) by the duration of autocrine Shh signaling, which is governed by the proliferative expansion and then displacement of cells from the polarizing region (**Figure 2C**; specification of digit 1 is considered to be Shh-independent in the hind-limb). Consistent with the model, the restriction of paracrine signaling in a *Dispatched* mutant (see later section on Mechanisms of Shh signaling) resulted in loss of one digit, suggested to be digit 2. This model also gained support from the finding that when *Shh* expression was curtailed in the developing mouse limb, this resulted in only three digits developing. The authors identified these digits as being 1, 2, and 3 consistent with the prediction that digits should be lost in a posterior to anterior sequence (Scherz et al., 2007). A particular feature of this model is that it takes considerable time for all the antero-posterior positional values to be specified (**Figure 2C**), rather than over a short time in the early limb bud. Moreover, it does not take into account promotion through a transitory series of anterior to posterior positional values, which has been demonstrated to occur in the limb buds of the chick.

A later model was proposed by Zhu et al. (2008) based on the results of a more extensive set of experiments, in which *Shh* function was deleted at a series of different stages in mouse limb development. Again, digits were lost with progressively fewer digits developing when Shh function was deleted at earlier and earlier stages. However in this case, the authors suggested that the sequence of digit loss reflects the order in which digits form, with digits that form last being lost first. Thus, for example, they identified the digits in limbs with three digits as being 1, 2, and 4. If their identification of the digits is correct, a posterior digit has formed adjacent to an anterior digit, an outcome not predicted by any previous model. Based on their findings, they proposed a biphasic model for digit patterning—in which Shh has two functions (**Figure 2D**). In the first phase, Shh specifies positional values across the antero-posterior axis of the very early limb bud, possibly via a concentration gradient, while in the second phase Shh is required to support proliferation and survival of cells that will form the digits (Zhu et al., 2008). It is not clear whether this

latter function is a separate direct function of Shh signaling or reflects an essential role of Shh signaling in maintaining sufficient apical ectodermal ridge signaling. According to this model, the resultant digit patterns when Shh function is deleted are due to loss of Shh compromising survival and proliferation of specified digit progenitor cells rather than failure to specify antero-posterior positional values (Zhu et al., 2008). Furthermore, positional values would have to be specified in the early mouse limb bud over a period of approximately 6 h (based on *Ptch1* expression), which suggests that this process is not integrated with growth as in the chick wing.

The ability to observe promotion in chick limbs gives insights into the time required to specify positional values, but in the mouse limb, in which promotion is not readily observed, it is difficult to distinguish between the effects of Shh signaling on specification of positional values and survival and proliferation of the cells that will form the digit condensations. Indeed the time required for digit specification proposed by Zhu et al. does not appear consistent with a model in which antero-posterior positional values are promoted in response to the concentration and/or duration of Shh signaling. However, if one were to take promotion into account, a unifying model can be proposed (Towers et al., 2011). According to this proposal, positional values would be specified early in the mouse limb as suggested in the biphasic model. However, these would only be anterior positional values, which would then be promoted to posterior values by both paracrine and autocrine Shh signaling operating in parallel. Thus, the pattern of digits specified would depend on how far positional values have been promoted at the time at which *Shh* function is deleted in keeping with more conventional models for digit patterning. The digits that develop in the three-digit mouse limb when Shh signaling is curtailed would therefore be predicted to be 1, 2, and 2—a pattern that is readily observed in cyclopamine-treated chick legs (Towers et al., 2011), and occasionally in wings (Pickering and Towers, 2016). However, there are difficulties in applying a promotion model to the specification of digit 5 of the mouse limb as this would imply that it is the last digit of the pattern to be specified (**Figure 2F**), when in fact it forms before more-anterior digits (**Figure 2D**, see also discussion in Towers et al., 2011).

INTERACTION BETWEEN POSITIONAL INFORMATION AND A TURING-TYPE MECHANISM

Although, it has been shown that Shh is the critical signal in controlling development across the antero-posterior axis of the limb, there is evidence that the periodic condensation of cells that will form the digits depends on an underlying Turing type self-organization mechanism independent of graded Shh signaling. In the basic Turing model, diffusible signals—one operating as an inhibitor, the other as an activator—interact to produce the pattern of digits and interdigits. Positional information and self-organization have been presented as competing models of digit development, when in fact the power of both processes operating together has been long recognized (see (Wolpert, 1989) and for original paper on reaction-diffusion (Turing, 1952).

The first indications that such a self-organization mechanism might be involved in limb development came from experiments in which it was shown that recombinant limb buds formed from disaggregated single cells, re-aggregated and placed back in an ectodermal jacket could still form digits (Zwilling, 1964; Pautou, 1973). Indeed, based on this latter study, one of the first computer simulations of limb development was developed (Wilby and Ede, 1975). Further experiments showed that when recombinant limbs were made from chick mesenchyme cells from the anterior halves of early chick leg buds, which would not include a polarizing region, and which would not normally give rise to digits, two or three morphologically similar digit-like structures developed (Hardy et al., 1995; Elisa Piedra et al., 2000). When a polarizing region was grafted into such recombinant limbs, however, the digits that developed had recognizable identities (MacCabe et al., 1973). These experiments elegantly revealed that positional information and self-organization are integrated in limb development. There is evidence that a self-organization mechanism also operates in mouse limb buds, as the limbs of mutant mouse embryos in which the Shh signaling pathway is non-functional have many morphologically similar digits (Litingtung et al., 2002; te Welscher et al., 2002; see Section–Measurement of Shh concentration and duration of signaling). Indeed, recent studies in the mouse limb have suggested that this mechanism is based on WNT signals acting as inhibitors and BMP signals as activators, that together, converge on the transcription factor Sox9 to generate a repeated series of digit condensations (Raspopovic et al., 2014).

Since digits 2–5 have similar morphologies in the mouse limb, particularly in regard to phalangeal count, one proposal is that self-organization plays a dominant process (Delgado and Torres, 2016). This scenario could for account for difficulties in applying a positional information model to the five digits of the mouse limb. Moreover, a recent study on developing chick wings has revealed how positional information and self-organization can interact and this could be relevant to understanding how the mouse digit pattern is specified. If chick wing buds are treated with cyclopamine under conditions in which the promotion of antero-posterior values is truncated, a series of morphologically similar digit 2s in a pattern 1-2-2-2 can develop by self-organization (Pickering and Towers, 2016). It should be noted that the digit 2s were not of identical morphologies and sizes suggesting other factors control these finer aspects of development. In wings with multiple digit 2s, the most-posterior of these digits arises from cells of the polarizing region. An interpretation of these findings is that antero-posterior expansion mediated by a posteriorly extended apical ectodermal ridge has enabled a small pool of cells specified with the same positional value to produce a series of digit 2s by self-organization (Pickering and Towers, 2016). In extrapolating these data to the mouse limb, it has been suggested that a similar mechanism could account for the patterning of digits 1 through to 4 (Pickering and Towers, 2016; **Figure 2F**). In addition, the apical ectodermal ridge of the mouse limb completely overlies the polarizing region (Pickering and Towers, 2016), and an

intriguing suggestion is that this could enable the cells of the polarizing region to expand sufficiently to give rise to two digits (4 and 5) by self-organization (**Figure 2F**; Pickering and Towers, 2016). The specification of the same positional value during mouse limb development could occur if cells become refractory to the levels/duration of Shh signaling at a certain point (**Figure 2F**). In support of such a mechanism operating in the mouse, there is not a simple linear relationship between position and level of positive Shh signaling in the limb bud as expected in a classical positional information model (Ahn and Joyner, 2004). However, even though mouse digits 2–5 are morphologically similar, it is clear that they still have different identities, with the cells of digit 4 being characterized by having many more receptors for both testosterone and estrogen than digit 2 thus determining the sexual dimorphism in digit length (Zheng and Cohn, 2011). Indeed, digit 5 in particular, has quite a different morphology to the other digits. Taken together, even if the cells that give rise to mouse digits 2–5 are specified with the same positional value that is interpreted so that they have the same phalanx number, other factors operate to give the digits their individual morphologies and hence identities. Additional support for a model in which loss of Shh signaling can increase digit number and also result in posterior digits developing with anterior traits has been provided by work on the fore-limbs and hind-limbs of the amphibian *Xenopus tropicalis*. Inhibition of Shh signaling at a series of developmental stages resulted in fore-limbs occasionally developing with five digits rather than four (Stopper et al., 2016). In addition, hind-limbs often developed terminal claws on all five digits whereas in normal development claws are only present on digits 1, 2, and 3. Additional work is required to determine if other characteristics of these posterior digits are anteriorised such as phalange number.

The work of Pickering and Towers further highlights the complex relationship between the polarizing region and the apical ridge already mentioned (Niswander et al., 1994), and the importance of short-range reciprocal signaling between these structures in the formation of posterior digits in particular as observed in the mouse limb (Zuniga et al., 1999; Bouldin et al., 2010). Thus, in the chick wing, Shh signaling inhibits the overlying apical ridge and the polarizing region fails to produce digits, yet in the mouse limb, the overlying apical ridge is less sensitive to Shh signaling than in the chick wing (see also (Bouldin et al., 2010), and in persisting posteriorly, allows two digits to form—the chick leg appears to have an intermediate relationship allowing one digit to form. Such dynamic interplay between the polarizing region and apical ridge could have contributed to patterns of posterior digit loss during limb evolution (see Section on Evolutionary aspects of Shh signaling in the limb).

MECHANISMS OF Shh SIGNALING

As indicated in the models outlined above, positional values in developing limbs are specified by paracrine Shh signaling, in which Shh acts as a long-range graded signal and in a concentration/time dependent fashion, or by the duration of autocrine Shh signaling. Therefore, the crucial questions are how a graded distribution of Shh arises, how the range of Shh signaling is controlled and how cells measure the concentration of Shh and the duration of Shh signaling.

Long-Range Shh Signaling and Gradient Formation

Studies in developing mouse limbs have revealed general mechanisms that modulate the distribution of Shh protein in tissues. One factor is the addition of lipids. Following its autocatalytic conversion, Shh is secreted by cells as a modified form of ShhN with cholesterol added at the C-terminus and a palmitoyl group (as part of a thiol ester) at the N-terminus (known as ShhNp; p indicating that ShhN is processed; reviewed (Lee et al., 2016). In limb buds of mouse embryos in which the C-terminal processing domain of *Shh* is conditionally deleted so that the polarizing region produces ShhN instead of ShhNp, ShhN spreads further across the limb bud and additional digits develop anteriorly (Li et al., 2006). It should be noted that previous analyses also suggested that cholesterol modification extends the range of paracrine Shh signaling. Thus, mice limbs expressing ShhN that lacks cholesterol failed to form digits 2 and 3 (Lewis et al., 2001) consistent with a role for paracrine Shh signaling in specifying these digits (Harfe et al., 2004). Other data however are consistent with cholesterol modification restricting the spread of Shh. Thus, mice deficient in SREBP-2 that encodes a sterol regulatory element binding protein that regulates cholesterol production failed to up-regulate *Ptch1*, consistent with impaired Shh transport (Vergnes et al., 2016). Similar studies on mutant mice that are unable to palmitoylate Shh show that this modification is essential for long range signaling (Chen et al., 2004). Intriguingly, cholesterol has also recently been shown to be the endogenous activator of Smoothened (Huang P. X. et al., 2016). Because cholesterol plays such important roles in Shh signaling, changes in the availability of cholesterol can impact on the development of the limb and might explain the subtle alterations in the spacing of the digits that have been observed in the limbs of mice with a mutation in a gene encoding a protein required for cholesterol metabolism (Schmidt et al., 2009) and in the limbs of rat embryos treated with triparanol, an inhibitor of cholesterol biosynthesis (Gofflot et al., 2003). The membrane protein Dispatched1 is required for paracrine signaling by cholesterol–modified Shh (Tian et al., 2005). The restriction of the spread of the ligand in a *Dispatched1* mouse mutant resulted in the loss of a digit, which was interpreted as being digit 2, and as already mentioned, provided crucial evidence for the temporal expansion model (Harfe et al., 2004).

Another mechanism that influences the range of Shh signaling is the binding of Shh to cell surface and extracellular proteins. A generic response to Shh in all tissues is transcriptional up-regulation of genes encoding cell surface proteins such as *Ptch1* and *Hhip* that bind Shh. The resultant increase in their expression in response to Shh creates negative feedback loops, that not only limit the spread of Shh by sequestering it at the cell surface, but also, in the case of Ptch1, because it inhibits Smoothened activity, dampens activation of the Shh pathway. In mice in which *Ptch1*

is conditionally inactivated in the limbs (Butterfield et al., 2009), and therefore the signaling pathway is activated independently of Shh, the hind-limbs have extra digits, but the fore-limbs have fewer digits. This difference between hind-limbs and fore-limbs appears to be due to the timing of activation of the signaling pathway, which is earlier in the mutant fore- limbs (Zhulyn et al., 2014).

In contrast to *Ptch1* and *Hhip1*, the genes *Cdo* (*CAM-related/downregulated by oncogene*), *Boc* (*brother of Cdo*) and *Gas1* (*growth arrest specific 1*) encoding membrane associated proteins that bind Shh, are expressed in the anterior region of early limb buds and their expression is negatively regulated by Shh. Analysis of limb development in single or double mouse mutants suggest that Gas1 and Boc sustain paracrine Shh signaling at a distance from the polarizing region (Allen et al., 2007). ShhNp can also bind to heparan sulfate proteoglycans and the distribution of these and other extracellular proteins in the developing limb will affect the distribution of Shh. In *Drosophila,* the hydrolase notum that cleaves glypicans, a sub-family of heparan sulfate proteoglycans, promotes high-level Hh signaling in the wing. Interestingly, in the chick wing bud, *Notum* was identified in microarray experiments as being downstream of Shh signaling (Bangs et al., 2010), suggesting possible functional conservation.

One way in which Shh could spread across the limb bud is by diffusion (see Muller et al., 2013, for discussion on mechanisms of morphogen transport), although it has been questioned whether simple diffusion would be a sufficiently robust mechanism to generate a stable concentration gradient (Kerszberg and Wolpert, 2007). Mathematical modeling however showed that specification of positional values for the three digits of the chick wing can be simulated by simple diffusion of Shh from the polarizing region (Woolley et al., 2014). In the model, based on the results of (Drossopoulou et al., 2000), Shh specifies the initial size of the domain that will give rise to the digits and then provides positional information. The model incorporates promotion of positional values in a dose-dependent fashion over the observed time frame in a growing domain of the correct dimensions as determined experimentally (Towers et al., 2008). The model can be extended successfully to the specification of the positional values in the chick leg, even though digit 4 arises from the polarizing region. However, it is unclear whether Shh levels in the polarizing region could reach the predicted concentration required to specify digit 4 (assumed to be double that required to specify digit 3) and whether indeed there is a simple graded response to Shh signaling in the leg. It is therefore more plausible that digit 4 is specified by length of time that cells express *Shh*. The model cannot however be extended further to simulate easily specification of the fifth digit of the mouse limb.

Live imaging of chick wing buds showed that Shh can be transported along the external surface of specialized filopodia (similar structures in insects are called cytonemes). These filopodia extend up to 150 microns away from the polarizing region and a similar distance away from the receiving cell (Sanders et al., 2013) equating to about 300 microns, the initial size of the chick wing digit-forming field (Vargesson et al., 1997; Towers et al., 2008). Thus, direct cell-cell contacts can span the required range of Shh signaling. Furthermore, Boc and Cdo have been visualized in discrete microdomains on a subset of filopodia extending from Shh-responding cells. However, it is not clear whether this transport mechanism could produce robust graded signaling and indeed whether filopodia are required. The involvement of filopodia could however explain the apparently anomalous finding that grafts of cells expressing a membrane-tethered form of Shh (generated by fusing the integral membrane protein CD4 to the C-terminus of ShhN) can duplicate digits in the chick wing (Yang et al., 1997).

Measurement of Shh Concentration and Duration of Signaling

It has been proposed that limb bud cells respond to paracrine Shh signaling in a concentration dependent fashion although length of exposure to the Shh signal also plays a role. So how do cells measure the concentration of Shh? The mechanism depends on the Shh-dependent processing of full-length Gli proteins, which act as transcriptional activators; in the absence of Shh signaling, Gli proteins are processed to short forms, which act as transcriptional repressors (reviewed in Lee et al., 2016). In normal chick and mouse limb buds, anterior cells not exposed to Shh contain high levels of Gli repressor, while in the posterior region of the limb, there is a gradient in the ratio of Gli activator/Gli repressor, higher posteriorly than anteriorly, reflecting the response to the Shh gradient across this part of the limb (Wang et al., 2000). There are three *Gli* genes, *Gli1*, *Gli2,* and *Gli3* with the protein encoded by *Gli1* acting exclusively as an transcriptional activator as it does not undergo processing into a repressor form. While functional inactivation of *Gli1* and *Gli2* in mice has little effect on limb development (Mo et al., 1997; Park et al., 2000), when *Gli3*, the major contributor to transcriptional repression, is functionally inactivated, *Shh* is expressed anteriorly and several additional morphologically similar digits form anteriorly while posterior digits are less affected (Wang et al., 2000). Unexpectedly, the limbs of *Gli3* and *Shh* double knockout embryos are identical to the $Gli3^{-/-}$ limb buds showing that the function of Shh in the limb is to relieve repression by Gli3 and allow a patterned set of digits to develop from the posterior part of the limb (Litingtung et al., 2002; te Welscher et al., 2002). In the mouse limb, the gradient of Gli3 activity could only specify at most digits 1, 2, and 3 because *Gli3* is not expressed in the polarizing region itself (Buscher and Ruther, 1998). Instead the initial response to autocrine Shh signaling would have to be mediated by Gli2, and consistent with this hypothesis, removing the function of *Gli2* in a *Gli3* mutant background, thus effectively inactivating all Gli function, results in the digits appearing morphologically similar (Bowers et al., 2012). This suggests that Gli3 mediates the response of cells in the limb bud to paracrine Shh signaling and Gli2 to autocrine Shh signaling. It should also be noted that the digits that form in single $Gli3^{-/-}$ mouse limbs (and also in compound $Shh^{-/-}/Gli3^{-/-}$ mouse limbs) are thinner and more closely spaced together than in normal limbs, suggesting that Gli3 plays a role in regulating the digit period (Sheth et al., 2012, see section Interaction Between Positional Information and a Turing-type Mechanism). 5′Hoxa/d

function also seems to be involved since the progressive titration of 5′*Hox* genes in the *Gli3*$^{-/-}$ background increases digit number and decreases the digit period still further (Sheth et al., 2012).

Surprisingly, chemical mutagenic screens to identify mutations causing polydactyly in mouse identified genes required for formation and functioning of primary cilia (Huangfu et al., 2003; Weatherbee et al., 2009; Ashe et al., 2012). In such mutants, many morphologically similar digits develop and this is because Gli processing takes place on primary cilia in vertebrate cells. Thus, absence of cilia is equivalent to functional inactivation of all three Gli genes. The classical chicken mutant, *talpid3*, with a range of defects including polydactylous limbs (Ede and Kelly, 1964) was found to have a mutation in a gene encoding a centrosomal protein required for formation of a primary cilium (Davey et al., 2006), and functionally inactivating the *talpid3* gene in a mouse limb, leads to the development of many morphologically similar digits (Bangs et al., 2011). Another chicken mutant, *talpid2*, with the same range of defects including polydactylous limbs, was found to have a mutation in a gene encoding another ciliary protein—C2CD3 (Chang et al., 2014).

For autocrine Shh signaling, the duration of signaling is the most important parameter. Timing appears to be a general way of specifying positional values, but how cells in embryos measure time is little understood. Interestingly, a timing mechanism involving a cell cycle clock has been proposed to specify proximo-distal positional values in the chick wing bud (Saiz-Lopez et al., 2015), although the most proximal positional values may be specified by retinoic acid signaling (Cooper et al., 2011; Rosello-Diez et al., 2011). The molecular nature of intrinsic timers is currently unknown and presents a widespread problem in developmental biology.

INITIATION OF *Shh* EXPRESSION

A key discovery in understanding how *Shh* expression is localized to the posterior margin of the limb bud was identification of a *cis*-regulatory element that controls limb-specific expression (Lettice et al., 2002). Analysis of *Sasquatch*, an insertional mouse mutant with limb polydactyly, in which *Shh* was expressed anteriorly as well as posteriorly in the limb, showed that the exogenous DNA construct had serendipitously disrupted an enhancer (Sharpe et al., 1999). This 1.7 Kb enhancer, which has become known as the ZRS (zone of polarizing activity regulatory sequence), is unexpectedly located in intron 5 of the *LMBR1* (*limb region 1*) gene, which is almost 1 MB upstream of the promoter of the *Shh* gene. It is still not clear why insertion of the transgene into this particular region of the ZRS in *Sasquatch* leads to anterior *Shh* expression in the limb bud. In contrast, deletion of the entire ZRS region in mouse embryos results in loss of *Shh* expression in the limb buds resulting in limb truncations similar to those found in mouse embryos lacking *Shh* function (Sagai et al., 2005). It should be noted however, that the many other defects seen in mouse embryos lacking *Shh* function, which reflect the widespread functions of Shh signaling in organogenesis, are not present in the mouse embryos in which the ZRS is deleted.

The ZRS is of general interest as an example of a long-range enhancer—a cluster of three similar long-range enhancers also regulates *Shh* expression in the epithelial linings of the pharynx, the lung and the gut respectively (Sagai et al., 2009). 3D FISH and chromatin configuration assays showed close associations between the ZRS and the *Shh* locus in mouse limb bud cells compared to cells from other tissues (Amano et al., 2009). Curiously, transcriptional activity was not seen in all polarizing region cells suggesting that the cells may express *Shh* in pulses. One possibility is that *Shh* is expressed periodically during the cell cycle. In support of this, *Shh* expression is lost in chick wing buds treated with aphidicolin—an inhibitor of progression through S-phase (Ohsugi et al., 1997). More recently FISH and chromatin configuration assays together with super-resolution microscopy have revealed that the *Shh* locus loops out of its chromosome territory to make contacts with the ZRS in polarizing region cells in the mouse limb bud at the time *Shh* expression is activated (Williamson et al., 2016).

The ZRS provides an excellent reference point for deciphering the gene network that controls *Shh* expression in the limb and contains binding sites for the transcription factors, Hand2 (heart and neural crest derivatives 2; (Galli et al., 2010) and 5′ Hoxd proteins. The genes encoding these transcription factors are expressed in the posterior region of the early limb bud and when they are deleted in the mouse limb, *Shh* is not expressed. Conversely, when *Hoxd13* is expressed throughout the mouse limb bud, there is an ectopic *Shh* domain and polydactylous limbs result (Zakany et al., 2004).

Expression of *Hand2* and *Hoxd* genes is restricted to the posterior part of mouse limb buds by Gli3. In the mouse fore-limb-forming region, *Hand2* expression is also repressed anteriorly by the *Hox5* paralogous group genes (Xua et al., 2013), while *Hand2* expression in the posterior region of the fore-limb-forming region is dependent on the *Hox9* paralogous group genes, thus providing antero-posterior polarity prior to the transcriptional activation of the *Shh* gene (Xu and Wellik, 2011). Recently, it has emerged that GATA family transcription factors also contribute to supressing anterior expression of *Shh* (Kozhemyakina et al., 2014) as conditional removal of *Gata4/6* in limbs of mouse embryos results in pre-axial polydactyly. Two distinct mechanisms have been proposed. One is that GATA transcription factors in complex with FOG co-factors bind directly to the ZRS enhancer while the other is that GATA6 may interact directly with GLI3 to promote repression of the vertebrate Hedgehog pathway and this may explain the formation of an additional anterior digit in the hindlimb (Hayashi et al., 2016).

Shh expression in the polarizing region is also controlled by FGF signaling from the apical ridge and FGF signaling has been shown to regulate the expression of the genes encoding the ETS translocation variant transcription factors ETV4 and ETV5. The genes encoding these transcription factors are expressed beneath the entire extent of the apical ectodermal ridge and suppress *Shh* expression outside of the polarizing region. These ETV transcription factors bind directly to sites in the ZRS. In the

polarizing region, posteriorly expressed ETS1/GABPα binds to other sites in the ZRS and over-rides this inhibition and allows expression of *Shh* (Lettice et al., 2012). Wnt7a signaling from the dorsal ectoderm also contributes to controlling *Shh* expression but the mechanism is not yet known (Yang and Niswander, 1995).

The activity of the ZRS not only determines the location of cells expressing *Shh* in the developing limb bud but also the size of the *Shh* expression domain. In addition, an autoregulatory mechanism has been discovered in which Shh controls the number of polarizing region cells by regulating the size of the posterior necrotic zone (Sanz-Ezquerro and Tickle, 2000) via BMP2 signaling (Bastida et al., 2009) Taken together these mechanisms have the crucial function of controlling the levels of Shh signaling.

Lastly, retinoic acid derived from the flank also appears to be required for initiating *Shh* expression in limb buds. *Shh* expression is greatly reduced in the limb buds of vitamin A deficient quails (Stratford et al., 1999) and in chick wing buds following treatment with inhibitors of retinoic acid synthesis (Stratford et al., 1996). Mouse embryos in which a gene encoding an enzyme that generates retinoic acid was functionally inactivated died early and lacked fore-limbs. When these embryos were provided with retinoic acid so that development can proceed further, *Shh* was not restricted posteriorly in the rescued fore-limb buds suggesting that retinoic acid plays a role in determining antero-posterior polarity prior to activation of *Shh* expression (Niederreither et al., 2002; Zhao et al., 2009).

TERMINATION OF *Shh* EXPRESSION

The failure of the positive feedback loop between the polarizing region and the apical ectodermal ridge has been proposed to terminate the duration of *Shh* expression in the chick wing. In this model, Shh up-regulates *Grem1* by paracrine signaling, but cells displaced from the polarizing region by proliferative expansion are then unable to up-regulate *Grem1* (the apical ridge maintenance factor; Scherz et al., 2004). This is proposed to create a tissue barrier that results in Shh being no longer able to up-regulate *Grem1* at a distance, leading to de-repressed BMP signaling suppressing *Fgf4* expression in the apical ectodermal ridge, that in turn, leads to loss of *Shh* expression in the polarizing region (Scherz et al., 2004). Tbx2 is proposed to be the factor that suppresses the posterior up-regulation of *Grem1* in and around the polarizing region (Farin et al., 2013). In the absence of Tbx2, *Grem1* expression expands posteriorly resulting in prolonged *Shh* expression and extra tissue growth indicated by the bifurcation of digit 4. It is unclear why this only occurs in the hind-limbs of these *Tbx2* knockout mice. An alternative model for the mouse limb is that increased FGF signaling inhibits *Grem1* expression leading to termination of the feedback loop (Verheyden and Sun, 2008).

A clock linked with the cell cycle has also been shown to be involved in timing the duration of *Shh* expression in the polarizing region of the chick wing bud with the clock being set once retinoic acid concentrations fall below a certain level. Thus, tissue transplantation experiments have shown that the chick wing polarizing region intrinsically times the duration of *Shh* expression irrespective of the extrinsic signaling environment (Chinnaiya et al., 2014). Indeed, *Shh* expression has been shown to terminate on time if the separation of *Grem1* and *Shh* expressing cells is prevented (Towers et al., 2008). Furthermore, the inhibition of Shh signaling with cyclopamine in the chick wing leads to the premature loss of *Shh* expression in the presence of an *Fgf4*-expressing apical ectodermal ridge and *Grem1* expression extending into the posterior part of the wing bud, thus suggesting that Shh autoregulates its own transcription in the polarizing region (Pickering and Towers, 2016). The mechanism by which this is achieved has not yet been elucidated.

RESPONSE TO Shh SIGNALING IN THE LIMB

Many studies have provided information about the expression of individual genes that are affected by Shh signaling in the limb. For example, changes in gene expression have been observed in chick limb buds treated with Shh or cyclopamine, and in mouse limb buds in which *Shh* or *Gli3* is functionally inactivated, or in which Gli3 processing does not occur, e.g., mutants with defective cilia. Microarray analyses have been carried out in both chick and mouse limbs (Vokes et al., 2008; Bangs et al., 2010). It has been estimated from one microarray study that 10% of the genes expressed in the early limb bud (about 1,000 genes) are downstream of Shh signaling (Bangs et al., 2010). Putative direct targets of Gli3 repression have been identified by ChIP seq analysis of limb bud nuclear extracts using transgenic mice expressing a tagged form of the Gli3 protein (Vokes et al., 2008). Further analysis has involved RNAseq (Lewandowski et al., 2015).

Analysis of this information has begun to uncover the gene regulatory network underlying the response to the Shh signaling pathway in the limb in addition to the generic suite of genes that encode proteins that enable or modulate Shh signaling. The genes in the network include those that are expressed posteriorly either due to positive regulation by Shh or because Shh relieves Gli3 repression; also those that are expressed anteriorly either due to negative regulation by Shh or because they are downstream of Gli3 repression (Bangs et al., 2010). A study involving analysis of gene expression patterns in the limb buds of $Shh^{-/-}$, $Gli3^{-/-}$ double mouse mutants indicated that the expression of nearly all the putative Gli target genes identified by ChIP seq in the posterior mesenchyme of E10.5 mouse limb buds depends on Gli repressor activity rather than Gli activator activity (Lewandowski et al., 2015).

One generic class of potential target genes already mentioned comprises genes encoding cell cycle regulators such as *N-myc* and *Cyclin D1* that are predominantly expressed posteriorly and *Cyclin D2* that is expressed in the polarizing region, and that are likely mediate the effects of Shh on proliferation (Towers et al., 2008; Welten et al., 2011). Shh has also been shown to promote vascularisation of the chick wing bud via regulating expression genes encoding pro-angiogenic factors such as VEGF (Davey et al., 2007). There is evidence in the mouse limb, that transcription factor genes including 5′ genes in the *Hoxa*

and *Hoxd* clusters, *Sall1*, and *Tbx2/Tbx3* are putative direct targets of Shh and would be predicted to encode the positional information conferred by the autocrine/paracrine Shh signaling (Vokes et al., 2008). Experiments with cultured mouse limb buds suggest that Shh signaling is required for robust and continued expression of 5′members of the *Hoxd* cluster (Panman et al., 2006; Lewandowski et al., 2015) while mis-expression of *Tbx2* and *Tbx3* genes in the chick leg bud in the embryo has been reported to change digit identity (Suzuki et al., 2004).

Other putative direct Gli3 targets are genes involved in BMP signaling; *Gremlin* encoding the apical ridge maintenance factor and *Bmp 2* expressed together with *Bmp7,* in the posterior region of the early limb bud (Vokes et al., 2008). There is a close relationship between *Shh* and *Bmp2* expression elsewhere in vertebrate embryos, which is also conserved in *Drosophila*. For instance in the *Drosophila* wing imaginal disc, Hh secreted from the posterior compartment induces expression of the *Bmp2* orthologue, *Dpp*, that encodes a long range signaling molecule regulating position-dependent expression of transcription factors such as *Spalt* and *Omb*, orthologues of *Sall1* and *Tbx2/3* respectively. Experiments in chick wing buds show that Bmp-soaked beads placed at the anterior margin of a chick limb do not induce digit duplications (Drossopoulou et al., 2000). However, when a bead soaked in a BMP antagonist was implanted at the anterior margin of the wing bud following implantation of an Shh-soaked bead, a series of morphologically similar digits developed anteriorly suggesting that BMP signaling is involved in digit promotion (Drossopoulou et al., 2000). In chick leg buds, BMP signaling is graded across the tip of the bud at the stage at which the digit condensations form in the so-called phalanx-forming region (PFR—Suzuki et al., 2008). Grafting interdigital tissue to different positions between digit condensations and manipulating BMP signaling alters the morphology of the digits in terms of phalange number suggesting that it is BMPs produced by interdigital regions that are directly responsible for realizing digit-specific morphology (Dahn and Fallon, 2000). Recently, evidence has been presented that interdigital signaling may also be involved in regulating the morphogenesis of the digit condensations in mouse limbs (Huang B. L. et al., 2016).

CLINICAL ASPECTS OF Shh SIGNALING IN THE LIMB

The increasing understanding of the molecular basis of antero-posterior pattern formation has led to insights into congenital malformations that affect the limb. Unsurprisingly, defects in Shh function have been found to underlie several inherited disorders. In particular, these include polydactyly: pre-axial polydactyly in which additional digits arise from the thumb-side of the hand, and post-axial polydactyly in which the additional digits arise from the little finger-side (Biesecker, 2011). Often these conditions are associated with syndactyly (fusion of the soft tissues between the digits).

Alterations in the coding sequence of the *SHH* locus are not known to form the basis of any congenital malformation of the limb—presumably because such lesions are not compatible with the development of other tissues. However, point mutations in the ZRS enhancer that would be predicted to lead to ectopic *SHH* expression specifically in the limb bud are found in human patients with pre-axial polydactyly type 1 (PPD1—OMIM 174400) and triphalangeal thumb polysyndactyly syndrome (TPTPS OMIM 174500) (see review Hill and Lettice, 2013). In TPTPS, additional digits can arise post-axially as well as pre-axially, suggesting that the normal regulation of *SHH* expression at the posterior margin of the limb is also perturbed. It remains to be determined how these point mutations affect the regulation of endogenous *SHH* expression. One possibility is that the levels and/or duration of *SHH* expression are increased and these lead not only to an additional digit pre-axially but also to overgrowth of the polarizing region and its subsequent development into additional post-axial digits—perhaps by self-organization (see section on Interaction between positional information and a Turing-type mechanism). A point mutation at a particular position in the ZRS is associated with Werner mesomelic syndrome in which there are distal arm and leg bone defects in addition to extra digits (VanderMeer et al., 2014). Unexpectedly, duplications of the ZRS have also been reported in individuals with TPTPS as well as the related condition Haas-type polysyndactyly (OMIM 186200). Microduplications of the ZRS have also been detected in patients with Laurin-Sandrow syndrome OMIM 13750); the limb phenotype of these patients overlaps with the Haas-type polysyndactyly phenotype but can be distinguished by mirror-image polysyndactyly of the feet and duplication of the fibula (Lohan et al., 2014). In contrast, patients with a deletion involving exon 4 and portions of introns 3 and 4 of the *LMBR1* gene, a region distinct from the ZRS, have a condition known as acheiropodia (OMIM 200500) in which elements distal to the elbow/knee fail to form in all four limbs. This condition not only resembles the phenotype of the limb buds of mouse embryos lacking Shh function but also that of the limbs of *Ozd* mutant chickens in which it has now been shown that a large part of the ZRS sequence is deleted (Maas and Fallon, 2004). Inborn errors in cholesterol metabolism can lead to limb anomalies, as might be expected given the importance of cholesterol in Shh signaling as already discussed. For example, post-axial polydactyly is found in patients with Smith-Lemli-Opitz syndrome (OMIM 270400) in which a mutation deactivates the function of 7-dehydrocholesterol reductase, which is the final enzyme in the metabolic pathway that generates cholesterol. Post-axial polydactyly is also seen at low frequencies in patients with other syndromes in which cholesterol biosynthesis is altered (Gofflot et al., 2003). Why post-axial polydactlyly occurs however is not clear.

Defects in the response to Shh signaling are found in syndromes that include polydactyly. For instance, the Pallister-Hall (OMIM 146510, Hill et al., 2007) and Grieg Cephalopolysyndactyly (OMIM 175700- Kalff-Suske et al., 1999) syndromes present with pre-axial and post-axial polydactyly and are caused by mutations in the *GLI3* gene. The effects of these mutations are likely due to the de-repression of the Shh signaling pathway in the anterior part of the limb. Since the processing of full-length Gli3, occurs in primary cilia, syndromes known as ciliopathies, in which cilia function/structure is

compromised, include polydactyly as part of their spectrum of defects—examples being, Bardet-Biedl syndrome (BBS–OMIM 209900, Forsythe and Beales, 2013) and Meckel-Gruber syndrome (OMIM 249000, Shaheen et al., 2013). Recently mutations in the *TALPID3* gene, required for formation of cilia have been discovered in patients with Joubert syndrome (OMIM 21330) although these patients rarely show limb defects (Roosing et al., 2015; Stephen et al., 2015). Homozygous mutations in the *TALPID3* gene have however been found in families affected by lethal ciliopathies associated with polydactyly (Alby et al., 2015), phenotypes more akin to those of the homozygous chicken mutants already mentioned in which the *talpid3* gene was first identified.

Several clinical conditions are associated with mutations in putative gene targets of Shh signaling in the limb (see previous section on Response to Shh signaling, also reviewed Pickering and Towers, 2014). *Sall1* encoding a transcription factor is expressed in the posterior region of the early chick and mouse limb buds but more widely at the base of the digital plate at later stages (Buck et al., 2001; Fisher et al., 2011). Mutations in *SALL1* that produce a truncated protein with dominant negative activity have been detected in patients with Townes-Brockes syndrome characterized in the limb by pre-axial polydactyly and triphalangeal thumb (Kohlhase et al., 1998). A transgenic mouse model in which a truncated SALL1 protein is produced mimics the human limb phenotype (Kiefer et al., 2003). Inactivating mutations in the gene encoding the transcription factor Tbx3, which is expressed at high levels in stripes at both anterior and posterior margins of early chick and mouse limb buds (Tumpel et al., 2002; Emechebe et al., 2016) are seen in patients with Ulnar-mammary syndrome (OMIM 181450); the defects affect the development of posterior structures in the upper limb and include missing ulna, missing posterior digits and post-axial polydactyly. The same limb phenotype is seen in mouse *Tbx3* mutant embryos (Davenport et al., 2003; Emechebe et al., 2016). Finally mutations in *HOXD13* are associated with many clinical conditions in which there are digital abnormalities including polydactyly, syndactyly (fused digits) and brachydactly (short digits). *Hoxd13* is another putative gene target of Shh signaling identified in the mouse limb and is expressed in the posterior region of early chick and mouse limb buds and then throughout the digital plate at later stages (Nelson et al., 1996). A complex spectrum of mutations in *HOXD13*-polyanaline tract expansions, truncating mutations and point mutations leading to amino acid substitutions have been identified (reviewed Goodman, 2002). *Hoxd13* is likely to have several roles in digit development and the challenge is to understand how a particular genetic change leads to a particular phenotype.

Shh SIGNALING AND LIMB REGENERATION

Adult urodele amphibians (newts and salamanders) can regenerate their limbs after amputation. *Shh* signaling occurs in adult urodele limbs during regeneration and understanding how *Shh* expression is activated in these adult tissues may be relevant in the context of stimulating growth and repair of tissues in damaged limbs. Following amputation of a newt limb, a mound of undifferentiated cells called the blastema forms at the stump surface and proliferation of blastemal cells replenishes the missing limb structures. *Shh* is expressed in posterior part of the newt limb blastema recapitulating embryonic expression in the limb bud (Imokawa and Yoshizato, 1997), and when regenerating salamander limbs were treated with cyclopamine, only one digit-like structure formed—similar to hind-limbs of *Shh* mutant mice (Chiang et al., 1996). Recently, it has been demonstrated that *Shh*, which is expressed in the posterior part of the salamander blastema is part of a reciprocal feedback loop via *Grem1* and *Fgf8* that are expressed in the anterior part of the blastema (Nacu et al., 2016). This feedback loop is required for outgrowth of the blastema and closely recapitulates the epithelial-mesenchyme signaling network that drives embryonic limb development. The demonstration that two signals, which can act at a distance—Shh and Fgf8—drive limb regeneration is at odds with a long standing model in which direct cell-cell interactions stimulate intercalary growth to even out disparate positional identities between anterior and posterior parts of the blastema (French et al., 1976). The size of the limb blastema is about 10 times that of embryonic limb buds, therefore it is not clear whether these signals could indeed act over the large distances involved.

Fate maps of the blastema showing which cells give rise to the digits and experiments addressing timing of specification of antero-posterior positional values could give important insights into whether digit regeneration is comparable to embryonic development. One possibility is that cells within a blastema maintain memory of their position along the antero-posterior axis and restore missing structures by a timing mechanism linked to proliferation. Evidence for such a cellular memory based on epigenetic modifications has been obtained in regenerating limb buds of Xenopus embryos (Hayashi et al., 2015). A timing model would dispense with difficulties in scaling long range gradients over considerable distances to restore missing positional values during regeneration and the role of Shh and Fgfs would be to maintain the outgrowth and the width of the blastema. It would also be useful to know the fate of polarizing region cells from embryonic urodele limb buds in adult limbs and regenerating limbs.

Unlike urodeles, anuran amphibians can only regenerate their limbs during embryonic stages. Interestingly, increased methylation of the ZRS enhancer during *Xenopus* development correlates with reduced capacity to regenerate the limb in the adult suggesting that epigenetic mechanisms limit this process by preventing re-expression of *Shh* (Yakushiji et al., 2007).

EVOLUTIONARY ASPECTS OF Shh SIGNALING IN THE LIMB

The ZRS element located in the fifth intron of *Lmbr1* gene that drives limb-specific *Shh* expression is well conserved at the sequence level in many vertebrates. The ZRS is an excellent candidate for evolutionary modifications that have resulted in

changes in limb morphology because the rich diversity of limb morphologies could have evolved without affecting other features of the body plan. In support of this, mutations in the ZRS at a conserved ETS1 binding site in pythons have been described that appear to be responsible for the early loss of *Shh* expression and subsequent failure of limb bud outgrowth (Kvon, 2016; Leal, 2016). CRISPR/CAS9 gene editing approaches, in which the mouse ZRS was replaced by the python ZRS sequence, resulted in limb truncations similar to those obtained upon the complete removal of *Shh* function in the mouse limb (Kvon, 2016). As in pythons, *Shh* fails to be up-regulated in the hind-limbs of the spotted dolphin and is associated with reduced outgrowth, although the molecular basis of this has not been examined (Thewissen et al., 2006). Many described ZRS mutations to date, however, result in ectopic expression of *Shh* in the anterior part of the limb, and therefore the development of additional digits as in domesticated animals; for instance, Dorking's (Bouldin and Harfe, 2009) and Silkie chickens (Dunn et al., 2011) have an additional anterior digit in the leg and dogs and cats (notably Hemingway cats) have extra anterior digits in their fore-paws (Lettice et al., 2008).

Limbs with more than five digits have not been selected for during evolution suggesting there is little benefit in increasing digit number. Interestingly, the limbs of the earliest Devonian tetrapods such as *Acanthostega* and *Icthyostega* had up to eight digits (Clack, 2002). The mechanism by which such digit patterns would have been specified is of considerable interest. In having several digits, the limbs of such tetrapods superficially resemble the limbs of mouse *Gli* mutants, which have many digits that form by self-organization. However, the digits in these Devonian tetrapods display differences in phalangeal number suggestive of antero-posterior positional values specified by Shh in the early limb bud. Once pentadactyly was established in tetrapods, this has remained the basic plan, although occasionally limbs with so-called "sixth digits" have evolved. These sixth digits are in fact, adaptations of other limb bones, such as the overgrown wrist bone in the case of the mole's "paddle-like" limb (Mitgutsch et al., 2012). The chick leg has retained the basic pentadactyl phalangeal pattern in digits 1–4 and therefore is of special interest to the evolution of digit patterns. As we discussed earlier, a model in which Shh signaling specifies different positional values is sufficient to explain chick leg patterning. Thus, any deviations away from this model in the mouse limb would therefore suggest a derived mode of patterning digits 1–4 in the mammalian lineage.

Digit loss has commonly occurred over the course of evolution and alterations in *Shh* expression and response to Shh have been implicated. A striking example is seen in the wings of birds and the fore-limbs of their basal theropod dinosaur ancestors in which two digits have been lost during evolution (Sereno, 1999). Understanding this mode of digit loss has puzzled investigators for over 150 years because theropods appeared to have had digit identities 1, 2, and 3, but in the embryo at least, bird digits appear to arise from positions 2, 3, and 4 (Burke and Feduccia, 1997). Therefore, it was suggested in the so-called "frameshift" model that digits with the identities 1, 2, and 3 arise from positions 2, 3, and 4 of the bird wing (Wagner and Gauthier, 1999; Tamura et al., 2011), perhaps due to reduced Shh signaling levels/duration in limbs of the theropod ancestors of birds (Vargas and Wagner, 2009). However, the Green Fluorescent Protein fate-mapping experiments in chick wings (see **Figure 1c**) showed that in fact digits with the identities 1, 2, and 3 arise from embryonic positions 1, 2, and 3 that are found in tissue adjacent to the polarizing region (Towers et al., 2011). Therefore, it is not necessary to invoke a frameshift and suggests that the digits 4 and 5 of the dinosaur hand were simply lost and that bird wing digits should be numbered 1, 2, and 3 in line with the fossil record, as is now generally accepted. As already mentioned, in the chick wing bud, the posterior necrotic zone overlaps with the polarizing region. In the chick wing bud, the posterior necrotic zone is much larger than the corresponding zone in chick leg and mouse limb buds (Fernandez-Teran et al., 2006). Therefore, the loss of the two posterior digits in birds might be based on evolutionary changes in Shh signaling, in particular the autoregulatory mechanisms by which Shh signaling regulates apoptosis in the posterior necrotic zone of the wing bud (Sanz-Ezquerro and Tickle, 2000) and also proliferation (Chinnaiya et al., 2014). Interestingly, a recent study showed that an extension of the posterior part of the apical ectodermal ridge in the absence of Shh signaling was sufficient to enable the polarizing region to give rise to a digit in the chick wing. In such buds, the posterior necrotic zone was lost and this was accompanied by a dramatic increase in proliferation of polarizing region cells (Pickering and Towers, 2016).

Shh has also been implicated in digit loss in cow limbs in which only two digits form (3 and 4). It was revealed that *Ptch1* is expressed in the very posterior of the bud and at low levels in response to Shh signaling, because of the degeneration of a cis-regulatory enhancer. As a consequence, it is suggested that Shh fails to be sequestered and restricted to the posterior part of the cow limb bud resulting in more-or-less uniform Shh signaling which results in symmetrical and distally restricted antero-posterior gene expression patterns (Lopez-Rios et al., 2014). As a result, the two digits of the cow limb are also symmetrical and lateral digits are lost because the apical ectodermal ridge fails to extend sufficiently to support their outgrowth. Similarly, *Ptch1* is also restricted to the posterior of the limb buds of pigs that develop four digits, two of which are prominent (digits 3 and 4; Cooper et al., 2014). However, camels do not display a posterior restriction and down-regulation of *Ptch1* in their developing limb buds although they also produce two digits (3 and 4), suggesting another mechanism of digit loss (Cooper et al., 2014). An additional case of digit loss involves the limbs of different species of the Australian skink, *Hemiergis* (Shapiro, 2002). The shortened duration of *Shh* expression in these lizards correlates well with the extent of digit reduction—species with five digits express higher levels of *Shh* for a longer time than those with only two digits (Shapiro et al., 2003). Interestingly, digit reduction correlates with a reduction in cell proliferation. One possibility is that factors other than reduced Shh signaling could be involved. As yet no mutations have been reported in ZRS sequences of various *Hemiergis* clades. However, as further studies are required to understand how positional values are specified by Shh signaling in mammals and lizards, this means that it is difficult to interpret some of the patterns of evolutionary digit loss discussed in this section.

FUTURE DIRECTIONS

It is now established that Shh has a pivotal function in vertebrate limb development and many details have been uncovered. Surprisingly however, there is still no consensus about how Shh specifies antero-posterior positional values in the limb. It remains possible that different combinations of transcription factors govern antero-posterior positional values, but it has been difficult to identify them because all the digits are made up of the same differentiated cell types. Therefore, a gene-regulatory network such as one operating downstream of Shh in the neural tube to specify distinct neural fates is unlikely to operate during limb development (Balaskas et al., 2012). It is also likely that the temporal regulation of the same sets of genes could contribute to specifying positional values. For instance, there is a clear relationship between *Hoxd* expression and thumb (digit 1) development, with cells that give rise to thumb the only cells that express *Hoxd13* and not *Hoxd12* (Vargas and Fallon, 2005). Therefore, since the cells that give rise to all the other digits express *Hoxd12* and *Hoxd13*, a simple *Hox* code is unlikely to specify the digits, and perhaps timing of expression is the important determinant. Another challenge is to understand how the positional information conferred by Shh signaling is remembered and then interpreted so that digits with different identities arise in the proper places in the limb. In chick limbs, it is clear that the concentration/duration of Shh is sufficient to specify digit identity, however, this is not readily apparent in mammalian limbs because the digits are morphologically similar—at least in terms of phalangeal number. It will be important to fill this gap in knowledge in order to apply the principles to developing human limbs and gain deeper insights into the basis of congenital limb defects and to evolutionary alteration in digit pattern. The analysis of the function of Shh in new animal models of limb development could help resolve issues regarding the relationship between positional values and digit identity. Further development of the CRISPR/Cas9 system should facilitate this.

An issue of general relevance is the mode of Shh transport in the limb and how a graded distribution of Shh is established. This may require further refinement of *in vivo* imaging techniques to visualize directly the distribution of Shh in real time. It also seems clear that the timing of *Shh* expression is another critical parameter that still needs to be addressed. Disentangling the relationship between autoregulatory mechanisms of intrinsic timing of Shh expression and extrinsic mechanisms could shed light on processes that ensure robustness of limb development and pattern scaling between different species.

AUTHOR CONTRIBUTIONS

MT and CT jointly wrote the manuscript, MT formatted the Figures.

ACKNOWLEDGMENTS

MT is supported by the Wellcome Trust (Grant number 202756/Z/16/Z).

REFERENCES

Ahn, S., and Joyner, A. L. (2004). Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell* 118, 505–516. doi: 10.1016/j.cell.2004.07.023

Alby, C., Piquand, K., Huber, C., Megarbane, A., Ichkou, A., Legendre, M., et al. (2015). Mutations in KIAA0586 Cause Lethal Ciliopathies Ranging from a Hydrolethalus Phenotype to Short-Rib Polydactyly Syndrome. *Am. J. Hum. Genet.* 97, 311–318. doi: 10.1016/j.ajhg.2015.06.003

Allen, B. L., Tenzen, T., and McMahon, A. P. (2007). The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. *Genes Dev.* 21, 1244–1257. doi: 10.1101/gad.1543607

Amano, T., Sagai, T., Tanabe, H., Mizushina, Y., Nakazawa, H., and Shiroishi, T. (2009). Chromosomal dynamics at the Shh locus: limb bud-specific differential regulation of competence and active transcription. *Dev. Cell* 16, 47–57. doi: 10.1016/j.devcel.2008.11.011

Anderson, C., Williams, V. C., Moyon, B., Daubas, P., Tajbakhsh, S., Buckingham, M. E., et al. (2012). Sonic hedgehog acts cell-autonomously on muscle precursor cells to generate limb muscle diversity. *Genes Dev.* 26, 2103–2117. doi: 10.1101/gad.187807.112

Ashe, A., Butterfield, N. C., Town, L., Courtney, A. D., Cooper, A. N., Ferguson, C., et al. (2012). Mutations in mouse Ift144 model the craniofacial, limb and rib defects in skeletal ciliopathies. *Hum. Mol. Genet.* 21, 1808–1823. doi: 10.1093/hmg/ddr613

Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K. M., et al. (2012). Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell* 148, 273–284. doi: 10.1016/j.cell.2011.10.047

Bangs, F., Antonio, N., Thongnuek, P., Welten, M., Davey, M. G., Briscoe, J., et al. (2011). Generation of mice with functional inactivation of talpid3, a gene first identified in chicken. *Development* 138, 3261–3272. doi: 10.1242/dev.063602

Bangs, F., Welten, M., Davey, M. G., Fisher, M., Yin, Y., Downie, H., et al. (2010). Identification of genes downstream of the Shh signalling in the developing chick wing and syn-expressed with Hoxd13 using microarray and 3D computational analysis. *Mech. Dev.* 127, 428–441. doi: 10.1016/j.mod.2010.08.001

Bastida, M. F., Sheth, R., and Ros, M. A. (2009). A BMP-Shh negative-feedback loop restricts Shh expression during limb development. *Development* 136, 3779–3789. doi: 10.1242/dev.036418

Biesecker, L. G. (2011). Polydactyly: how many disorders and how many genes? 2010 update. *Dev. Dyn.* 240, 931–942. doi: 10.1002/dvdy.22609

Bouldin, C. M., Gritli-Linde, A., Ahn, S., and Harfe, B. D. (2010). Shh pathway activation is present and required within the vertebrate limb bud apical ectodermal ridge for normal autopod patterning. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5489–5494. doi: 10.1073/pnas.0912818107

Bouldin, C. M., and Harfe, B. D. (2009). Aberrant FGF signaling, independent of ectopic hedgehog signaling, initiates preaxial polydactyly in Dorking chickens. *Dev. Biol.* 334, 133–141. doi: 10.1016/j.ydbio.2009.07.009

Bowers, M., Eng, L., Lao, Z., Turnbull, R. K., Bao, X., Riedel, E., et al. (2012). Limb anterior-posterior polarity integrates activator and repressor functions of GLI2 as well as GLI3. *Dev. Biol.* 370, 110–124. doi: 10.1016/j.ydbio.2012.07.017

Buck, A., Kispert, A., and Kohlhase, J. (2001). Embryonic expression of the murine homologue of SALL1, the gene mutated in Townes-Brocks syndrome. *Mech. Dev.* 104, 143–146. doi: 10.1016/S0925-4773(01)00364-1

Burke, A., and Feduccia, A. (1997). Developmental Patterns and the Identification of Homologies in the Avian Hand. *Science* 278, 666–668. doi: 10.1126/science.278.5338.666

Buscher, D., and Ruther, U. (1998). Expression profile of Gli family members and Shh in normal and mutant mouse limb development. *Develop. Dyn.* 211, 88–96.

Butterfield, N. C., Metzis, V., McGlinn, E., Bruce, S. J., Wainwright, B. J., and Wicking, C. (2009). Patched 1 is a crucial determinant of asymmetry

and digit number in the vertebrate limb. *Development* 136, 3515–3524. doi: 10.1242/dev.037507

Chang, C. F., Schock, E. N., OHare, E. A., Dodgson, J., Cheng, H. H., Muir, W. M., et al. (2014). The cellular and molecular etiology of the craniofacial defects in the avian ciliopathic mutant talpid2. *Development* 141, 3003–3012. doi: 10.1242/dev.105924

Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M., and Chuang, P. T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* 18, 641–659. doi: 10.1101/gad.1185804

Chevalier, A. K. M., and Mauger, A. (1977). Limb-somite relationship: origin of the limb musculature. *J. Embryol. Exp. Morphol.* 41, 245–258.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., et al. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383, 407–413. doi: 10.1038/383407a0

Chinnaiya, K., Tickle, C., and Towers, M. (2014). Sonic hedgehog-expressing cells in the developing limb measure time by an intrinsic cell cycle clock. *Nat. Commun.* 5:4230. doi: 10.1038/ncomms5230

Clack, J. A. (2002). An early tetrapod from 'Romer's Gap'. *Nature* 418, 72–76. doi: 10.1038/nature00824

Cooke, J., and Summerbell, D. (1980). Cell cycle and experimental pattern duplication in the chick wing during embryonic development. *Nature* 287, 697–701. doi: 10.1038/287697a0

Cooper, K. L., Hu, J. K., ten Berge, D., Fernandez-Teran, M., Ros, M. A., and Tabin, C. J. (2011). Initiation of proximal-distal patterning in the vertebrate limb by signals and growth. *Science* 332, 1083–1086. doi: 10.1126/science.1199499

Cooper, K. L., Sears, K. E., Uygur, A., Maier, J., Baczkowski, K. S., Brosnahan, M., et al. (2014). Patterning and post-patterning modes of evolutionary digit loss in mammals. *Nature* 511, 41–45. doi: 10.1038/nature13496

Dahn, R. D., Davis, M. C., Pappano, W. N., and Shubin, N. H. (2007). Sonic hedgehog function in chondrichthyan fins and the evolution of appendage patterning. *Nature* 445, 311–314. doi: 10.1038/nature05436

Dahn, R. D., and Fallon, J. F. (2000). Interdigital regulation of digit identity and homeotic transformation by modulated BMP signaling. *Science* 289, 438–441. doi: 10.1126/science.289.5478.438

Davenport, T. G., Jerome-Majewska, L. A., and Papaioannou, V. E. (2003). Mammary gland, limb and yolk sac defects in mice lacking Tbx3, the gene mutated in human ulnar mammary syndrome. *Development* 130, 2263–2273. doi: 10.1242/dev.00431

Davey, M. G., James, J., Paton, I. R., Burt, D. W., and Tickle, C. (2007). Analysis of talpid3 and wild-type chicken embryos reveals roles for Hedgehog signalling in development of the limb bud vasculature. *Dev. Biol.* 301, 155–165. doi: 10.1016/j.ydbio.2006.08.017

Davey, M. G., Paton, I. R., Yin, Y., Schmidt, M., Bangs, F. K., Morrice, D. R., et al. (2006). The chicken talpid3 gene encodes a novel protein essential for Hedgehog signaling. *Genes Dev.* 20, 1365–1377. doi: 10.1101/gad.369106

Delgado, I., and Torres, M. (2016). Gradients, waves and timers, an overview of limb patterning models. *Semin. Cell Dev. Biol.* 49, 109–115. doi: 10.1016/j.semcdb.2015.12.016

Drossopoulou, G., Lewis, K. E., Sanz-Ezquerro, J. J., Nikbakht, N., McMahon, A. P., Hofmann, C., et al. (2000). A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling. *Development* 127, 1337–1348.

Dunn, I. C., Paton, I. R., Clelland, A. K., Sebastian, S., Johnson, E. J., McTeir, L., et al. (2011). The chicken polydactyly (Po) locus causes allelic imbalance and ectopic expression of Shh during limb development. *Dev. Dyn.* 240, 1163–1172. doi: 10.1002/dvdy.22623

Duprez, D., Lapointe, F., Edom-Vovard, F., Kostakopoulou, K., and Robson, L. (1999). Sonic Hedgehog (SHH) specifies muscle pattern at tissue and cellular chick level, in the chick limb bud. *Mech. Dev.* 82, 151–163.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., et al. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75, 1417–1430. doi: 10.1016/0092-8674(93)90627-3

Ede, D. A., and Kelly, W. A. (1964). Developmental abnormalities in the trunk and limbs of the Talpid3 mutant of the fowl. *J. Embryol. Exp. Morphol.* 12, 339–356.

Elisa Piedra, M., Borja Rivero, F., Fernandez-Teran, M., and Ros, M. A. (2000). Pattern formation and regulation of gene expressions in chick recombinant limbs. *Mech. Dev.* 90, 167–179. doi: 10.1016/S0925-4773(99)00247-6

Emechebe, U., Kumar, P. P., Rozenberg, J. M., Moore, B., Firment, A., Mirshahi, T., et al. (2016). T-box3 is a ciliary protein and regulates stability of the Gli3 transcription factor to control digit number. *Elife* 5. doi: 10.7554/elife.07897

Fallon, J. F., and Crosby, G. M. (1977). "Polarizing region zone activity in the limb buds of amniotes," in *Vertebrate Limb and Somite Morphogenesis,* eds D. A. Ede, J. R. Hinchliffe, and M. Balls (Cambridge: Cambridge University Press), 55–70.

Farin, H. F., Lütdke, T. H., Schmidt, M. K., Placzko, S., Schuster-Gossler, K., Petry, M., et al. (2013), Tbx2 terminates shh/fgf signaling in the developing mouse limb bud by direct repression of *gremlin1*. *PLoS Genet*. 9:e1003467. doi: 10.1371/journal.pgen.1003467

Fernandez-Teran, M. A., Hinchliffe, J. R., and Ros, M. A. (2006). Birth and death of cells in limb development: a mapping study. *Dev. Dyn.* 235, 2521–2537. doi: 10.1002/dvdy.20916

Fisher, M., Downie, H., Welten, M. C. M., Delgado, I., Bain, A., Planzer, T., et al. (2011). Comparative analysis of 3D expression patterns of transcription factor genes and digit fate maps in the developing chick wing. *PLoS ONE* 6:e18661. doi: 10.1371/journal.pone.0018661

Forsythe, E., and Beales, P. L. (2013). Bardet-Biedl syndrome. *Eur. J. Hum. Genet.* 21, 8–13. doi: 10.1038/ejhg.2012.115

French, V., Bryant, P. J., and Bryant, S. V. (1976). Pattern formation in epimorphic fields. *Science* 2547, 969–981. doi: 10.1126/science.948762

Galli, A., Robay, D., Osterwalder, M., Bao, X., Benazet, J. D., Tariq, M., et al. (2010). Distinct roles of Hand2 in initiating polarity and posterior Shh expression during the onset of mouse limb bud development. *PLoS Genet.* 6:e1000901. doi: 10.1371/journal.pgen.1000901

Gofflot, F., Hars, C., Illien, F., Chevy, F., Wolf, C., Picard, J. J., et al. (2003). Molecular mechanisms underlying limb anomalies associated with cholesterol deficiency during gestation: implications of Hedgehog signaling. *Hum. Mol. Genet.* 12, 1187–1198. doi: 10.1093/hmg/ddg129

Goodman, F. R. (2002). Limb malformations and the human HOX genes. *Am. J. Med. Genet.* 112, 256–265. doi: 10.1002/ajmg.10776

Gritli-Linde, A., Lewis, P., McMahon, A. P., and Linde, A. (2001). The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. *Dev. Biol.* 236, 364–386. doi: 10.1006/dbio.2001.0336

Gurdon, J. B., Mitchell, A., and Mahony, D. (1995). Direct and continuous assessment by cells of their position in a morphogen gradient. *Nature* 376, 520–521. doi: 10.1038/376520a0

Hardy, A., Richardson, M. K., Francis-West, P. H., Rodriguez, C., Izpisua-Belmonte, J. C., Duprez, D., et al. (1995). Gene expression, polarising activity and skeletal patterning in reaggregated hind limb mesenchyme. *Development* 121, 4329–4337.

Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P., and Tabin, C. J. (2004). Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* 118, 517–528. doi: 10.1016/j.cell.2004.07.024

Hayashi, S., Akiyama, R., Wong, J., Tahara, N., Kawakami, H., and Kawakami, Y. (2016). Gata6-dependent GLI3 repressor function is essential in anterior limb progenitor cells for proper limb development. *PLoS Genet.* 12:e1006138. doi: 10.1371/journal.pgen.1006138

Hayashi, S., Kawaguchi, A., Uchiyama, I., Kawasumi-Kita, A., Kobayashi, T., Nishide, H., et al. (2015). Epigenetic modification maintains intrinsic limb-cell identity in Xenopus limb bud regeneration. *Dev. Biol.* 406, 271–282. doi: 10.1016/j.ydbio.2015.08.013

Hill, P., Wang, B., and Ruther, U. (2007). The molecular basis of Pallister Hall associated polydactyly. *Hum. Mol. Genet.* 16, 2089–2096. doi: 10.1093/hmg/ddm156

Hill, R. E., and Lettice, L. A. (2013). Alterations to the remote control of Shh gene expression cause congenital abnormalities. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 368:20120357. doi: 10.1098/rstb.2012.0357

Honig, L. S. (1981). Positional signal transmission in the developing chick limb. *Nature* 291, 72–73. doi: 10.1038/291072a0

Hu, J. K. H., McGlinn, E., Harfe, B. D., Kardon, G., and Tabin, C. J. (2012). Autonomous and nonautonomous roles of Hedgehog signaling in regulating limb muscle formation. *Genes Dev.* 26, 2088–2102. doi: 10.1101/gad.187385.112

Huang, B. L., Trofka, A., Furusawa, A., Norrie, J. L., Rabinowitz, A. H., Vokes, S. A., et al. (2016). An interdigit signalling centre instructs coordinate phalanx-joint formation governed by 5' Hoxd-Gli3 antagonism. *Nat. Communications* 7. doi: 10.1038/ncomms12903

Huang, P. X., Nedelcu, D., Watanabe, M., Jao, C., Kim, Y., Liu, J., et al. (2016). Cellular cholesterol directly activates smoothened in hedgehog signaling. *Cell* 166, 1176–1184. doi: 10.1016/j.cell.2016.08.003

Huangfu, D., Liu, A., Rakeman, A. S., Murcia, N. S., Niswander, L., and Anderson, K. V. (2003). Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* 426, 83–87. doi: 10.1038/nature02061

Imokawa, Y., and Yoshizato, K. (1997). Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proc. Natl. Acad. Sci. U.S.A.* 94, 9159–9164. doi: 10.1073/pnas.94.17.9159

Kalff-Suske, M., Wild, A., Topp, J., Wessling, M., Jacobsen, E. M., Bornholdt, D., et al. (1999). Point mutations throughout the GLI3 gene cause Greig cephalopolysyndactyly syndrome. *Hum. Mol. Genet.* 8, 1769–1777. doi: 10.1093/hmg/8.9.1769

Kerszberg, M., and Wolpert, L. (2007). Specifying positional information in the embryo: looking beyond morphogens. *Cell* 130, 205–209. doi: 10.1016/j.cell.2007.06.038

Kiefer, S. M., Ohlemiller, K. K., Yang, J., McDill, B. W., Kohlhase, J., and Rauchman, M. (2003). Expression of a truncated Sall1 transcriptional repressor is responsible for Townes-Brocks syndrome birth defects. *Hum. Mol. Genet.* 12, 2221–2227. doi: 10.1093/hmg/ddg233

Kohlhase, J., Wischermann, A., Reichenbach, H., Froster, U., and Engel, W. (1998). Mutations in the SALL1 putative transcription factor gene cause Townes-Brocks syndrome. *Nat. Genet.* 18, 81–83. doi: 10.1038/ng0198-81

Kozhemyakina, E., Ionescu, A., and Lassar, A. B. (2014). GATA6 is a crucial regulator of Shh in the Limb Bud. *PLoS Genet.* 10:e1004072. doi: 10.1371/journal.pgen.1004072

Kruger, M., Mennerich, D., Fees, S., Schafer, R., Mundlos, S., and Braun, T. (2001). Sonic hedgehog is a survival factor for hypaxial muscles during mouse development. *Development* 128, 743–752.

Kvon, K. V. V. A. (2016). Progressive loss of function in a limb enhancer during snake evolution. *Cell* 633–642. doi: 10.1016/j.cell.2016.09.028

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A., and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 79, 993–1003. doi: 10.1016/0092-8674(94)90030-2

Leal, F. C. M. (2016). Loss and re-emergence of legs in snakes by modular evolution of sonic hedgehog and HOXD enhancers. *Curr. Biol.* 2966–2973. doi: 10.1016/j.cub.2016.09.020

Lee, R. T. H., Zhao, Z. H., and Ingham, P. W. (2016). Hedgehog signalling. *Development* 143, 367–372. doi: 10.1242/dev.120154

Lettice, L. A., Hill, A. E., Devenney, P. S., and Hill, R. E. (2008). Point mutations in a distant sonic hedgehog cis-regulator generate a variable regulatory output responsible for preaxial polydactyly. *Hum. Mol. Genet.* 17, 978–985. doi: 10.1093/hmg/ddm370

Lettice, L. A., Horikoshi, T., Heaney, S. J., van Baren, M. J., van der Linde, H. C., Breedveld, G. J., et al. (2002). Disruption of a long-range cis-acting regulator for Shh causes preaxial polydactyly. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7548–7553. doi: 10.1073/pnas.112212199

Lettice, L. A., Williamson, I., Wiltshire, J. H., Peluso, S., Devenney, P. S., Hill, A. E., et al. (2012). Opposing functions of the ETS factor family define Shh spatial expression in limb buds and underlie polydactyly. *Dev. Cell* 22, 459–467. doi: 10.1016/j.devcel.2011.12.010

Lewandowski, J. P., Du, F., Zhang, S. L., Powell, M. B., Falkenstein, K. N., Ji, H. K., et al. (2015). Spatiotemporal regulation of GLI target genes in the mammalian limb bud. *Dev. Biol.* 406, 92–103. doi: 10.1016/j.ydbio.2015.07.022

Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B., et al. (2001). Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* 105, 599–612. doi: 10.1016/S0092-8674(01)00369-5

Li, Y., Zhang, H., Litingtung, Y., and Chiang, C. (2006). Cholesterol modification restricts the spread of Shh gradient in the limb bud. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6548–6553. doi: 10.1073/pnas.0600124103

Litingtung, Y., Dahn, R. D., Li, Y., Fallon, J. F., and Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* 418, 979–983. doi: 10.1038/nature01033

Lohan, S., Spielmann, M., Doelken, S. C., Flottmann, R., Muhammad, F., Baig, S. M., et al. (2014). Microduplications encompassing the Sonic hedgehog limb enhancer ZRS are associated with Haas-type polysyndactyly and Laurin-Sandrow syndrome. *Clin. Genet.* 86, 318–325. doi: 10.1111/cge.12352

Lopez-Rios, J., Duchesne, A., Speziale, D., Andrey, G., Peterson, K. A., Germann, P., et al. (2014). Attenuated sensing of SHH by Ptch1 underlies evolution of bovine limbs. *Nature* 511, 46–51. doi: 10.1038/nature13289

Maas, S. A., and Fallon, J. F. (2004). Isolation of the chicken Lmbr1 coding sequence and characterization of its role during chick limb development. *Dev. Dyn.* 229, 520–528. doi: 10.1002/dvdy.10502

MacCabe, J. A., Saunders, J. W. Jr. and Pickett, M. (1973). The control of the anteroposterior and dorsoventral axes in embryonic chick limbs constructed of dissociated and reaggregated limb-bud mesoderm. *Dev. Biol.* 31, 323–335. doi: 10.1016/0012-1606(73)90269-8

Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V., and Tabin, C. J. (1996). Conservation in hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* 122, 1225–1233.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K., and Shiroishi, T. (1995). A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* 9, 1645–1653. doi: 10.1101/gad.9.13.1645

Mitgutsch, C., Richardson, M. K., Jimenez, R., Martin, J. E., Kondrashov, P., de Bakker, M. A., et al. (2012). Circumventing the polydactyly 'constraint': the mole's 'thumb'. *Biol. Lett.* 8, 74–77. doi: 10.1098/rsbl.2011.0494

Mo, R., Freer, A. M., Zinyk, D. L., Crackower, M. A., Michaud, J., Heng, H. H., et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* 124, 113–123.

Muller, P., Rogers, K. W., Yu, S. Z. R., Brand, M., and Schier, A. F. (2013). Morphogen transport. *Development* 140, 1621–1638. doi: 10.1242/dev.083519

Nacu, E., Gromberg, E., Oliveira, C. R., Drechsel, D., and Tanaka, E. M. (2016). FGF8 and SHH substitute for anterior-posterior tissue interactions to induce limb regeneration. *Nature* 533, 407–410. doi: 10.1038/nature17972

Nelson, C. E., Morgan, B. A., Burke, A. C., Laufer, E., DiMambro, E., Murtaugh, L. C., et al. (1996). Analysis of Hox gene expression in the chick limb bud. *Development* 122, 1449–1466.

Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P., and Dolle, P. (2002). Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. *Development* 129, 3563–3574.

Niswander, L., Jeffrey, S., Martin, G. R., and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371, 609–612. doi: 10.1038/371609a0

Noji, S., Nohno, T., Koyama, E., Muto, K., Ohyama, K., Aoki, Y., et al. (1991). Retinoic acid induces polarizing activity but is unlikely to be a morphogen in the chick limb bud. *Nature* 350, 83–86. doi: 10.1038/350083a0

Odent, S., Attie-Bitach, T., Blayau, M., Mathieu, M., Auge, J., Delezoide, A. L., et al. (1999). Expression of the Sonic hedgehog (SHH) gene during early human development and phenotypic expression of new mutations causing holoprosencephaly. *Hum. Mol. Genet.* 8, 1683–1689. doi: 10.1093/hmg/8.9.1683

Ohsugi, K., Gardiner, D. M., and Bryant, S. V. (1997). Cell cycle length affects gene expression and pattern formation in limbs. *Dev. Biol.* 189, 13–21. doi: 10.1006/dbio.1997.8665

Pagan, S. M., Ros, M. A., Tabin, C., and Fallon, J. F. (1996). Surgical removal of limb bud Sonic hedgehog results in posterior skeletal defects. *Dev. Biol.* 180, 35–40. doi: 10.1006/dbio.1996.0282

Panman, L., Galli, A., Lagarde, N., Michos, O., Soete, G., Zuniga, A., et al. (2006). Differential regulation of gene expression in the digit forming area of the mouse limb bud by SHH and gremlin 1/FGF-mediated epithelial-mesenchymal signalling. *Development* 133, 3419–3428. doi: 10.1242/dev.02529

Park, H. L., Bai, C., Platt, K. A., Matise, M. P., Beeghly, A., Hui, C. C., et al. (2000). Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. *Development* 127, 1593–1605.

Parr, B. A., and McMahon, A. P. (1995). Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* 374, 350–353. doi: 10.1038/374350a0

Pautou, M. P. (1973). Morphogenesis of the feet of birds using interspecific cellular mixtures. I. Morphological study. *J. Embryol. Exp. Morphol.* 29, 175–196.

Pickering, J., and Towers, M. (2014). *Molecular Genetics of Human Congenital Limb Malformations.* Chichester: eLS John Wiley & Sons Ltd.

Pickering, J., and Towers, M. (2016). Inhibition of Shh signalling in the chick wing gives insights into digit patterning and evolution. *Development* 143, 3514–3521. doi: 10.1242/dev.137398

Raspopovic, J., Marcon, L., Russo, L., and Sharpe, J. (2014). Modeling digits. Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science* 345, 566–570. doi: 10.1126/science.1252960

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75, 1401–1416. doi: 10.1016/0092-8674(93)90626-2

Robson, L. G., Kara, T., Crawley, A., and Tickle, C. (1994). Tissue and cellular patterning of the musculature in chick wings. *Development* 120, 1265–1276.

Roosing, S., Hofree, M., Kim, S., Scott, E., Copeland, B., Romani, M., et al. (2015). Functional genome-wide siRNA screen identifies KIAA0586 as mutated in Joubert syndrome. *Elife* 4. doi: 10.7554/eLife.06602

Ros, M. A., Dahn, R. D., Fernandez-Teran, M., Rashka, K., Caruccio, N. C., Hasso, S. M., et al. (2003). The chick oligozeugodactyly (ozd) mutant lacks sonic hedgehog function in the limb. *Development* 130, 527–537. doi: 10.1242/dev.00245

Rosello-Diez, A., Ros, M. A., and Torres, M. (2011). Diffusible signals, not autonomous mechanisms, determine the main proximodistal limb subdivision. *Science* 332, 1086–1088. doi: 10.1126/science.1199489

Sagai, T., Amano, T., Tamura, M., Mizushina, Y., Sumiyama, K., and Shiroishi, T. (2009). A cluster of three long-range enhancers directs regional Shh expression in the epithelial linings. *Development* 136, 1665–1674. doi: 10.1242/dev.032714

Sagai, T., Hosoya, M., Mizushina, Y., Tamura, M., and Shiroishi, T. (2005). Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb. *Development* 132, 797–803. doi: 10.1242/dev.01613

Saiz-Lopez, P., Chinnaiya, K., Campa, V. M., Delgado, I., Ros, M. A., and Towers, M. (2015). An intrinsic timer specifies distal structures of the vertebrate limb. *Nat. Commun.* 6:8108. doi: 10.1038/ncomms9108

Sanders, T. A., Llagostera, E., and Barna, M. (2013). Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. *Nature* 497, 628–632. doi: 10.1038/nature12157

Sanz-Ezquerro, J. J., and Tickle, C. (2000). Autoregulation of Shh expression and Shh induction of cell death suggest a mechanism for modulating polarising activity during chick limb development. *Development* 127, 4811–4823.

Saunders, J. W. Jr. and Gasseling, M. T. (1962). Cellular death in morphogenesis of the avian wing. *Dev. Biol.* 5, 147–178. doi: 10.1016/0012-1606(62)90008-8

Saunders, J. W., and Gasseling, M. T. (1968). "Ectodermal-mesenchymal interactions in the origin of limb symmetry," in *Mesenchymal-Epithelial Interactions,* eds R. Fleischmeyer and R. E. Billingham (Baltimore, MD: Williams and Wilkins), 78–97.

Scherz, P. J., Harfe, B. D., McMahon, A. P., and Tabin, C. J. (2004). The limb bud Shh-Fgf feedback loop is terminated by expansion of former ZPA cells. *Science* 305, 396–399. doi: 10.1126/science.1096966

Scherz, P. J., McGlinn, E., Nissim, S., and Tabin, C. J. (2007). Extended exposure to Sonic hedgehog is required for patterning the posterior digits of the vertebrate limb. *Dev. Biol.* 308, 343–354. doi: 10.1016/j.ydbio.2007.05.030

Schmidt, K., Hughes, C., Chudek, J. A., Goodyear, S. R., Aspden, R. M., Talbot, R., et al. (2009). Cholesterol metabolism: the main pathway acting downstream of Cytochrome P450 oxidoreductase in skeletal development of the limb. *Mol. Cell. Biol.* 29, 2716–2729. doi: 10.1128/MCB.01638-08

Sereno, P. C. (1999). The evolution of dinosaurs. *Science* 284, 2137–2147. doi: 10.1126/science.284.5423.2137

Shaheen, R., Ansari, S., Mardawi, E. A., Alshammari, M. J., and Alkuraya, F. S. (2013). Mutations in TMEM231 cause Meckel-Gruber syndrome. *J. Med. Genet.* 50, 160–162. doi: 10.1136/jmedgenet-2012-101431

Shapiro, M. D. (2002). Developmental morphology of limb reduction in Hemiergis (Squamata: Scincidae): chondrogenesis, osteogenesis, and heterochrony. *J. Morphol.* 254, 211–231. doi: 10.1002/jmor.10027

Shapiro, M. D., Hanken, J., and Rosenthal, N. (2003). Developmental basis of evolutionary digit loss in the Australian lizard Hemiergis. *J. Exp. Zool.* 297B, 48–56. doi: 10.1002/jez.b.19

Sharpe, J., Lettice, L., Hecksher-Sorensen, J., Fox, M., Hill, R., and Krumlauf, R. (1999). Identification of sonic hedgehog as a candidate gene responsible for the polydactylous mouse mutant Sasquatch. *Curr. Biol.* 9, 97–100. doi: 10.1016/S0960-9822(99)80022-0

Shellswell, G., and B., Wolpert, L. (1977). "The pattern of muscle development in the chick wing," in *Vertebrate Limb and Somite Morphogenesis,* eds D. A. Ede, J. R. Hinchliffe, and M. Balls (Cambridge: Cambridge University Press), 71–87.

Sheth, R., Marcon, L., Bastida, M. F., Junco, M., Quintana, L., Dahn, R., et al. (2012). Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. *Science* 338, 1476–1480. doi: 10.1126/science.1226804

Smith, J. C. (1980). The time required for positional signalling in the chick wing bud. *J. Embryol. Exp. Morphol.* 60, 321–328.

Smith, J. C., and Wolpert, L. (1981). Pattern formation along the anteroposterior axis of the chick wing: the increase in width following a polarizing region graft and the effect of X-irradiation. *J. Embryol. Exp. Morphol.* 63, 127–144.

Stephen, L. A., Tawamie, H., Davis, G. M., Tebbe, L., Nurnberg, P., Nurnberg, G., et al. (2015). TALPID3 controls centrosome and cell polarity and the human ortholog KIAA0586 is mutated in Joubert syndrome (JBTS23). *Elife* 4. doi: 10.7554/eLife.08077

Stopper, G. F., Richards-Hrdlicka, K. L., and Wagner, G. P. (2016). Hedgehog inhibition causes complete loss of limb outgrowth and transformation of digit identity in *Xenopus tropicalis*. *J. Exp. Zool. B Mol. Dev. Evol.* 326, 110–124. doi: 10.1002/jez.b.22669

Stratford, T., Horton, C., and Maden, M. (1996). Retinoic acid is required for the initiation of outgrowth in the chick limb bud. *Curr. Biol.* 6, 1124–1133. doi: 10.1016/S0960-9822(02)70679-9

Stratford, T., Logan, C., Zile, M., and Maden, M. (1999). Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. *Mech. Dev.* 81, 115–125. doi: 10.1016/S0925-4773(98)00231-7

Summerbell, D. (1974). Interaction between the proximo-distal and antero-posterior co-ordinates of positional value during the specification of positional information in the early development of the chick limb-bud. *J. Embryol. Exp. Morphol.* 32, 227–237.

Summerbell, D., and Tickle, C. (1977). "Pattern formation along the anterior- posterior axis of the chick limb bud," in *Vertebrate Limb and Somite Morphogenesis,* eds D. A. Ede, J. R. Hinchliffe, and M. Balls (Cambridge:Cambridge University Press), 41–53.

Suzuki, T., Hasso, S. M., and Fallon, J. F. (2008). Unique SMAD1/5/8 activity at the phalanx-forming region determined digit identity. *Proc. Natl. Acad. Sci. U.S.A.* 11, 4185–4190. doi: 10.1073/pnas.0707899105

Suzuki, T., Takeuchi, J., Koshiba-Takeuchi, K., and Ogura, T. (2004). Tbx genes specify posterior digit identity through Shh and BMP signaling. *Dev. Cell* 6, 43–53. doi: 10.1016/S1534-5807(03)00401-5

Tamura, K., Nomura, N., Seki, R., Yonei-Tamura, S., and Yokoyama, H. (2011). Embryological evidence identifies wing digits in birds as digits 1, 2, and 3. *Science* 331, 753–757. doi: 10.1126/science.1198229

te Welscher, P., Zuniga, A., Kuijper, S., Drenth, T., Goedemans, H. J., Meijlink, F., et al. (2002). Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* 298, 827–830. doi: 10.1126/science.1075620

Thewissen, J. G. M., Cohn, M. J., Stevens, L. S., Bajpai, S., Heyning, J., and Horton, W. E. (2006). Developmental basis for hind-limb loss in dolphins and origin of the cetacean bodyplan. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8414–8418. doi: 10.1073/pnas.0602920103

Tian, H., Jeong, J. H., Harfe, B. D., Tabin, C. J., and McMahon, A. P. (2005). Mouse Disp1 is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand. *Development* 132, 133–142. doi: 10.1242/dev.01563

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* 289, 295–298. doi: 10.1038/289295a0

Tickle, C. (1995). Vertebrate limb development. *Curr. Opin. Genet. Dev.* 5, 478–484. doi: 10.1016/0959-437X(95)90052-I

Tickle, C., Alberts, B., Wolpert, L., and Lee, J. (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature* 296, 564–566. doi: 10.1038/296564a0

Tickle, C., Lee, J., and Eichele, G. (1985). A quantitative analysis of the effect of all-trans-retinoic acid on the pattern of chick wing development. *Dev. Biol.* 109, 82–95. doi: 10.1016/0012-1606(85)90348-3

Tickle, C., Shellswell, G., Crawley, A., and Wolpert, L. (1976). Positional signalling by mouse limb polarising region in the chick wing bud. *Nature* 259, 396–397. doi: 10.1038/259396a0

Tickle, C., Summerbell, D., and Wolpert, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* 254, 199–202. doi: 10.1038/254199a0

Towers, M., Mahood, R., Yin, Y., and Tickle, C. (2008). Integration of growth and specification in chick wing digit-patterning. *Nature* 452, 882–886. doi: 10.1038/nature06718

Towers, M., Signolet, J., Sherman, A., Sang, H., and Tickle, C. (2011). Insights into bird wing evolution and digit specification from polarizing region fate maps. *Nat. Commun.* 2:426. doi: 10.1038/ncomms1437

Towers, M., and Tickle, C. (2009). Growing models of vertebrate limb development. *Development* 136, 179–190. doi: 10.1242/dev.024158

Tumpel, S., Sanz-Ezquerro, J. J., Isaac, A., Eblaghie, M. C., Dobson, J., and Tickle, C. (2002). Regulation of Tbx3 expression by anteroposterior signalling in vertebrate limb development. *Dev. Biol.* 250, 251–262. doi: 10.1006/dbio.2002.0762

Turing, A. M. (1952). The chemical basis of morphogenesis. *Philos. Trans. R. Soc. Lond. B* 237, 37–72. doi: 10.1098/rstb.1952.0012

VanderMeer, J. E., Lozano, R., Sun, M., Xue, Y., Daentl, D., Jabs, E. W., et al. (2014). A novel ZRS Mutation Leads to Preaxial Polydactyly Type 2 in a Heterozygous Form and Werner Mesomelic Syndrome in a Homozygous Form. *Hum. Mutat.* 35, 945–948. doi: 10.1002/humu.22581

Vargas, A. O., and Fallon, J. F. (2005). Birds have dinosaur wings: the molecular evidence. *J. Exp. Zool. B Mol. Dev. Evol.* 304, 86–90. doi: 10.1002/jez.b.21023

Vargas, A. O., and Wagner, G. P. (2009). Frame-shifts of digit identity in bird evolution and Cyclopamine-treated wings. *Evol. Dev.* 11, 163–169. doi: 10.1111/j.1525-142X.2009.00317.x

Vargesson, N., Clarke, J. D., Vincent, K., Coles, C., Wolpert, L., and Tickle, C. (1997). Cell fate in the chick limb bud and relationship to gene expression. *Development* 124, 1909–1918.

Vergnes, L., Chin, R. G., Vallim, T. D., Fong, L. G., Osborne, T. F., Young, S. G., et al. (2016). SREBP-2-deficient and hypomorphic mice reveal roles for SREBP-2 in embryonic development and SREBP-1c expression. *J. Lipid Res.* 57, 410–421. doi: 10.1194/jlr.M064022

Verheyden, J. M., and Sun, X. (2008). An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. *Nature* 454, 638–641. doi: 10.1038/nature07085

Vokes, S. A., Ji, H., Wong, W. H., and McMahon, A. P. (2008). A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev.* 22, 2651–2663. doi: 10.1101/gad.1693008

Wagner, G. P., and Gauthier, J. A. (1999). 1,2,3 = 2,3,4: a solution to the problem of the homology of the digits in the avian hand. *Proc. Natl. Acad. Sci. U.S.A.* 96, 5111–5116. doi: 10.1073/pnas.96.9.5111

Wanek, N., Gardiner, D. M., Muneoka, K., and Bryant, S. V. (1991). Conversion by retinoic acid of anterior cells into ZPA cells in the chick wing bud. *Nature* 350, 81–83. doi: 10.1038/350081a0

Wang, B., Fallon, J. F., and Beachy, P. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* 100, 423–434. doi: 10.1016/S0092-8674(00)80678-9

Weatherbee, S. D., Niswander, L. A., and Anderson, K. V. (2009). A mouse model for Meckel syndrome reveals Mks1 is required for ciliogenesis and Hedgehog signaling. *Hum. Mol. Genet.* 18, 4565–4575. doi: 10.1093/hmg/ddp422

Welten, M., Pavlovska, G., Chen, Y., Teruoka, Y., Fisher, M., Bangs, F., et al. (2011). 3D expression patterns of cell cycle genes in the developing chick wing and comparison with expression patterns of genes implicated in digit specification. *Dev. Dyn.* 240, 1278–1288. doi: 10.1002/dvdy.22633

Wilby, O. K., and Ede, D. A. (1975). A model generating the pattern of cartilage skeletal elements in the embryonic chick limb. *J. Theor. Biol.* 52, 199–217. doi: 10.1016/0022-5193(75)90051-X

Williamson, I., Lettice, L., Hill, R. E., and Bickmore, W. A. (2016). Super-resolution imaging and 5C reveals Shh/ZRS spatial proximity and limb ZPA-specific co-localisation. *Development* 16, 2994–3001. doi: 10.1242/dev.139188

Wolpert, L. (1969). Positional information and the spatial pattern of cellular formation. *J. Theoret. Biol.* 25, 1–47. doi: 10.1016/S0022-5193(69)80016-0

Wolpert, L. (1989). Positional information revisited. *Development* 107, 3–12.

Woolley, T. B., R., Maini, P., Tickle, C., and Towers, M. (2014). Mathematical modelling of digit specification by an Sonic hedgehog gradient. *Dev. Dyn.* 293, 290–298. doi: 10.1002/dvdy.24068

Xu, B., and Wellik, D. M. (2011). Axial Hox9 activity establishes the posterior field in the developing forelimb. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4888–4891. doi: 10.1073/pnas.1018161108

Xua, B., Hrycaj, S. M., McIntyre, D. C., Baker, N. C., Takeuchi, J. K., Jeannotte, L., et al. (2013). Hox5 interacts with Plzf to restrict Shh expression in the developing forelimb. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19438–19443. doi: 10.1073/pnas.1315075110

Yakushiji, N., Suzuki, M., Satoh, A., Sagai, T., Shiroishi, T., Kobayashi, H., et al. (2007). Correlation between Shh expression and DNA methylation status of the limb-specific Shh enhancer region during limb regeneration in amphibians. *Dev. Biol.* 312, 171–182. doi: 10.1016/j.ydbio.2007.09.022

Yang, Y., Drossopoulou, G., Chuang, P. T., Duprez, D., Marti, E., Bumcrot, D., et al. (1997). Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. *Development* 124, 4393–4404.

Yang, Y., and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* 80, 939–947. doi: 10.1016/0092-8674(95)90297-X

Zakany, J., Kmita, M., and Duboule, D. (2004). A dual role for Hox genes in limb anterior-posterior asymmetry. *Science* 304, 1669–1672. doi: 10.1126/science.1096049

Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J. Jr., Schreiner, C. M., and Robbins, D. J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* 411, 716–720. doi: 10.1038/35079648

Zhao, X., Sirbu, I. O., Mic, F. A., Molotkova, N., Molotkov, A., Kumar, S., et al. (2009). Retinoic acid promotes limb induction through effects on body axis extension but is unnecessary for limb patterning. *Curr. Biol.* 19, 1050–1057. doi: 10.1016/j.cub.2009.04.059

Zheng, Z. G., and Cohn, M. J. (2011). Developmental basis of sexually dimorphic digit ratios. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16289–16294. doi: 10.1073/pnas.1108312108

Zhu, J., Nakamura, E., Nguyen, M. T., Bao, X., Akiyama, H., and Mackem, S. (2008). Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. *Dev. Cell* 14, 624–632. doi: 10.1016/j.devcel.2008.01.008

Zhulyn, O., Li, D. Y., Deimling, S., Vakili, N. A., Mo, R., Puviindran, V., et al. (2014). A switch from low to high Shh activity regulates establishment of limb progenitors and signaling centers. *Dev. Cell* 29, 241–249. doi: 10.1016/j.devcel.2014.03.002

Zuniga, A., Haramis, A. P., McMahon, A. P., and Zeller, R. (1999). Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 401, 598–602. doi: 10.1038/44157

Zwilling, E. (1964). Development of fragmented and of dissociated limb bud mesoderm. *Dev. Biol.* 89: 20–37. doi: 10.1016/0012-1606(64)90012-0

Zwilling, E., and Hansborough, L. (1956). Interactions between limb bud ectoderm and mesoderm in the chick embryo. III. Experiments with polydactylous limbs. *J. Exp. Zool.* 132, 219–239. doi: 10.1002/jez.1401320203

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

PERSPECTIVE
published: 23 March 2017
doi: 10.3389/fcell.2017.00023

Sox9 Expression in Amniotes: Species-Specific Differences in the Formation of Digits

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In tetrapods the digit pattern has evolved to adapt to distinct locomotive strategies. The number of digits varies between species or even between hindlimb and forelimb within the same species. These facts illustrate the plasticity of embryonic limb autopods. *Sox9* is a precocious marker of skeletal differentiation of limb mesenchymal cells. Its pattern of expression in the developing limb has been widely studied and reflects the activity of signaling cascades responsible for skeletogenesis. In this assay we stress previously overlooked differences in the pattern of expression of Sox9 in limbs of avian, mouse and turtle embryos which may reflect signaling differences associated with distinct limb skeletal morphologies observed in these species. Furthermore, we show that *Sox9* gene expression is higher and maintained in the interdigital region in species with webbed digits in comparison with free digit animals.

Keywords: limb development, interdigit regression, chondrogenesis, skeletal progenitors, SOX9 transcription factor

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Consejo Superior de Investigaciones Científicas, Spain

Reviewed by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *03 January 2017*
Accepted: *07 March 2017*
Published: *23 March 2017*

Citation:
Montero JA, Lorda-Diez CI, Francisco-Morcillo J, Chimal-Monroy J, Garcia-Porrero JA and Hurle JM (2017) Sox9 Expression in Amniotes: Species-Specific Differences in the Formation of Digits. Front. Cell Dev. Biol. 5:23. doi: 10.3389/fcell.2017.00023

The limb is an excellent model system to study the molecular basis of morphogenesis (Hinchliffe, 2002; Fabrezi et al., 2007). The skeletal pattern of the limb is conserved in tetrapods, yet differences in bone morphology are remarkable among different species (Kavanagh et al., 2013). Interpretations of skeletal limb diversification has been largely based on comparative developmental studies using histochemical or radiolabeling markers of initial stages of cartilage differentiation. From these approaches it has been proposed that the limb skeleton in tetrapods is generated by sequential branching and segmentation of a basic pattern representative of the distal segment of the fish fins, termed the "metapterygial axis." The advent and progress of molecular biology has provided new insights about the diversification of the limb skeletal morphology. For example, it has been shown that activation of signals responsible for skeletogenesis may be differentially regulated by transcriptional enhancer DNA sequences that are species-specific (Kvon et al., 2016). These studies explain major skeletal differences in evolutionary distant species such as the absence of limbs in snakes. However, differences between the fore- and the hind-limb in the same species or skeletal differences observed among closely related tetrapods might be regulated in a different fashion, such as timing differences in the expression of signaling molecules (Richardson et al., 2009; Moore et al., 2015; Zuniga, 2015).

Sox9 is a well known marker of the skeleton that precedes the appearance of cartilage blastemas (Wright et al., 1995; Healy et al., 1999; Chimal-Monroy et al., 2003; Kawakami et al., 2005; Lorda-Diez et al., 2011; Sensiate et al., 2014).

Hence, Sox9 is expressed even in domains that represent skeletal pieces lost in the course of evolution of specialized species (de Bakker et al., 2013). Silencing Sox9 in mouse embryos causes loss of appendicular skeleton and increases programmed cell death (Akiyama et al., 2002). Sox9 overexpression promotes polydactyly (Akiyama et al., 2007). Furthermore, Sox9 along with BMP and WNT signaling are considered key regulators of digits formation through a self-organizing Turing mechanism (Raspopovic et al., 2014). Overall, such findings make Sox9 an excellent marker to detect signaling differences, later transduced into specific patterns of chondrification (Richardson et al., 2009), responsible for variations in the morphology of the appendicular skeleton. Based on the observation of *in situ* hybridizations, we have revised the pattern of Sox9 gene expression during digit development in reptilian (Mauremys turtle), avian (chick and duck), and mammal (mouse) species with different autopodial morphology to uncover signaling differences of potential interest to explain digit morphogenesis.

In chick embryos the expression of *Sox9* shows differences between the wing (**Figures 1A–G**) and the leg bud (**Figures 1H–M**). In wing buds at stage HH22 (3.5 id) the expression of *Sox9* marks the primary axis of the appendicular skeleton. In next stages, the initial domain extends proximally and distally (**Figures 1A–C**). Proximally, the domain forms the humerus primordium, and distally it shows a branching that establishes the primordium of the radius (**Figures 1C,D**). By stage HH24 (4 id) the primary axis is continued distally by the digital arch oriented toward the anterior margin of the bud. Between stages HH26-HH28 the digital arch undergoes a branching process to form each digit (**Figures 1E–G**). First branching forms digit 3 and a common branch that bifurcates to form digit 4, and a reduced domain reminiscent of a digit 5. The latest, is progressively reduced in size and expression intensity. The most anterior digit, is formed distally and aligned with the radial domain (**Figures 1F,G**).

In the leg bud the initial expression of *Sox9* at stage HH22 appears divided into a posterior (primary axis) and an anterior domain for the tibia (**Figures 1H,I**). The femur is identifiable at stage HH25 coupled between the proximal end of the fibular and tibial domains (**Figure 1K**). The appearance of these skeletal domains at stage HH23 is accompanied by the formation of a nascent digital arch that occupies a posterior and distal position (**Figures 1K,L**). Initially, the expression is uniform and limited to the posterior half of the autopod but, in the following stages (HH25 and HH26), the expression progresses anteriorly and digits became identifiable as patches of higher expression (**Figures 1J–M**). Digits 3 and 4 are the most prominent at these stages while digits 2 and 5 are poorly defined areas where the expression of Sox9 is not very intense. Interestingly, the most anterior part of the autopod lacks *Sox9* transcripts until stage HH26-HH27.

Both in the wing and in the leg bud, concomitantly with the intensification of *Sox9* expression at stage HH26 in the digit blastemas, a carpal/tarsal arch of lower *Sox9* expression level is formed. Carpal and tarsal pre-cartilages are individualized when digit blastemas are defined.

Expression of *Sox9* in the mouse is similar in fore- and hind-limbs (**Figures 1N–S**). Initial expression of *Sox9* occupies the whole central region of the early bud (**Figures 1N,O**).

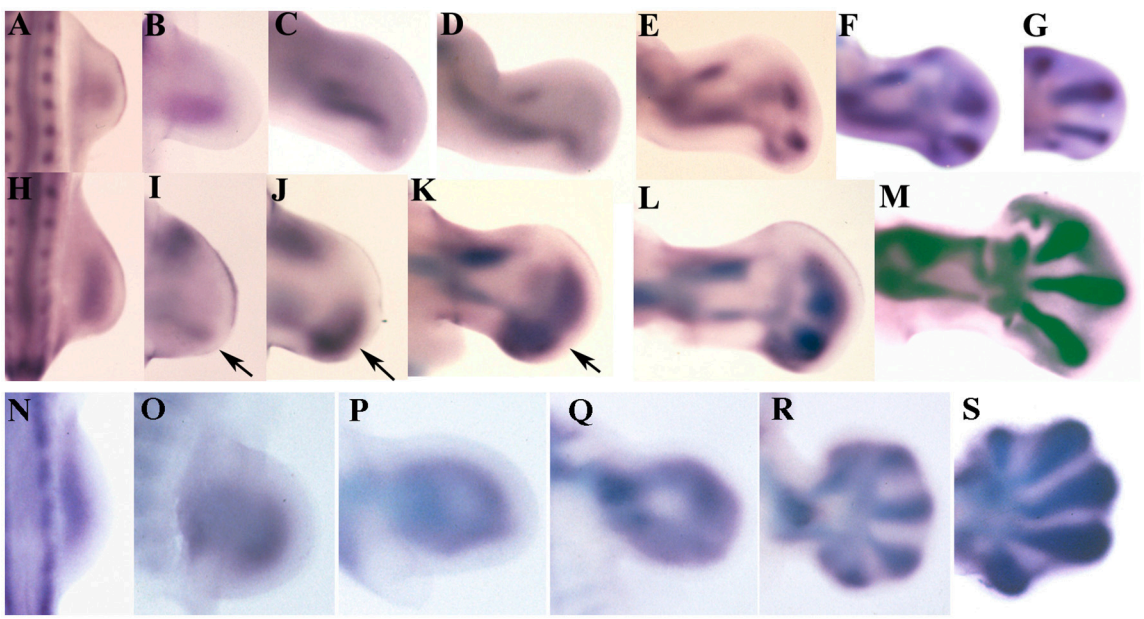

FIGURE 1 | *Sox9* expression during limb development of chicken and mouse embryos. (A–G) Embryonic chicken wing buds at stages HH20 **(A)**, HH22 **(B)**, HH24 **(C)**, HH 25 **(D)**, HH26 **(E)**, HH27 **(F)**, and HH28 **(G)**. **(H–M)** Chicken leg buds at stages HH20 **(H)**, HH22 **(I)**, HH23 **(J)**, HH25 **(K)**, HH27 **(L)**, HH28 **(M)**. Arrows indicate the position of the digital arch domain. **(N–S)** Mouse forelimbs illustrating the sequence of *Sox9* expression at stages E9,5 **(N)**, E10 **(O)**, E10,5 **(P)**, E11 **(Q)**, E12 **(R)**, and E13 **(S)**. Anterior is to the top and distal to the right in all images.

Regionalization of this domain in stylopod, zeugopod and digital arch is due to the loss of transcripts from the central region at 10.5 pc (**Figures 1P,Q**). Due to this process, expression of *Sox9* appears as a loop where the distal curved region constitutes the digital arch. The proximal part of the loop lengthens marking the position of the stylopod. The zeugopodial elements are identified as the lateral regions of the loop. In next stages the digit primordia appear as elongated domains of intensified *Sox9* gene expression (**Figures 1R,S**). Digits 3 and 4 are the first to appear.

The skeletal domains of *Sox9* in the Mauremys turtle are similar in fore and hind-limbs (**Figure 2**). At the beginning, a central ill-defined domain is transformed into a triangular domain with a posterior elongated vertex, which marks the stylopod (**Figure 2C**). The sides of the triangle form the zeugopodial domains, and the base corresponds with the digital arch. The expression of Sox9 in the digital arch becomes progressively intensified at discrete regions to form digit primordia (**Figures 2D,E**). Digits 3 and 4 are the most precociously identifiable while digit 1 is the last to appear, preceded by digit 5 (**Figures 2E–I**). In the course of digit development, the expression of Sox9 is progressively restricted to the digit tip and to the developing joints (**Figures 2E–I**).

Remarkably, the Mauremys turtle interdigital regions retain considerable levels of *Sox9* expression not observed in chick and mouse embryos (**Figures 2F–I**). To ascertain if interdigital expression of *Sox9* associates with the absence of interdigit

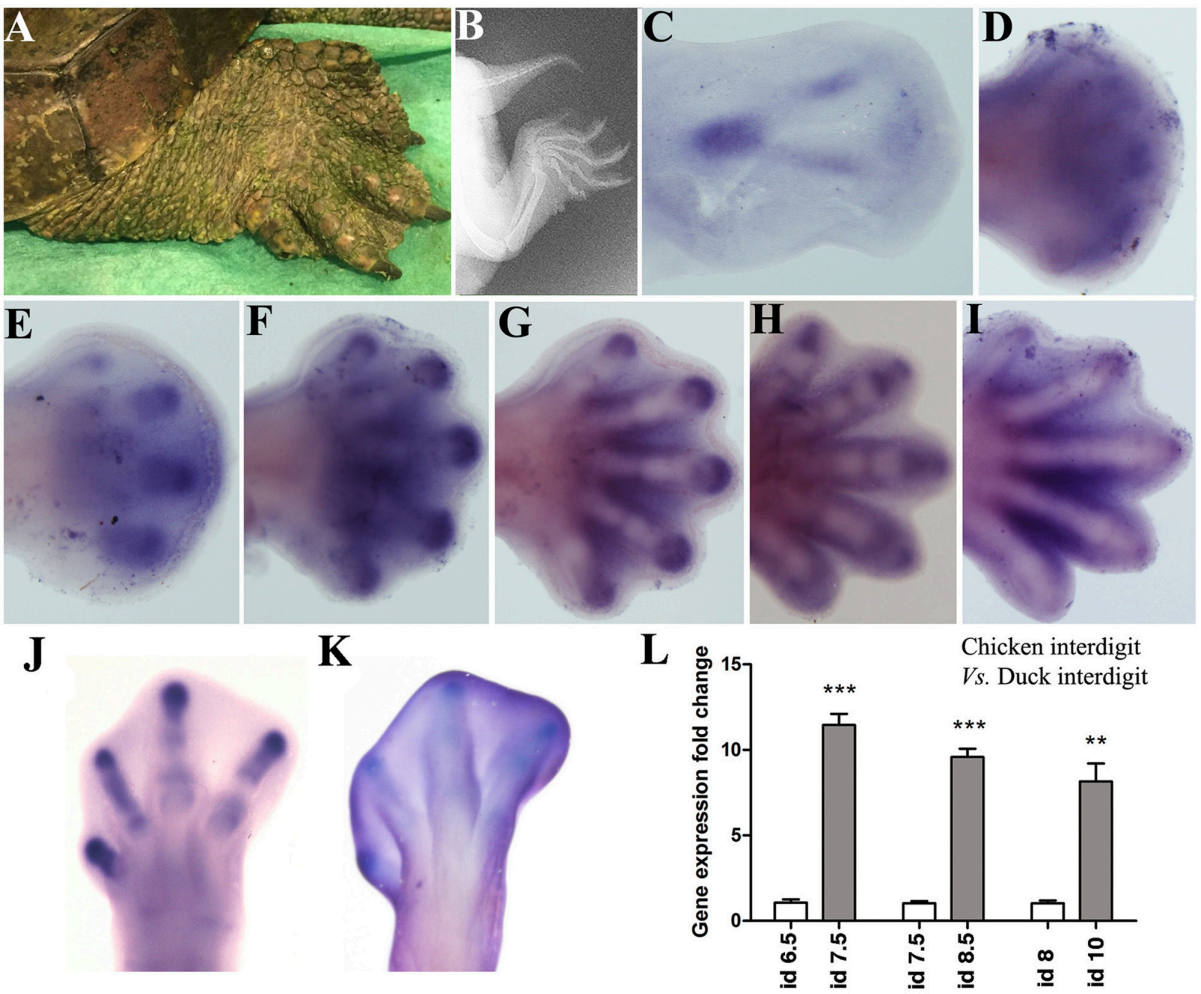

FIGURE 2 | *Sox9* expression during limb development in the turtle (*Mauremys Leprosa*). Stages were established according to the developmental series of Yntema (1968). **(A,B)** adult limb in a live picture **(A)** and a radiographic image **(B)** to illustrate the presence of interdigital membranes in this species. **(C–I)** *Sox9* expression at stages Y14 **(C)**, Y15 **(D)**, Y16 **(E)**, Y17 **(F)**, Y18 **(G)**, Y19 **(H)**, and Y20 **(I)**. **(J–L)** Comparative analysis of *Sox9* expression in chick and duck interdigits. **(J,K)** *Sox9* expression in the chicken and duck autopod at day 6.5 and 8 of incubation respectively. **(L)** QPCR comparison of *Sox9* expression level in the developing third interdigit of the leg bud of chicken (white bars) and duck (gray bars) embryos at equivalent developmental stages. Each value represents the mean of three samples of 12 interdigits and statistical significance was set at $P < 0.05$. Incubation days (id) from left to right: chicken id 6.5 vs. duck id 7.5; chicken id 7.5 vs. duck 8.5; and chicken id 8 vs. duck id 10. Q-PCR specific primers were designed searching for identical homologous sequences in the duck and chicken for Sox9 and GAPDH genes. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

remodeling in the Mauremys turtle (**Figure 2A**), we compared the level of expression of *Sox9* in the third interdigit of the leg bud of chick and duck embryos, as characteristic models of species with free and webbed digits respectively. As shown in **Figures 2J–L**, expression of *Sox9* in the non-regressing interdigit of the duck was much higher than that of the chick embryo.

Detailed analysis of phylogenetically related but phenotypically different species have provided important cues about the mechanisms accounting for limb morphogenesis (Moore et al., 2015). Gene expression computational modeling have also provided insights on the molecular bases responsible for differences in limb skeletogenesis among vertebrates (Uzkudun et al., 2015). The consideration of the subtle *Sox9* expression differences highlighted in this "perspective" assay, are consistent with heterochrony detected in the stages of chondrification (Richardson et al., 2009), and may help to improve our understanding of how digits differ in morphology. In all species, the expression of *Sox9* marks the successive appearance of the stylopod, zeugopod, and autopod along the proximo-distal axis of the limb. The autopod includes the mesopodium (carpi/tarsi) and the acropod (digits); the specification of zeugopod and the acropod has been proposed to determine the mesopodial intermediate domain in between (Woltering and Duboule, 2010). Consistent with this hypothesis, the appearance of intensified expression of Sox9 marking the nascent digits precedes that of the carpal/tarsal domains that lie in the concavity of the digit arch.

The formation and expansion of the digit arch in the chick embryo is clearly distinct from that observed in mouse and turtle embryos. Consistent with the evolutionary model proposed by Shubin and Alberch (1986), in the avian limb the progressive appearance of the digit expression domains follows a polarized sequence from posterior to anterior, which is more accentuated in the wing bud. In contrast, the digital arch domain in mouse and turtle limbs appears occupying a central position in the autopod, and in the course of development expands uniformly to the margins of the bud. These differences raise doubts about the validity of current thought, which considers independent identities for each of the digits in the hand/foot of vertebrates. The consideration of such identities have implications for evolutionary hypothesis that consider digit 1, as the most distal element of a conserved skeletal axis modified in the course of evolution through branching and segmentation processes (see Cohn et al., 2002, for discussion). Sox9 expression domains precede the appearance of prechondrogenic blastemas that were formerly employed in traditional comparative embryonic studies. Hence, the differences among species observed here, support mechanisms of skeletal diversification based on the combination of a distinct distribution of signals with differences in the intrinsic properties of the skeletal progenitors of the autopod, likely associated with differential epigenetic signatures (Sheth et al., 2016). Both in the wing and in the leg of avian embryos, digits are different along the antero-posterior axis justifying the consideration of different digit identities according to their position and number of phalanxes. In contrast, digits of mouse and turtle embryos, expands from the centrally located digital arch toward the margins of the limb bud. In this model of digit arch expansion, there are not morphological landmarks that allow to assign specific identities to the central digits (2,3,4). It must be taken into account that the carpal/tarsal domains of *Sox9* appear when the digit arch shows independent digit domains. Therefore, at these embryonic stages mesopodial domains cannot be taken as a primary reference to establish the identity of the digits. The only morphological differences observed among the pre-cartilaginous blastemas are located in the marginal digits (digits 1 and 5) where *Sox9* domains exhibit a reduced size and appear at more advanced stages than the central digits.

The growth of the limb bud is regulated by a complex signaling network (Uzkudun et al., 2015), where *Shh* and *Gremlin1* genes play an important role in digit specification (Sanz-Ezquerro and Tickle, 2003a; Zhu et al., 2008). Evolutionary or genetic deregulations of the *Shh/Gremlin* loop causes polydactylous (Norrie et al., 2014) or oligodactylous (Lopez-Rios et al., 2014) autopods. Consistent with our interpretation, central digits in these mutants, regardless of its number, are identical and indistinguishable from each other (Norrie et al., 2014). These findings make plausible that digit formation result of the self-organization of the limb mesenchyme (Cooper, 2015), within an autopod of dimensions and shape finely tuned by regulatory genes responsible for growth (Zhu et al., 2008).

Sox9 is target of signals controlling proliferation and differentiation of the skeletal progenitors, including FGFs, BMPs, TGFbetas, and Retinoic acid (RA). These signals are themselves closely regulated by the AER and the ZPA, to establish the pattern of limb skeletogenesis as well as the number of digits in the autopod. BMPs up-regulate the expression of *Sox9* and promote differentiation of progenitors (Lorda-Diez et al., 2014; Norrie et al., 2014) and in conjunction with TGFβs and Activins induce the formation of extra-digits in the avian limb (Chimal-Monroy et al., 2003; Montero et al., 2008). FGFs are major determinants of digit size (Sanz-Ezquerro and Tickle, 2003b; Seki et al., 2015). FGFs inhibit chondrogenesis but expand the amount of Sox 9 positive skeletal progenitors and its overexpression in the limb results in the formation of extra cartilages, including extra-digits (Montero et al., 2001; Norrie et al., 2014), or extra-phalanxes (Sanz-Ezquerro and Tickle, 2003b). RA is a potent inhibitor of Sox9 gene expression (Weston et al., 2002) and RA inhibition in the autopod causes the formation of extra digits (Rodriguez-Leon et al., 1999). Hence, the pattern of Sox9 gene expression may reflect differences in the spatial distribution of signals within the limb bud mesoderm. According with this interpretation, avian digits may represent an evolutionary specialization of digit development consequence of a posterior polarization of signals responsible for limb outgrowth. In contrast, the pentadactyl autopod of mouse and turtle embryos may result from the uniform expansion (like opening a fan) of the signals that coordinate proliferation and differentiation of the skeletal progenitors.

ETHICS STATEMENT

The animal care and handling, and all the experimental procedures were in accordance with the guidelines of the

European Communities Council and the Spanish legislation and they were approved by the Service of Animal Health and Welfare of the Regional Government of Cantabria (Reference No. PI-10-15).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CL, JF, JG, JC, and JH. Performed the experiments: CL, JM, JF, JC, and JH. Analyzed the data: CL, JM, JG, JC, and JH. Writing of the manuscript: JM and JH.

ACKNOWLEDGMENTS

Thanks are due to Prof. Virginio García-Martínez for help and suggestions. Supported by Grants BFU2014-54026P from the Spanish Science and Innovation Ministry to JH and NextVal NVAL15-07 from the IDIVAL to CL. **Figure 1L** has been previously published by Chimal-Monroy et al. (2003).

REFERENCES

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* 16, 2813–2828. doi: 10.1101/gad.1017802

Akiyama, H., Stadler, H. S., Martin, J. F., Ishii, T. M., Beachy, P. A., Nakamura, T., et al. (2007). Misexpression of Sox9 in mouse limb bud mesenchyme induces polydactyly and rescues hypodactyly mice. *Matrix Biol.* 26, 224–233. doi: 10.1016/j.matbio.2006.12.002

Chimal-Monroy, J., Rodriguez-Leon, J., Montero, J. A., Gañan, Y., Macias, D., Merino, R., et al. (2003). Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: sox genes and BMP signaling. *Dev. Biol.* 257, 292–301. doi: 10.1016/S0012-1606(03)00066-6

Cohn, M. J., Lovejoy, C. O., Wolpert, L., and Coates, M. I. (2002). Branching, segmentation and the metapterygial axis: pattern versus process in the vertebrate limb. *Bioessays* 24, 460–465. doi: 10.1002/bies.10088

Cooper, K. L. (2015). Self-organization in the limb: a turing mechanism for digit development. *Curr. Opin. Genet. Dev.* 32, 92–97. doi: 10.1016/j.gde.2015.02.001

de Bakker, M. A., Fowler, D. A., den Oude, K., Dondorp, E. M., Navas, M. C., Horbanczuk, J. O., et al. (2013). Digit loss in archosaur evolution and the interplay between selection and constraints. *Nature* 500, 445–448. doi: 10.1038/nature12336

Fabrezi, M., Abdala, V., and Oliver, M. I. (2007). Developmental basis of limb homology in lizards. *Anat. Rec. (Hoboken)* 290, 900–912. doi: 10.1002/ar.20522

Healy, C., Uwanogho, D., and Sharpe, P. T. (1999). Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* 215, 69–78. doi: 10.1002/(SICI)1097-0177(199905)215:1<69::AID-DVDY8>3.0.CO;2-N

Hinchliffe, J. R. (2002). Developmental basis of limb evolution. *Int. J. Dev. Biol.* 46, 835–845.

Kavanagh, K. D., Shoval, O., Winslow, B. B., Alon, U., Leary, B. P., Kan, A., et al. (2013). Developmental bias in the evolution of phalanges. *Proc. Natl. Acad. Sci. U.S.A.* 110, 18190–18195. doi: 10.1073/pnas.1315213110

Kawakami, Y., Tsuda, M., Takahashi, S., Taniguchi, N., Esteban, C. R., Zemmyo, M., et al. (2005). Transcriptional coactivator PGC-1alpha regulates chondrogenesis via association with Sox9. *Proc. Natl. Acad. Sci. U. S. A.* 102, 2414–2419. doi: 10.1073/pnas.0407510102

Kvon, E. Z., Kamneva, O. K., Melo, U. S., Barozzi, I., Osterwalder, M., Mannion, B. J., et al. (2016). Progressive loss of function in a limb enhancer during snake evolution. *Cell* 167, 633.e11–642.e11. doi: 10.1016/j.cell.2016.09.028

Lopez-Rios, J., Duchesne, A., Speziale, D., Andrey, G., Peterson, K. A., Germann, P., et al. (2014). Attenuated sensing of SHH by Ptch1 underlies evolution of bovine limbs. *Nature* 511, 46–51. doi: 10.1038/nature13289

Lorda-Diez, C. I., Montero, J. A., Choe, S., Garcia-Porrero, J. A., and Hurle, J. M. (2014). Ligand- and stage-dependent divergent functions of BMP signaling in the differentiation of embryonic skeletogenic progenitors *in vitro*. *J. Bone Miner. Res.* 29, 735–748. doi: 10.1002/jbmr.2077

Lorda-Diez, C. I., Montero, J. A., Diaz-Mendoza, M. J., Garcia-Porrero, J. A., and Hurle, J. M. (2011). Defining the earliest transcriptional steps of chondrogenic progenitor specification during the formation of the digits in the embryonic limb. *PLoS ONE* 6:e24546. doi: 10.1371/journal.pone.0024546

Montero, J. A., Gañan, Y., Macias, D., Rodriguez-Leon, J., Sanz-Ezquerro, J. J., Merino, R., et al. (2001). Role of FGFs in the control of programmed cell death during limb development. *Development* 128, 2075–2084.

Montero, J. A., Lorda-Diez, C. I., Gañan, Y., Macias, D., and Hurle, J. M. (2008). Activin/TGFbeta and BMP crosstalk determines digit chondrogenesis. *Dev. Biol.* 321, 343–356. doi: 10.1016/j.ydbio.2008.06.022

Moore, T. Y., Organ, C. L., Edwards, S. V., Biewener, A. A., Tabin, C. J., Jenkins, F. A. Jr., et al. (2015). Multiple phylogenetically distinct events shaped the evolution of limb skeletal morphologies associated with bipedalism in the jerboas. *Curr. Biol.* 25, 2785–2794. doi: 10.1016/j.cub.2015.09.037

Norrie, J. L., Lewandowski, J. P., Bouldin, C. M., Amarnath, S., Li, Q., Vokes, M. S., et al. (2014). Dynamics of BMP signaling in limb bud mesenchyme and polydactyly. *Dev. Biol.* 393, 270–281. doi: 10.1016/j.ydbio.2014.07.003

Raspopovic, J., Marcon, L., Russo, L., and Sharpe, J. (2014). Modeling digits. Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science* 345, 566–570. doi: 10.1126/science.1252960

Richardson, M. K., Gobes, S. M., van Leeuwen, A. C., Polman, J. A., Pieau, C., and Sánchez-Villagra, M. R. (2009). Heterochrony in limb evolution: developmental mechanisms and natural selection. *J. Exp. Zool. B Mol. Dev. Evol.* 312, 639–364. doi: 10.1002/jez.b.21250

Rodriguez-Leon, J., Merino, R., Macias, D., Gañan, Y., Santesteban, E., and Hurle, J. M. (1999). Retinoic acid regulates programmed cell death through BMP signalling. *Nat. Cell Biol.* 1, 125–126.

Sanz-Ezquerro, J. J., and Tickle, C. (2003b). Fgf signaling controls the number of phalanges and tip formation in developing digits. 13, 1830–1836. doi: 10.1016/j.cub.2003.09.040

Sanz-Ezquerro, J. J., and Tickle, C. (2003a). Digital development and morphogenesis. *J. Anat.* 202, 51–58. doi: 10.1046/j.1469-7580.2003.00134.x

Seki, R., Kitajima, K., Matsubara, H., Suzuki, T., Saito, D., Yokoyama, H., et al. (2015). AP-2β is a transcriptional regulator for determination of digit length in tetrapods. *Dev. Biol.* 407, 75–89. doi: 10.1016/j.ydbio.2015.08.006

Sensiate, L. A., Sobreira, D. R., Da Veiga, F. C., Peterlini, D. J., Pedrosa, A. V., Rirsch, T., et al. (2014). Dact gene expression profiles suggest a role for this gene family in integrating Wnt and TGF-β signaling pathways during chicken limb development. *Dev. Dyn.* 243, 428–439. doi: 10.1002/dvdy.23948

Sheth, R., Barozzi, I., Langlais, D., Osterwalder, M., Nemec, S., Carlson, H. L., et al. (2016). Distal limb patterning requires modulation of cis-regulatory activities by HOX13. *Cell Rep.* 17, 2913–2926. doi: 10.1016/j.celrep.2016.11.039

Shubin, N. H., and Alberch, P. (1986). A morphogenetic approach to the origin and basic origanization of the tetrapod limb. *Evol. Biol.* 20, 319–387.

Uzkudun, M., Marcon, L., and Sharpe, J. (2015). Data-driven modelling of a gene regulatory network for cell fate decisions in the growing limb bud. *Mol. Syst. Biol.* 11:815. doi: 10.15252/msb.20145882

Weston, A. D., Chandraratna, R. A., Torchia, J., and Underhill, T. M. (2002). Requirement for RAR-mediated gene repression in skeletal

progenitor differentiation. *J. Cell Biol.* 158, 39–51. doi: 10.1083/jcb.200112029

Woltering, J. M., and Duboule, D. (2010). The origin of digits: expression patterns versus regulatory mechanisms. *Dev. Cell* 18, 526–532. doi: 10.1016/j.devcel.2010.04.002

Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., et al. (1995). The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nat. Genet.* 9, 15–20. doi: 10.1038/ng0195-15

Yntema, C. L. (1968). A series of stages in the embryonic development of chelydra serpentina. *J. Morphol.* 125, 219–251. doi: 10.1002/jmor.1051250207

Zhu, J., Nakamura, E., Nguyen, M. T., Bao, X., Akiyama, H., and Mackem, S. (2008). Uncoupling sonic hedgehog control of pattern and expansion of the developing limb bud. *Dev. Cell* 14, 624–632. doi: 10.1016/j.devcel.2008.01.008

Zuniga, A. (2015). Next generation limb development and evolution: old questions, new perspectives. *Development* 142, 3810–3820. doi: 10.1242/dev.125757

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 16 March 2017
doi: 10.3389/fcell.2017.00020

CaMKII Signaling Stimulates Mef2c Activity *In Vitro* but Only Minimally Affects Murine Long Bone Development *in vivo*

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The long bones of vertebrate limbs form by endochondral ossification, whereby mesenchymal cells differentiate into chondrogenic progenitors, which then differentiate into chondrocytes. Chondrocytes undergo further differentiation from proliferating to prehypertrophic, and finally to hypertrophic chondrocytes. Several signaling pathways and transcription factors regulate this process. Previously, we and others have shown in chicken that overexpression of an activated form of Calcium/calmodulin-dependent kinase II (CaMKII) results in ectopic chondrocyte maturation. Here, we show that this is not the case in the mouse. Although, *in vitro* Mef2c activity was upregulated by about 55-fold in response to expression of an activated form of CaMKII (DACaMKII), transgenic mice that expressed a dominant-active form of CaMKII under the control of the Col2a1 regulatory elements display only a very transient and mild phenotype. Here, only the onset of chondrocyte hypertrophy at E12.5 is accelerated. It is also this early step in chondrocyte differentiation that is temporarily delayed around E13.5 in transgenic mice expressing the peptide inhibitor CaM-KIIN from rat (rKIIN) under the control of the Col2a1 regulatory elements. Yet, ultimately DACaMKII, as well as rKIIN transgenic mice are born with completely normal skeletal elements with regard to their length and growth plate organization. Hence, our *in vivo* analysis suggests that CaMKII signaling plays a minor role in chondrocyte maturation in mice.

Keywords: CaMKII, peptide inhibitor, chondrocyte maturation, hypertrophy, Mef2c, mouse model

OPEN ACCESS

Edited by:
Sigmar Stricker,
Free University of Berlin, Germany

Reviewed by:
Joy Richman,
University of British Columbia, Canada
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Masaryk University, Czechia

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *25 October 2016*
Accepted: *02 March 2017*
Published: *16 March 2017*

Citation:
Amara CS, Fabritius C, Houben A, Wolff LI and Hartmann C (2017) CaMKII Signaling Stimulates Mef2c Activity In Vitro but Only Minimally Affects Murine Long Bone Development in vivo. Front. Cell Dev. Biol. 5:20. doi: 10.3389/fcell.2017.00020

INTRODUCTION

Endochondral ossification is the process underlying the formation of the long bones in the vertebrate limbs (Erlebacher et al., 1995; Kronenberg, 2003). It starts with the condensation of mesenchymal cells that undergo chondrogenic differentiation, forming a cartilage template consisting of immature chondrocytes. These produce an extracellular matrix composed of proteoglycans, glycosaminoglycans, and glycoproteins. This template, which prefigures the future skeletal element, enlarges through chondrocyte proliferation (Akiyama and Lefebvre, 2011). In the next phase, chondrocytes in the center of the cartilage anlage stop proliferating and differentiate into prehypertrophic, *Indian hedgehog* (*Ihh*) expressing chondrocytes. Prehypertrophic chondrocytes enlarge further, becoming hypertrophic chondrocytes, which

initiate synthesis of extracellular matrix containing type X collagen, encoded by the *Col10a1* and *Col10a2* genes (Kronenberg, 2003). The transition of chondrocytes from proliferating to prehypertrophic and then to hypertrophic cells is a critical step in determining the growth rate and size of skeletal elements (Kronenberg, 2003).

Previous studies in chicken and mouse identified a complex network of signaling pathways and transcription factors that regulate the different steps during endochondral bone formation (reviewed in Karsenty, 2008; Hartmann, 2009; Lefebvre and Bhattaram, 2010). A central regulatory node in the chondrocyte differentiation program is the Ihh-PTHrP (parathyroid hormone-related peptide) feedback loop (Vortkamp et al., 1996). Ihh, which is part of this feedback loop, is considered a master regulator of chondrocyte maturation and has multiple functions (Kronenberg, 2003; Mak et al., 2008). Numerous transcription factors such as Runx2 and Runx3, Mef2c, and Mef2d, as well as transcriptional co-factors such as β-catenin, promote chondrocyte hypertrophy (Inada et al., 1999; Kim et al., 1999; Hartmann and Tabin, 2000; Yoshida et al., 2004; Arnold et al., 2007; Guo et al., 2009).

It is not yet well established how the activity of these transcription factors is regulated by signaling events. Ca^{2+}/calmodulin-dependent kinase II (CaMKII), is a calmodulin (CaM) binding serine/threonine kinase and important for Ca^{2+}-mediated signal transduction (Colbran et al., 1989). Most vertebrates possess four different CaMKII genes (α, β, γ, and δ) giving rise to at least 38 different splice variants (Tombes et al., 2003). Two hallmarks distinguish CaMKII from other kinases: firstly, it acts as a multimeric holoenzyme composed of 4–14 heteromeric or homomeric subunits of the different isoforms of the four genes and secondly, its ability to autophosphorylate on the threonine 286 residue upon Ca^{2+}/CaM binding (Soderling, 1996; Hudmon and Schulman, 2002; Colbran, 2004; Lantsman and Tombes, 2005; Rosenberg et al., 2006). Autophosphorylation relieves the enzyme from its Ca^{2+}/CaM dependence. Alternatively, CaMKII can be activated by methionine oxidation (Erickson et al., 2008).

Various studies suggest that CaMKII signaling may play a role in skeletogenesis. All four genes are expressed in chicken and mouse chondrocytes (Taschner et al., 2008; Li and Dudley, 2009). Studies on human articular chondrocytes have suggested that CaMKII is involved in N-methyl-D-Aspartic acid (NMDA) receptor signaling, which is important for maintaining matrix integrity of joints (Salter et al., 2004; Shimazaki et al., 2006). CaMKII signaling has also been implicated in osteoblast and osteoclast differentiation (Quinn et al., 2000; Zayzafoon et al., 2005). In the chicken, we demonstrated previously using a retroviral system that the expression of a dominant active form of CaMKII (DaCaMKII), which mimics the autophosphorylated form, caused premature ectopic chondrocyte maturation, while the inhibition of CaMKII activity by a peptide inhibitor (rKIIN) delayed the hypertrophic program (Taschner et al., 2008). Li and colleagues suggested that the increasing CaMKII activity in the chondrocytes during their transition from the proliferative to the prehypertrophic state regulates Runx2 and β-catenin activity and thereby promotes chondrocyte hypertrophy (Li et al., 2011).

Retroviral driven expression in the chick system has the disadvantage that all mitotically active cells get infected. So besides the chondrocytes also the soft-tissue is infected. This makes it difficult to distinguish between cell-autonomous and non-cell-autonomous effects. Using a transgene approach in the mouse allowed us to overcome this problem. Hence, in order to gain more specific insights into the potential role of CaMKII in endochondral bone formation, we expressed an activated form of CaMKII (DaCaMKII) or the peptide inhibitor rKIIN under the control of the *Col2a1* promoter primarily in chondrocytes. Based on our observations in the transgenic mouse models we conclude that modulation of CaMKII activity in the mouse has only an effect early in development at the onset of chondrocyte hypertrophy.

Abbreviations: CaMKII, Calcium/calmodulin-dependent kinase II; Gfp, Green fluorescent protein; ISH, in situ hybridizations; tg, transgenic; E, embryonic day.

MATERIALS AND METHODS

Generation of *Col2a1*-Transgenic Mice

For the dominant active CaMKII transgene a cassette containing CaMKII-T286D C-terminal fused to eGFP (Taschner et al., 2008) followed at the 3′ end by two SV40polyA sequences was inserted via blunt-end cloning into the BamHI site of a plasmid carrying the *Col2a1* promoter–rabbit β globin intron–*Col2a1* enhancer element (a gift from Yoshi Yamada; (Nakata et al., 1993)). For the rKIIN transgene, a peptide inhibitor for CaMKII from rat (Chang et al., 1998, 2001) fused with eGFP at its N-terminus (Taschner et al., 2008) was cloned accordingly. The final transgenic constructs (**Figures 1A,B**) were excised with the restriction enzymes AflII and SwaI, purified on agarose gel and eluted with DNA microinjection buffer. The linear transgene cassettes were microinjected into the pronucleus of $B6CBAF_1$ zygotes in the transgene facility of the IMP, Vienna, Austria (Hogan et al., 1994). The zygotes were implanted into pseudo-pregnant mice to obtain transgenic founder lines. To maintain the transgenic lines in a pure genetic background, the founder lines were crossed with C57Bl/6J females and the subsequent generations were backcrossed with C57Bl/6J animals over at least eight generations. Genotyping of transgenic mice and embryos was performed by PCR using ear biopsies and material from the embryonic tail respectively, in combination with transgene-specific primer pairs (listed in Supplementary Table 1). Transgene-copy numbers were determined using genomic DNA from ear biopsies of the different transgenic lines (two independent samples per line) by qPCR and normalized to actin and control genomic DNA from mice carrying one copy of the transgene in the Rosa26 locus (Amara and Hartmann, unpublished; Ballester et al., 2004). Primers are listed in Supplementary Table 2. Mouse experiments were performed in accordance with local, institutional and national regulations under the following licenses (84-02.05.2012.260, 84-02.04.2015.A278, 84-02.05.50.15.022).

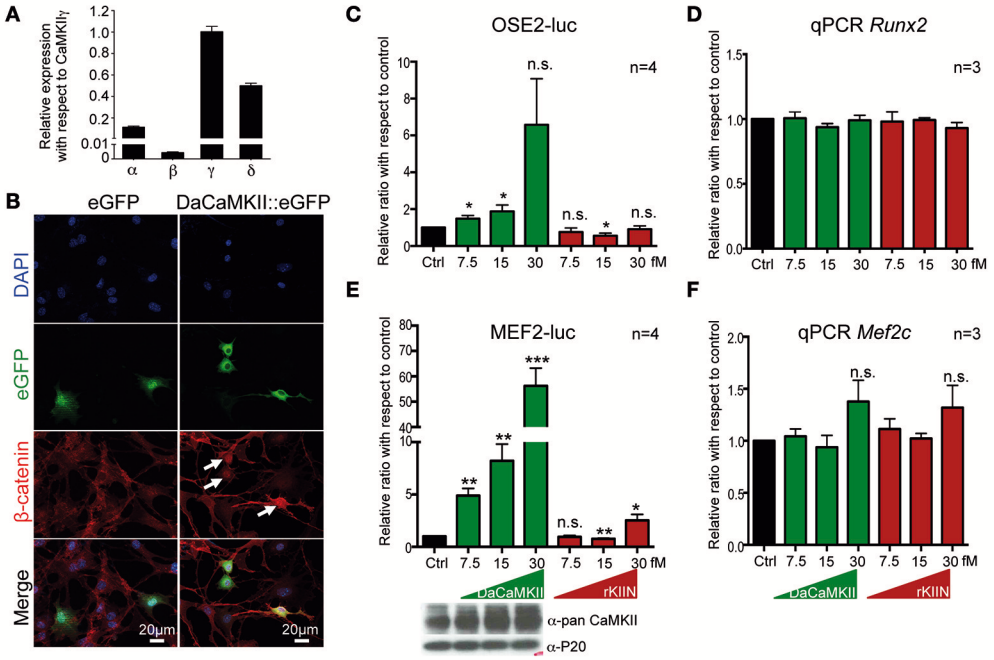

FIGURE 1 | CaMKII activity translocates β-catenin into the nucleus and regulates Runx2 and Mef2c activity in a dose dependent manner. (A) qPCR showing the expression levels of the four Camk2 isoforms, α, β, γ, δ, in chondrocytes isolated from E12.5 skeletal elements. Expression levels were normalized to *Gapdh* and *Actb* and ploted relative to the expression of CaMKIIγ. $n = 3$. Error bars indicate ±SEM. **(B)** Epiphyseal chondrocytes from P6 wild-type limbs were transfected with an expression vector encoding DaCaMKII::eGFP or eGFP (control). Nuclear localization of β-catenin (white arrows) was observed in DaCaMKII transfected cells. **(C)** Primary chondrocytes from E13.5 appendicular skeletal elements were co-transfected with 6X Ose2 luciferase (OSE2-luc) reporter and TK-renilla reporter plus increasing amounts of the expression vector encoding DaCaMKII or rKIIN. Ratios of luciferase activity with respect to control (Ctrl, black bar) are plotted as bar charts: DaCaMKII (green bars) and rKIIN (red bars). **(D)** Relative *Runx2* expression levels, determined by qPCR, in primary chondrocytes from E13.5 appendicular skeletal elements co-transfected with increasing amounts of an expression vector encoding DaCaMKII or rKIIN used for the OSE2-Luc assay. **(E)** Primary chondrocytes from E13.5 wild-type appendicular skeletal elements were co-transfected with 3X Mef2 luciferase reporter (MEF2-Luc), TK-renilla reporter, and increasing amounts of the expression vector encoding DaCaMKII or rKIIN. Bar graph showing the ratio of luciferase activity with respect to control (Ctrl, black bar), DaCaMKII (green bars), and rKIIN (red bars) from four independent transfection experiments. The immunoblot below shows the corresponding increase in CaMKII levels. **(F)** Relative *Mef2c* expression levels, determined by qPCR, in primary chondrocytes from MEF2-luc assays co-transfected with increasing amounts of expression vector encoding DaCaMKII or rKIIN. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Error bars indicate ± SEM.

Mouse Husbandry and Embryo Processing

For timed pregnancies, heterozygous transgenic mice were interbred overnight and the plug day was designated as embryonic day 0.5 (E0.5). Embryos were harvested at the required stages, dissected and fixed overnight in 4% paraformaldehyde (PFA). For stages E18.5 and older, the skin above the limbs was removed prior to fixation. Fixed limbs were removed, washed in PBS, and tissue was dehydrated using increasing ethanol concentrations (25, 50, 75, 100%).

In situ Hybridization, Histology and Skeletal Preparations

For *in situ* hybridization (ISH) and histological staining on sections, limbs were processed into paraffin using the Excelsior ES Vacuum Infiltration Processor (Thermo Scientific), embedded in paraffin and sectioned at 5 μm. Non-radioactive ISH were performed using digoxigenin (DIG)-labeled anti-sense RNA probes as previously described (Murtaugh et al., 2001). Probes for type 2 collagen (*Col2a1*), indian hedgehog (*Ihh*), osteocalcin (*Ocn*), osteopontin (*Opn*), type 1 collagen (*Col1a1*), and type 10 collagen (*Col10a1*) have been published previously (Hill et al., 2005). The *Gfp* probe was generated using a plasmid containing the eGFP coding region. All probes are available upon request. Histological stainings such as alcian blue, alcian blue/von Kossa, and hematoxylin/eosin were performed as previously described (Houben et al., 2016). Skeletal preparations were performed on 6 day old pups which were sacrificed by decapitation, skinned, eviscerated, fixed in 95% EtOH, and double-stained for alcian blue and alizarin red (McLeod, 1980). The length of isolated humeri and femora was calculated by using Zeiss image analysis software.

RNA Isolation and qPCR

The stylo- and zeugopod regions of forelimbs were isolated from embryos at the required stage. Skin and soft tissue was removed and skeletal elements were dispersed using the Polytron PT 1200 E manual disperser with the aggregate PT-DA 03/2 EC-E50 (Kinematica). The RNA was isolated using the QIAGEN RNeasy micro kit according to the manufacturer's instructions. A total of 500 ng RNA was reverse transcribed using PrimeScript RT

reagent kit (Takara, #RR037A) with oligo dT primer. For qPCR cDNA was diluted 1:10 in water. 3 µl of diluted cDNA were mixed with 1 µl of primer mix and 10 µl SYBR Premix Ex TaqII (Tli RNaseH plus) (Takara, #RR820Q) in a final volume of 20 µl. Gene expression was monitored using the BioRad CFX96 cycler and the following protocol: 95°C 10 min, 45 × (95°C 30 s, 60°C 30 s, 72°C 45 s + plate read), 72°C 5 min, melting curve: 55°C–99°C in 0.5°C increments for 5 s + plate read. qPCR analysis was performed in duplicates and results were produced from at least three independent samples. Expression values were calculated using the comparative ΔC(t) method and normalized to *Gapdh* and *Actb* expression. For primer sequences and product sizes see Supplementary Table 3.

Isolation and Cultivation of Primary Chondrocytes

E13.5 appendicular skeletal elements from the stylo- and zeugopod were dissected in PBS supplemented with 1% P/S followed by a digestion with 0.1% type II collagenase (Gibco, #17101-015) and 0.3% dispase (Gibco, #17105-041) in medium (DMEM/F-12; 1% P/S) for a period of 60 min shaking at 100 rpm in a CO_2 incubator. Cells were filtered through a cell strainer, centrifuged and washed with culture medium (DMEM/F-12, 1% P/S, and 10% FCS). Cell pellets were then resuspended in culture medium, plated at 2.5–3 × 10^4 cells/cm^2 in tissue culture flasks and grown for 5 days.

Luciferase Assays

Primary chondrocytes were seeded at a density of 3 × 10^4 cells/well in 48 well plates 1 day prior to transfection. Cells were transfected with the pGL4.23-Mef2-luc (a gift from Eric Olson) or the pGL4.10-Ose2-luc reporter plasmid (a gift from G. Karsenty) in combination with pRL-TK (Promega) as a control and pCDNA3.1-DaCaMKII or pCDNA3.1-rKIIN expression vectors. Total amounts of transfected plasmids (equivalent to 52 fM) in each group were adjusted by adding empty vector (pCDNA3.1+). Transfection was performed using jetPRIME™ (Polyplus transfection) according to manufacturer's instructions. Luciferase activities were measured 48 h after transfection following the protocol by (Hampf and Gossen, 2006). Luciferase measurements were normalized to the corresponding renilla activities for transfection efficiency. Experiments were performed in triplicates and repeated at least three times.

Immunofluorescence

For immunofluorescent staining on cells, primary chondrocytes isolated from P6 C57Bl/6J knee epiphyseal regions were seeded on 0.1% gelatin coated glass coverslips. Cells were transfected with DaCaMKII::eGFP and eGFP expression vectors (equivalent to 0.12 pmol) using jetPRIME™ (Polyplus transfection). Forty-Eight hours after transfection, cells were fixed with 4% PFA/PBS for 20 min, treated with 0.5% TritonX-100 for 5 min and blocked with 10% normal goat serum (NGS) in PBS for 1 h at room temperature (RT). Incubation with β-catenin antibody (1:200 in 2% NGS/PBS, BD Biosciences, #610154, RRID:AB_397555) was performed overnight at 4°C. Coverslips were subsequently incubated with Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes) at 1:200 in 2% NGS/PBS. Nuclei were counterstained with DAPI.

Immunoprecipitation by Magnetic Beads

HEK293 cells were co-transfected with Mef2c-Myc and DaCaMKII::eGFP at 4:3 ratio or FLAG-HDAC4 and DaCaMKII::eGFP at 1:3 ratio using $CaPO_4$ transfection method. Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 with proteinase and phosphatase inhibitors). After centrifugation, a total amount of 150 µg of protein from the supernatant was subjected to immunoprecipitation using 25 µl magnetic beads coupled with GFP monoclonal antibody (MBL, #D153-11) under the following conditions: 15 min at 4°C on a rotating wheel (10 rpm), washed 4 times with wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40) for 5 min at RT. For the pull-down of FLAG-HDAC4 using GFP-magnetic beads the conditions were as following: 500 µg of protein from the supernatant was subjected to immunoprecipitation using 50 µl magnetic beads and bound for 45 min at 4°C on a rotating wheel (10 rpm), followed by 3 washes (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40) for 30 s at RT. Magnetic beads were then boiled for 2 min in 25 µl Laemmli's sample buffer and supernatant of the beads was loaded on a 10% SDS-PAGE to separate the immunoprecipitated proteins.

Immunoblots

Transfected primary chondrocytes were lysed with ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 with proteinase and phosphatase inhibitors). 50 µg of protein lysates were run on a 10% SDS-PAGE and transferred to a 0.45 µm PVDF-membrane by semi-dry transfer (PerfectBlue™, PeqLab). The membrane was blocked with 5% milk and incubated with the appropriate primary antibodies anti-GFP (1:15000, Abcam, #ab13970, RRID:AB_300798), anti-Myc (1:1000, Cell Signaling Technology, mAb #2276, RRID:AB_331783), pan-CaMKII (1:1000, Cell Signaling Technology, mAb #4436S, RRID:AB_10545451), anti-Flag (1:1000, Sigma-Aldrich, #F1804, RRID:AB_262044), and proteasome 20 (P20) (1:15000, Abcam, #ab3325, RRID:AB_303706), followed by incubation with the respective, species-specific HRP-coupled secondary antibodies (1:1000 and 1:5000). ECL substrate was used for signal development (Amersham, #RPN2106) on X-ray film (Amersham).

Image Acquisition

Histological images were acquired using Zeiss AxioImager M2 equipped with an AxioCam MRc 6.45 µm color camera (Zeiss, Jena). Images of embryos were acquired using a Zeiss Stereo discovery V8 equipped with Zeiss plan Apo S, 0.63X lens. Immunofluorescent images were acquired using Zeiss AxioImager M2 equipped with an Apotome2 and an AxioCam MRm 6.45 µm monochromatic camera (Zeiss, Jena) using the Zen software (Zeiss, Jena).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software 6.0 (RRID: SCR_002798). Data are displayed as mean values ± standard error of the mean (SEM). Statistical significance of differences (*P*-value) was determined by the two-tailed, unpaired Student's *t*-test.

RESULTS

In Vitro: CaMKII Signaling Affects β-Catenin Localization and Alters Runx2 and Mef2c Activity

Based on previous studies in chicken, potential molecular mechanisms of how CaMKII signaling regulates chondrocyte hypertrophy have been suggested (Li et al., 2011). These included increased nuclear localization of β-catenin and Runx2 in chondrocytes with activated CaMKII signaling (Li et al., 2011). Thus, we investigated whether the molecular mechanism in mouse chondrocytes may be similar. Mouse chondrocytes, like chicken chondrocytes, express all four CaMKII isoforms, and also here the CaMKIIβ isoform was expressed at lower levels than the other three isoforms (**Figure 1A**, see also Taschner et al., 2008; Li et al., 2011). Similar to what was observed in chicken, β-catenin was localized in the nucleus in mouse chondrocytes transfected with a DaCaMKII::GFP expression plasmid (**Figure 1B**, white arrows). This was not observed in the GFP-transfected control cultures (**Figure 1B**, left panel). We also analyzed whether Runx2 transcriptional activity was altered by the presence of DaCaMKII or rKIIN in mouse chondrocytes. For this, the Runx2-dependent OSE2-luc reporter (Ducy and Karsenty, 1995) was co-transfected into primary chondrocytes with increasing amounts of either DaCaMKII or rKIIN expression plasmid. OSE2-luc reporter activity was upregulated in response to increasing amounts of DaCaMKII, while expression of rKIIN led to a downregulation of the reporter activity in a concentration independent manner (**Figure 1C**). To rule out the possiblity that modulation of CaMKII activity affects the expression levels of Runx2 itself, we examined the *Runx2* expression levels in the OSE2-luc assay by qPCR. Here, *Runx2* expression was not affected in cells transfected with the DaCaMKII or rKIIN expression plasmids (**Figure 1D**).

Another transcription factor promoting hypertrophy is Mef2c (Arnold et al., 2007). The MEF2-luc reporter (Naya et al., 1999) was activated over 50-fold by DaCaMKII at the highest concentration used (**Figure 1E**). An increase in CaMKII protein levels corresponding to the increased amount of transfected expression plasmid was demonstrated by western blot (**Figure 1E**, lower panel). The DaCaMKII expression levels were also determined by qPCR using a primer that detects the mouse as well as the transgenic rat Camk2a transcript. Here, the Camk2a expression levels were normalized to the endogenous Camk2a expression levels in control-transfected chondrocytes. This quantitative approach revealed an over 100-fold increase in Camk2a levels the cells transfected with 30 fM DaCaMKII (Supplementary Figure 1). Overexpression of rKIIN on the other hand, led, with the exception of the highest concentration, to a downregulation of the MEF2-luc reporter activity independent of the concentration (**Figure 1E**). To our surprise, at the highest concentration even a two-fold activation was observed (**Figure 1E**). Again, the endogenous *Mef2c* expression levels were not significantly affected by either DaCaMKII or rKIIN expression in the transfected primary chondrocytes (**Figure 1F**). Given the strong effect on Mef2c activity we tested whether CaMKII may directly interact with Mef2c. Co-immunoprecipitation assays in HEK293 cells using tagged proteins revealed that the activated form of CaMKII physically interacts with Mef2c (**Figure 2A**). Here we used GFP-magnetic beads and pulled on the DaCaMKII::GFP fusion protein and detected bound Mef2c::Myc (**Figure 2A**). Conversely, using Myc-magnetic beads and pulling on Mef2c::Myc the DaCaMKII::GFP fusion protein was detected by immunoblot in the bound fraction (data not shown).

Given previous findings that (a) the CaMKIIδ isoform can interact with and influence histone deacetylase 4 (Hdac4) activity in cardiomyocyte hypertrophy (Backs et al., 2006, 2009), (b) Hdac4 controls chondrocyte hypertrophy by interacting with and inhibiting the activity of Runx2 and Mef2c (Vega et al., 2004; Arnold et al., 2007), and (c) our observation that DaCaMKII modulates Mef2c as well as Runx2 activity, we addressed whether DaCaMKII also physically interacts with Hdac4 or whether it alters its repressive activity. For this, HEK293 cells were co-transfected with FLAG::Hdac4 and DaCaMKII::GFP. Co-IP studies with GFP-coupled magnetic beads revealed no physical interaction for Hdac4 in the pull-down lysate (**Figure 2B**). Luciferase assays on primary chondrocytes transfected with MEF2-luc, Mef2c and Hdac4 resulted in the inhibition of Mef2 activity. Yet, this inhibitory effect was not altered by the addition of increasing amounts of DaCaMKII (**Figure 2C**). This suggests that the DaCaMKII does not modulate Hdac4 activity. In essence, our results show that the activated form of CaMKII positively influences the activity of the transcription factors Mef2c and to a lesser extent Runx2 activity and that this effect is probably not mediated via an inhibitory effect on Hdac4. Furthermore, CaMKII physically interacts with Mef2c suggesting that it may possibly modulate its transcriptional activity by phosphorylation.

In Vivo: Generation of Chondrocyte Specific Transgenic Mice

In order to determine the *in vivo* role of CaMKII modulation in mouse endochondral ossification, we employed a transgenic approach using the *Col2a1* promoter/enhancer element to drive transgene expression in chondrocytes (**Figures 3A,B**). From previous studies in the chicken we knew that eGFP tagged transgenes are functional (Taschner et al., 2008). Hence, in order to visualize transgene expression and to facilitate the distinction between heterozygous and homozygous embryos, eGFP tagged transgenes were used. For the DaCaMKII::eGFP construct two independent transgenic founder lines (Tg1 and Tg2-DaCaMKII) were established and for the eGFP::rKIIN construct three independent founder lines were obtained (Tg1, Tg2, and Tg3-rKIIN; **Figures 3A,B**). All founder lines transmitted the transgene to the F1 generation. The transgenic lines

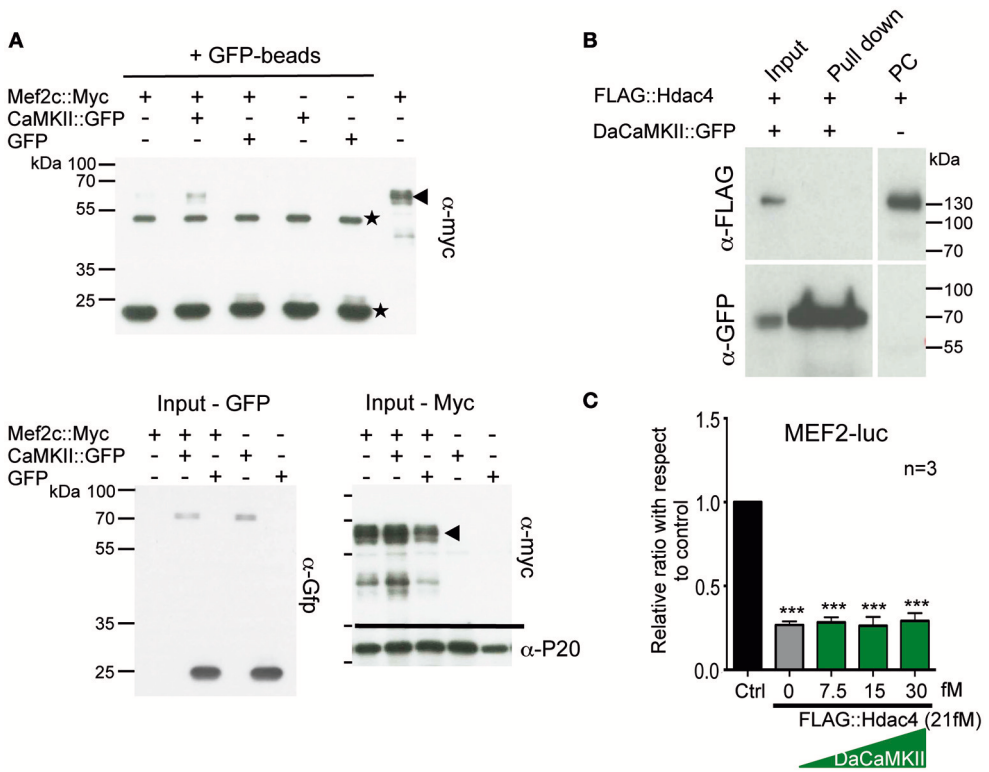

FIGURE 2 | CaMKII physically interacts with Mef2c but not with HDAC4 and does not modulate Hdac4 activity. (A) Representative immunoblot of a co-immunoprecipitation assay using GFP-magnetic beads and lysates from HEK293 cells co-transfected with Mef2c::Myc and CaMKII::GFP expression plasmids or Mef2c::Myc and GFP expression plasmids analyzed with anti-myc tag antibody ($n = 3$). An interaction was observed in the Co-IP of Mef2c::Myc with CaMKII::GFP (lane 2; indicated by the arrow at the hight of the Mef2c::Myc signal). The two unspecific signals due to the IgG heavy and light chains, are indicated by the stars. Below the input for the Gfp- and Myc-tagged proteins used in the co-IP and the loading control with anti-P20 are shown. **(B)** Representative immunoblot of a co-immunoprecipitation assay using GFP-magnetic beads on lysates from HEK293 cells co-transfected with FLAG::Hdac4 and CaMKII::GFP expression plasmids analyzed with anti-myc tag antibody. No interaction was observed between DaCaMKII::eGFP and Hdac4 (lane 2), $n = 2$. **(C)** Primary chondrocytes from E13.5 wild-type appendicular skeletal elements were co-transfected with MEF2-luc, TK-renilla reporter and expression vectors encoding FLAG-Hdac4, and DaCaMKII (increasing amounts). The ratio of luciferase activity with respect to control (Ctrl) is plotted in the bar graph: control (black bar), FLAG::Hdac4 (gray bar) and FLAG::Hdac4 together with DaCaMKII (green bars), $n = 3$. n refers to the number of independent biological samples. $^{***}p < 0.001$. Error bars indicate ± SEM.

differed in the number of transgenes integrated in the genome (**Figure 3A**). For a first characterization of the transgenic lines, heterozygous transgenic mice of the independent founder lines were intercrossed to isolate embryos at embryonic day (E) 15.5. Contrary to our expectations, no obvious gross morphological differences were detected within the offspring of the various intercrosses (**Figures 3C,D**). Genotyping of the offspring was performed examining the GFP intensity using a fluorescent stereomicroscope and confirmed by conventional PCR-based genotyping (**Figures 3C,D**; data not shown). Heterozygous and homozygous transgenic embryos were classified based on the fluorescence intensity (**Figures 3C,D**). As expected, GFP activity was detected in the cartilaginous regions of the skeletal elements, particularly visible in the skull and limbs at sites corresponding to *Col2a1* expression domains (**Figures 3C,D**; Cheah et al., 1991). This was confirmed by ISH with RNA antisense probes for *Col2a1* and *Gfp* on adjacent sections of transgenic limbs (**Figures 3E,F**). As expected, no *Gfp* signal was detected in humeri of non-transgenic (wild-type) littermates (**Figures 3E,F**). These results demonstrated that the transgene is indeed expressed in a *Col2a1*-promoter/enhancer-dependent manner. Furthermore, the GFP signal intensity in embryos as well as the *Gfp* ISH signal on sections allowed us to distinguish heterozygous and homozygous transgenic littermates.

In the chicken a subset of the chondrocytes that expressed DaCaMKII prematurely differentiated into prehypertrophic and subsequently into hypertrophic chondrocytes outside their normal expression domains (Taschner et al., 2008; Li et al., 2011). Hence, we performed ISH on E15.5 sections through the limbs using *Col2a1* as a marker for more immature chondrocytes, *Ihh* as a marker for prehypertrophic cells, and *Col10a1* as a marker for hypertrophic cells. Yet, no expression of the maturation markers *Ihh* or *Col10a1* was observed outside their normal expression domains and no obvious differences were observed with respect to the individual domain sizes, distance between the domains, or the expression levels of these three markers comparing non-transgenic with heterozygous and homozygous DaCaMKII-tg littermates (**Figures 4A,B**). Similar, negative results were

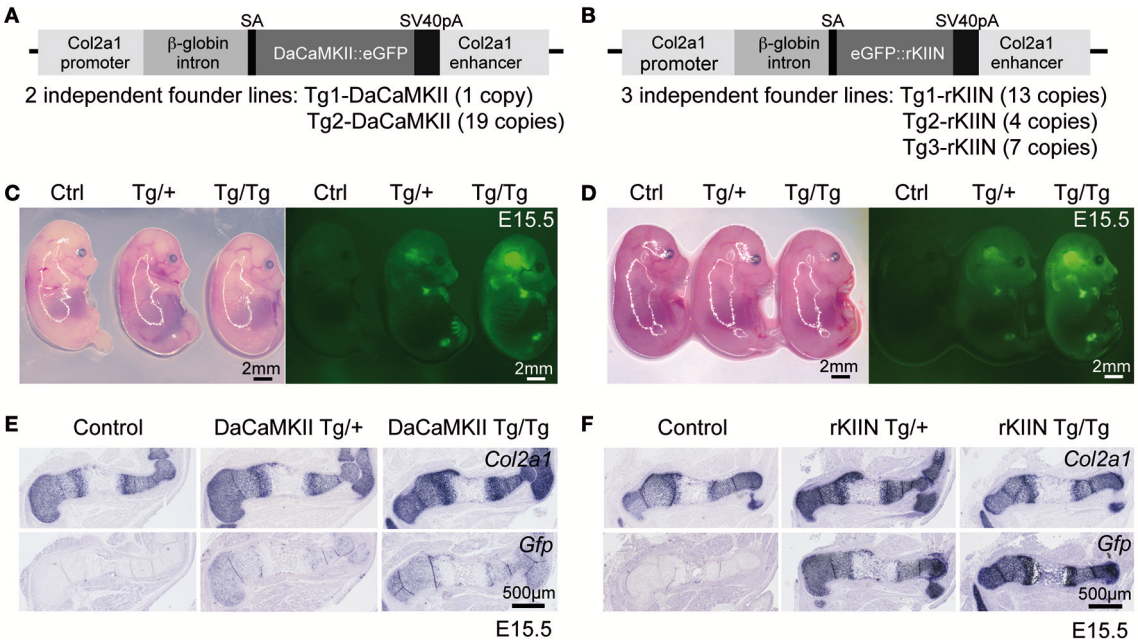

FIGURE 3 | Overview of transgenic constructs and their chondrocyte-specific expression in transgenic embryos. (A,B) Schematic view of constructs containing either the DaCaMKII::eGFP **(A)** or eGFP::rKIIN **(B)** downstream of the β-globin intron under the control of the *Col2a1* promoter/enhancer region. The number of independent transgenic lines achieved for each construct and the corresponding copy numbers in the genome are indicated below. **(C,D)** E15.5 littermates from intercrosses of DaCaMKII::eGFP transgenic mice **(C)** and from intercrosses of eGFP::rKIIN transgenic mice **(D)** imaged under white light and fluorescence. The GFP signal is detected in the cartilaginous skeletal elements of limbs, skull, and vertebrae. Note that the GFP signal is more intense in the homozygous (Tg/Tg) embryos compared to heterozygous (Tg/+) littermates, while only weak auto-fluorescence is detected in the control (non-transgenic, +/+) littermates. Images were taken at the same magnification under fluorescent stereomicroscope (Zeiss). **(E,F)** Non-radioactive *Col2a1* and *Gfp* ISH on E15.5 humeri isolated from DaCaMKII-tg mice **(E)** and isolated from rKIIN-tg mice **(F)** and corresponding control (non-transgenic, +/+) littermates showing that the *Gfp* riboprobe signal overlaps almost completely with that of *Col2a1*. Signal intensity for the *Gfp* anti-sense riboprobe differs between heterozygous and homozygous transgenic embryos. SA, splice acceptor site; pA, poly A.

obtained performing the analogous analysis on sections of E15.5 humeri from non-transgenic, heterozygous, and homozygous rKIIN-tg littermates (**Figures 4C,D**). Here, we did not observe an obvious delay in chondrocyte maturation, as one could have expected based on the effect of rKIIN overexpression in chicken.

The final step in endochondral ossification is the remodeling of the cartilage template into bone. Thus, in order to see whether this remodeling process was altered, we analyzed the longitudinal extension of the ossified zone by performing ISH analysis for the osteoblastic markers *collagen 1 (Col1a1), osteopontin (Opn),* and *osteocalcin (Ocn)* on sections of E18.5 DaCaMKII- and rKIIN-tg humeri and corresponding non-transgenic littermates. No significant alteration to the longitudinal extension of the ossified zone for either of the three analyzed markers was observed when comparing the humeri of the transgenic embryos expressing either DaCaMKII (**Figures 5A,B**) or rKIIN with humeri of their respective, non-transgenic controls (**Figures 5C,D**). Similar to what we had observed at E15.5, no morphological alterations with regard to the shape, organization or appearance of the prehypertrophic and hypertrophic chondrocytes were detected in the growth plates of the E18.5 DaCaMKII- and rKIIN-tg humeri compared to their corresponding non-transgenic littermate controls (Supplementary Figures 2A,B). In addition, the total length, as well as the length of the mineralized region was measured in alician blue/alizarin red stained humeri of 6-day-old pups. Here again, no differences regarding the two parameters were observed in comparison to non-transgenic littermate controls (Supplementary Figure 3).

Modulation of CaMKII Signaling Affects the Onset of Chondrocyte Maturation

As there were no obvious effects visible in the older mouse limbs, we addressed whether early steps of skeletogenesis would potentially be affected. In the forelimb the anlagen of humerus, radius and ulna are visible at E11.5. At this stage the cells in the center of the humerus begin to express *Ihh* (data not shown), while they start to express *Col10a1* around E12.5. In humeri of E12.5 DaCaMKII transgenic limbs the marker for prehypertrophic chondrocytes, *Ihh*, was expressed in a slightly broader domain compared to non-transgenic, control littermates (**Figures 6A–C**). The effect was more prominent in humeri of homozygous (6/8) than heterozygous (8/11). When we analyzed the expression of *Col10a1*, as a marker for hypertrophic chondrocyte differentiation, weak *Col10a1* expression was first detected in the humerus at E12.5-E13.0 in wild-type limbs. In the transgenic specimens, in which we had observed a broadend *Ihh* domain we noticed on adjacent sections a more intense staining for *Col10a1* in a broader domain (**Figure 6D**). Again

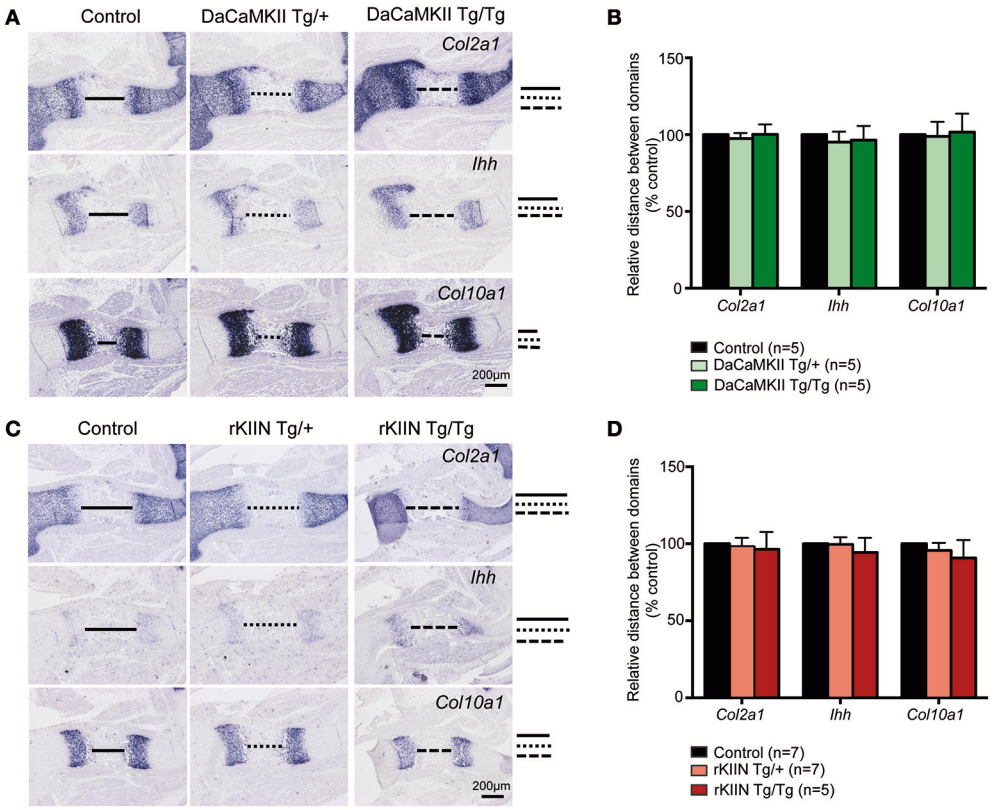

FIGURE 4 | Modulation of CaMKII activity has no effect on the progression of chondrocyte maturation. (A,C) Analysis of E15.5 DaCaMKII **(A)** and rKIIN **(C)** transgenic and respective control (non-transgenic) littermate humeri. **(A)** ISH for *Col2a1*, *Ihh,* and *Col10a1* on sections of E15.5 humeri from control, DaCaMKII hetero- (Tg/+) and homozygous (Tg/Tg) littermates, showing no major alterations in the expression levels or the sizes of the expression domains of these three markers. **(B)** Quantification of the relative distance between the *Col2a1*, *Ihh,* and *Col10a1* domains revealed no significant differences for these markers. **(C)** ISH for *Col2a1*, *Ihh,* and *Col10a1* on sections of E15.5 humeri from control, rKIIN hetero- (Tg/+) and homozygous (Tg/Tg) littermates revealed no obvious alterations. **(D)** Quantification of the relative distance between the *Col2a1*, the *Ihh,* and the *Col10a1* domains confirmed the absence of a significant difference. The distance between the domains in control was set to 100%. **(B,D)** The number of independent biological samples is referred to by *n*. The results are not significant. Error bars indicate ± SEM.

the effect was more obvious in the homozygous specimens. Next we asked whether the increased *Col10a1* expression was accompanied by the histological appearance of hypertrophic chondrocytes. For this, we examined E12.5 and E13.5 limbs by alcian blue staining. On alcian blue stained sections, the hypertrophic chondrocytes within the cartilage elements appear lighter in color due to their increase in size and vacuolization. Histological examination of E12.5 humeri ($n = 2$) revealed no apparent signs of hypertrophic chondrocyte differentiation in the transgenic limbs (Supplementary Figure S4A). In E13.5 DaCaMKII-tg humeri, a moderate size increase of the zone of hypertrophic chondrocytes was detected compared to the non-transgenic control (**Figure 6E**, $n = 3$). Consistent with the *in vivo* ISH results, qPCR analysis of material from E12.5 DaCaMKII transgenic and non-transgenic control limbs revealed an increase in *Ihh* and *Col10a1* expression levels in the transgenic limbs in comparison to the control (**Figure 6F**). These results suggest that the onset of chondrocyte maturation is slightly accelerated in the transgenic limbs.

We then analyzed rKIIN transgenic limbs at equivalent stages. At E12.5, no obvious differences were detected with respect to the size or intensity of the *Ihh* expression domains between transgenic and non-transgenic embryos (Supplementary Figure S4B). However, at E13.5 the two expression domains of *Ihh* were not yet separated in the humeri of homozygous transgenic animals and still closer together in the heterozygous transgenic animals compared to non-transgenic littermate controls (**Figure 6I**). Furthermore, the *Col10a1* expression domain was reduced in size in the humeri of the transgenic animals compared to non-transgenic littermate controls (**Figure 6J**). Together, his suggests that the onset of chondrocyte maturation is slightly delayed when CaMKII signaling is antagonized. Again, the effect was more pronounced in homozygous (9/9) than in heterozygous transgenic specimens (11/13) compared to non-transgenic specimens (**Figures 6I,J**). Histological examination of alcian blue stained E13.5 humeri confirmed that the decrease in the size of the *Col10a1* expression domain in homo- and heterozygous humeri was associated with reduced hypertrophic chondrocyte differentiation (**Figure 6K**). Quantification of *Ihh* and *Col10a1* expression levels by qPCR using material from E13.5 forelimbs corroborated the dose-dependent effects of CaMKII-signaling inhibition, which was statistically significant

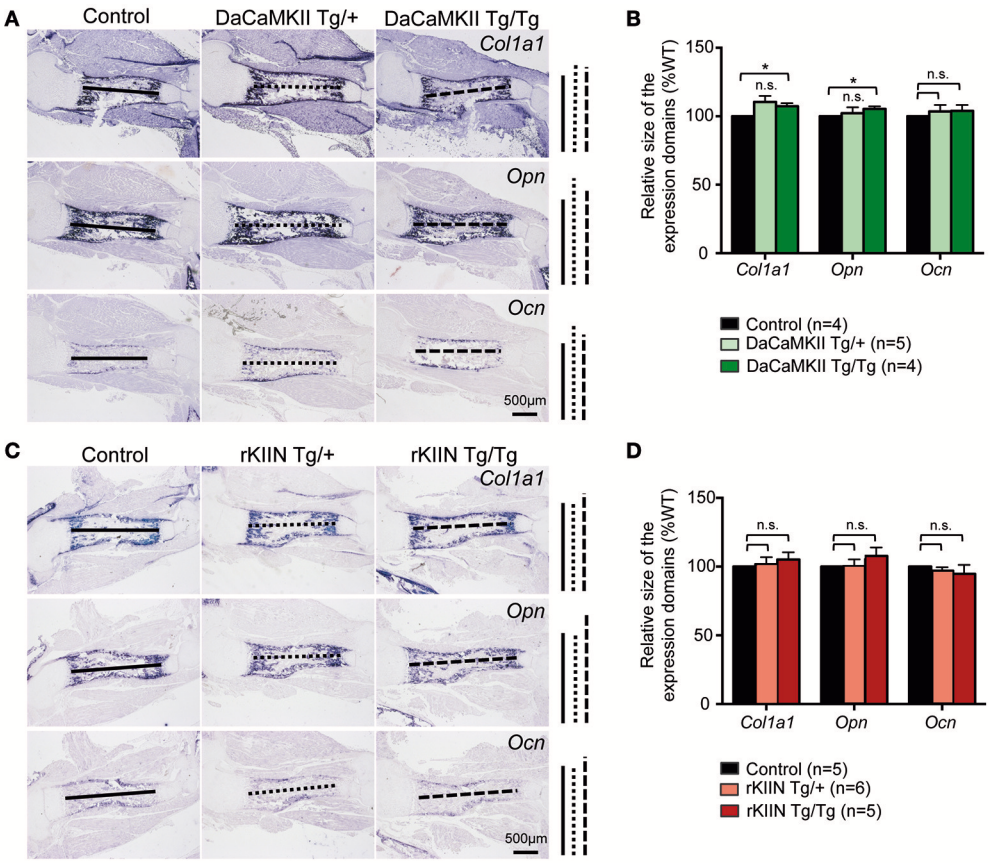

FIGURE 5 | Modulation of CaMKII activity does not affect the endochondral ossification process. (A,C) Representative images of ISH for the osteoblastic markers *Col1a1, Opn,* and *Ocn* on sections through E18.5 humeri of DaCaMKII- **(A)** and rKIIN-tg **(C),** and their respective littermate, non-transgenic controls. **(B,D)** Bar diagram representing relative size (longitudinal expansion) of the *Col1a1*, *Opn,* and *Ocn* expressing domains (zone of ossification) in the humeri of control (non-transgenic), DaCaMKII- **(B)** and rKIIN-tg **(D)** E18.5 embryos. **(B,D)** The length between the domains in the control humeri was set to 100% to account for developmental differences between litters. *n* refers to the number of independent biological samples. $^*p < 0.05$, n.s., not significant. Error bars indicate ± SEM.

with respect to *Col10a1* expression (**Figure 6L**). Together, our results suggest that modulation of CaMKII signaling in the mouse affects the onset of chondrocyte maturation during the early stages of endochondral ossification.

ISH with a *Gfp* riboprobe confirmed transgene expression at all stages examined and at least according to the ISH transgene expression was not silenced after E13.5 (Supplementary Figure 5). In order to determine why the expression of the DaCaMKII and rKIIN-transgenes may not lead to a major phenotype at later stages we examined the transgene expression level in the two DaCaMKII-transgenic lines, DaCaMKII-Tg1 (1 copy) and DaCaMKII -Tg2 (19 copies), using qPCR and RNA isolated from the chondrogenic elements of E12.5 limbs. Transgene expression levels were estimated by comparing the DaCaMKII::GFP transgene expression level to the endogenous expression levels of the four CaMKII isoforms and the levels were normalized to the *Camk2g* expression level. Here, we noticed that the transgene expression levels in the Tg1 line were similar to the *Camk2a* endogenous levels in the wild-type control. Yet, they were lower than the endogenous expression levels of the γ and δ isoforms. Interestingly, the *Camk2a* expression levels were increased significantly in the transgenic skeletal elements of the DaCaMKII-Tg1 line. In contrast, transgene expression level in the Tg2 line with 19 copies did not even reach the *Camk2a* endogenous levels. As such, the transgene copy number did not correlate with the transgene expression levels in the two independent lines (Supplementary Figures 6A,B).

DISCUSSION

Based on previous work in chicken it has been proposed that activation of the endogenous CaMKII activity controls the onset of the prehypertrophic and hypertrophic chondrogenic program. In proliferating chondrocytes, the phosphorylated form of CaMKII is not detectable and possibly constantly dephosphorylated under the influence of PTHrP signaling (Li et al., 2011). As a consequence, the phosphorylated form of CaMKII is limited to the prehypertrophic and hypertrophic chondrocytes in chicken and mouse (Li et al., 2011). In the chicken, ectopic expression of an activated form of CaMKII, was able to override the endogenous CaMKII activity or lack thereof in proliferating chondrocytes and led to premature and ectopic activation of the prehypertrophic/hypertrophic program in cells outside of the normal maturation zones (Taschner et al.,

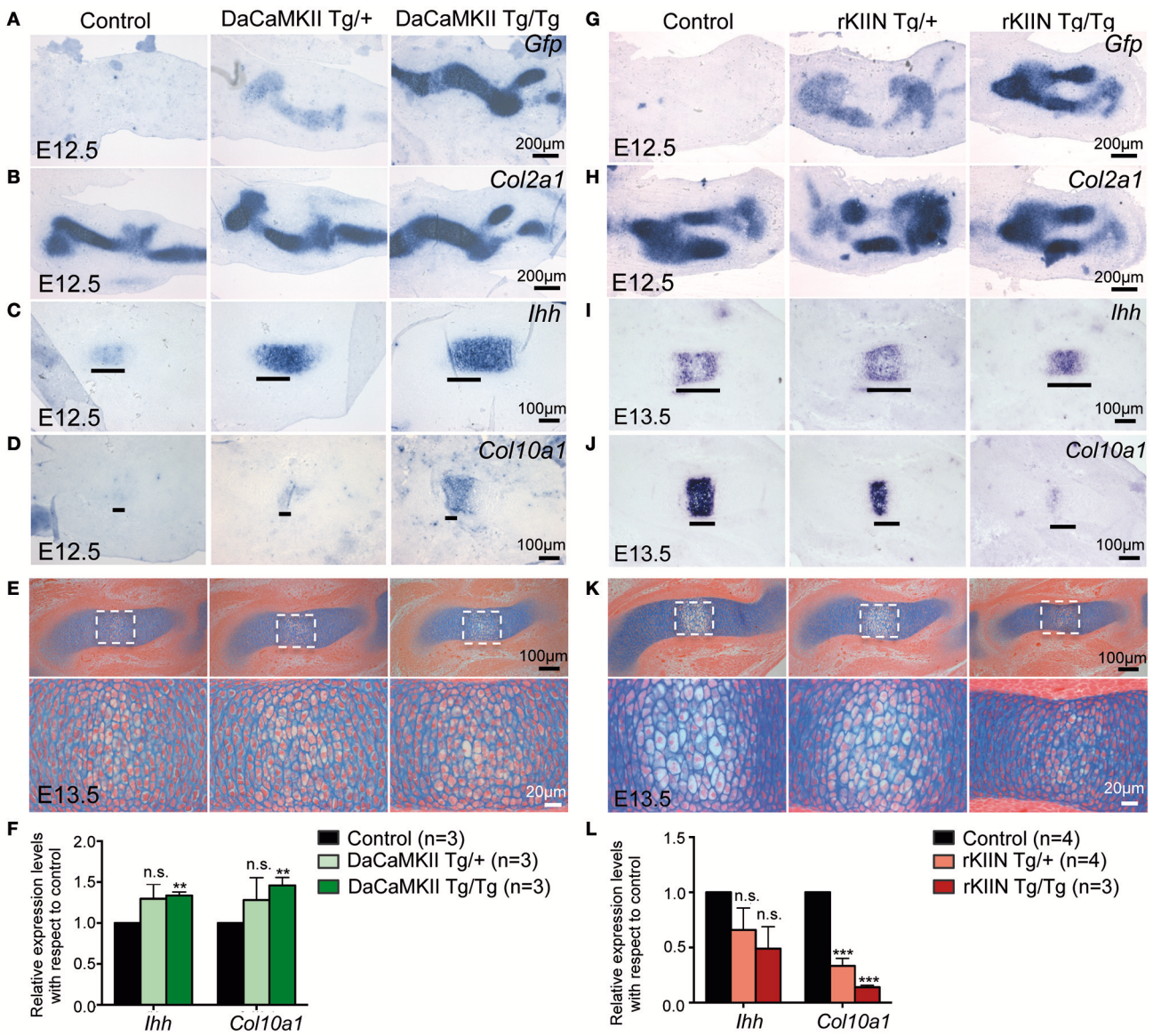

FIGURE 6 | Modulation of CaMKII signaling affects the onset of chondrocyte maturation in early skeletal development. (A–D) Representative, alternating sections through humeri of E12.5 control (non-transgenic, +/+), hetero- (Tg/+), and homozygous (Tg/Tg) DaCaMKII-tg littermates. **(A)** *Gfp* ISH revealing transgene expression in heterozygous and homozygous animals but not in non-transgenic littermate controls. **(B)** *Col2a1* ISH confirming that the transgene expression is restricted to the *Col2a1* positive domain. **(C)** *Ihh* ISH defining the prehypertrophic zone, which is enlarged in the homozygous and heterozygous transgenic embryos. **(D)** *Col10a1* ISH marking the onset of hypertrophic chondrocyte differentiation. *Col10a1* expression was more intense in the transgenic embryos compared to control (non-transgenic) littermates. **(E)** Alcian blue/eosin stained representative images of sections through E13.5 humeri of control (non-transgenic; +/+), hetero- (Tg/+), and homozygous (Tg/Tg) DaCaMKII-tg embryos. Images below show magnifications of the boxed areas in the panel above. Due to their increase in volume and vacuolization of the cells, the hypertrophic chondrocytes appear whiter. The region of hypertrophy is enlarged in the transgenic humeri compared to wild-type littermate control. **(F)** qPCR analysis for *Ihh* and *Col10a1* normalized to *Gapdh* and *Actb* using material from E12.5 control, hetero- and homozygous DaCaMKII-tg forelimbs reveals a dose-dependent increase in the expression of these two genes. Gene expression levels are plotted relative to control. **(G–J)** Representative, alternating sections through humeri of E12.5 **(G,H)** and E13.5 **(I,J)** control (non-transgenic; +/+) and hetero- (Tg/+), and homozygous (Tg/Tg) rKIIN-tg littermates. **(G)** *Gfp* ISH revealing transgene expression in heterozygous and homozygous animals but not in non-transgenic littermate controls. **(H)** *Col2a1* ISH confirming that the transgene expression is restricted to the *Col2a1* positive domain. **(I)** *Ihh* ISH defining the prehypertrophic zone, which is reduced in a transgene dose-dependent manner in the hetero- and homozygous transgenic embryos. **(J)** *Col10a1* ISH defining the hypertrophic zone, showing that hypertrophic differentiation is even more delayed. **(K)** Histological alcian blue/eosin stained representative images of sections from control, hetero- and homozygous rKIIN embryos: here, fewer and smaller hypertrophic, whiter cells are visible in hetero- and homozygous transgenic humeri compared to wild-type littermate controls. **(L)** qPCR analysis for *Ihh* and *Col10a1* normalized to *Gapdh* and *Actb* using material from E13.5 control, hetero- and homozygous rKIIN-tg forelimbs revealing a dose-dependent reduction in the expression of *Ihh* and *Col10a1*. Gene expression levels are plotted relative to control. **(F,L)** *n* refers to the number of independent biological samples. $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Error bars indicate ± SEM.

2008; Li and Dudley, 2009; Li et al., 2011). In mouse long bone development, the phenotypic effects of CaMKII activation in proliferating, type II collagen-expressing chondrocytes were very mild and *in vivo,* effects could only be detected developmentally around the onset of chondrocyte hypertrophy at E12.5–E13.5. Here, our data suggest that in agreement with previous findings in chicken, DaCaMKII in mouse activated the prehypertrophic/hypertrophic program prematurely at early stages of endochondral ossification, while down-regulation of endogenous CaMKII activity interfered with the onset of the prehypertrophic and hypertrophic program. Interestingly, the phenotypic changes caused by CaMKII activity modulation at the onset of chondrocyte maturation did not accumulate over time despite the fact that the transgene was continuously expressed in type II collagen-producing chondrocytes (Supplementary Figure 5). Yet, in contrast to the overexpression experiments in chicken, no premature maturation of chondrocytes outside their normal maturation zones or shortening of the limbs was observed. Hence, the phenotypic consequences of ectopic activation of CaMKII are quite distinct between chicken and mouse (Taschner et al., 2008; Li and Dudley, 2009; Li et al., 2011). Possible explanations for the phenotypic discrepancies between chicken and mouse could be that on the one hand in the chicken not only chondrocytes also the soft tissue is infected and that this could contribute to the phenotype. On the other hand, it is likely that the retroviral driven expression levels of the transgenes were much higher in chicken. For retroviral driven transgenes over 100-fold expression levels have been reported (Geetha-Loganathan et al., 2014; Nimmagadda et al., 2015). In the mouse, we did not detect phenotypic differences between the independent transgenic lines despite the fact that they varied in copy numbers (**Figure 3A**), which can be explained by the fact that transgene expression levels were similarly low in both cases (about one-fold of the Camk2a endogenous levels; Supplementary Figure 6). Hence, the lack of a major phenotype in the transgenic mice may be associated with the relatively low transgene expression levels compared to the expression levels that have been reached by retroviral expression in the chicken. Although, of course, the actual levels of retroviral-driven DaCaMKII transgene expression were not determined in the chicken experiments performed previously (Taschner et al., 2008; Li and Dudley, 2009; Li et al., 2011). A possible explanation for the observed transient phenotype at the early stages of skeletal development maybe that the onset of chondrocyte maturation may present a window of opportunity for the transgenes to excert a mild effect accelerating, respectively delaying chondrocyte maturation. And that this effect is later on compensated as the chondrocyte maturation program comes under the transcriptional control of many regulatory factors and feed back mechanisms as development progresses (Hartmann, 2009; Kozhemyakina et al., 2015).

At the molecular level, we observed *in vitro* that CaMKII robustly affects the transcriptional activity of the transcription factor Mef2c and to a lesser extent also Runx2 transcriptional activity. Mef2c and Runx2 start to be expressed as the chondrocytes undergo hypertrophy (Arnold et al., 2007). Mef2c and Runx2 activity are both negatively regulated by the class II histone deacetylase HDAC4 (Vega et al., 2004; Kozhemyakina et al., 2009; Correa et al., 2010). In vascular smooth muscle cells, CaMKIIdelta2 regulates Mef2 transcriptional activity through HDAC4/5 (Ginnan et al., 2012). Yet, our results indicate that activated CaMKII increases Mef2c activity by an HDAC4-independent mechanism. As the activated form of CaMKII physically interacts with Mef2c, the underlying mechanism may involve phosphorylation of Mef2c protein by CaMKII. In different cell types other kinases, such as p38 MAPK and ERK5, have also been shown to phosphorylate Mef2c enhancing its transcriptional activity (Han et al., 1997; Kato et al., 1997). Yet, there is the obvious discrepancy between the *in vitro* results where a robust stimulatory effect on Mef2c transcriptional activity was observed and the subtle *in vivo* effects. For the *in vitro* luciferase experiments an expression vector was used that drives DaCaMKII expression under the control of the CMV promoter, which drives high levels of expression in mammalian cells. The high transgene expression levels *in vitro* (Supplementary Figure 1) may be an explaination for the strong *in vitro* effect on Mef2c activity and in contrast to the only mild effect *in vivo* where transgene expression levels were at least a 100-fold lower. Furthermore, Li and colleagues proposed that the endogenous CaMKII activity is opposed by an inhibitory gradient, which is under the control of PTHrP signaling (Li et al., 2011). PTHrP signaling via cAMP and protein kinase A negatively regulates chondrocyte hypertrophy through HDAC4 mediated inhibition of Mef2c activity (Kozhemyakina et al., 2009). Yet, even *in vitro*, DaCaMKII signaling was not able to overcome or to partially revert the effect of HDAC4 on Mef2c activity (**Figure 2C**). The PTHrP expression itself is under the regulatory control of Ihh-signaling (Vortkamp et al., 1996; St-Jacques et al., 1999). As such the slightly increased Ihh expression observed in the E12.5 DaCaMKII transgenic limbs may upregulate PTHrP signaling and counteract a possibly direct, positive effect of DaCaMKII on the Mef2c activity via HDAC4. Hence, one possible scenario may be that increased levels of PTHrP signaling override the activation of Mef2c by DaCaMKII at later stages of development.

AUTHOR CONTRIBUTIONS

CA: Data collection, assembly, analyses, interpretation, and manuscript writing. CF: Performed the co-immunoprecipitation studies. AH: Performed qPCR studies determining transgene copy numbers and expression levels, and proof-read the manuscript. LW: Performed histological analysis on E18.5 material. CH: Conception and design, data interpretation, and manuscript writing.

FUNDING

Part of the work was conducted at the Institute of Molecular Pathology in Vienna which is funded by Boehringer Ingelheim. AH was supported by a grant from the Deutsche Forschungsgemeinsaft [HA 4767/2-1] and LIW is supported by a grant from the BMBF [Overload-PrevOP, SP07]. The funding sources had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Christian Theussl for pronuclear injections and Joanna Sherwood for checking the English in the manuscript. We also thank the reviewers for their valuable input.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2017.00020/full#supplementary-material

REFERENCES

Akiyama, H., and Lefebvre, V. (2011). Unraveling the transcriptional regulatory machinery in chondrogenesis. *J. Bone Miner. Metab.* 29, 390–395. doi: 10.1007/s00774-011-0273-9

Arnold, M. A., Kim, Y., Czubryt, M. P., Phan, D., McAnally, J., Qi, X., et al. (2007). MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev. Cell* 12, 377–389. doi: 10.1016/j.devcel.2007.02.004

Backs, J., Backs, T., Neef, S., Kreusser, M. M., Lehmann, L. H., Patrick, D. M., et al. (2009). The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2342–2347. doi: 10.1073/pnas.0813013106

Backs, J., Song, K., Bezprozvannaya, S., Chang, S., and Olson, E. N. (2006). CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. *J. Clin. Invest.* 116, 1853–1864. doi: 10.1172/JCI27438

Ballester, M., Castelló, A., Ibáñez, E., Sánchez, A., and Folch, J. M. (2004). Real-time quantitative PCR-based system for determining transgene copy number in transgenic animals. *Biotechniques* 37, 610–613.

Chang, B. H., Mukherji, S., and Soderling, T. R. (1998). Characterization of a calmodulin kinase II inhibitor protein in brain. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10890–10895. doi: 10.1073/pnas.95.18.10890

Chang, B. H., Mukherji, S., and Soderling, T. R. (2001). Calcium/calmodulin-dependent protein kinase II inhibitor protein: localization of isoforms in rat brain. *Neuroscience* 102, 767–777. doi: 10.1016/S0306-4522(00)00520-0

Cheah, K. S., Lau, E. T., Au, P. K., and Tam, P. P. (1991). Expression of the mouse alpha 1(II) collagen gene is not restricted to cartilage during development. *Development* 111, 945–953.

Colbran, R. J. (2004). Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem. J.* 378(Pt 1), 1–16. doi: 10.1042/bj20031547

Colbran, R. J., Smith, M. K., Schworer, C. M., Fong, Y. L., and Soderling, T. R. (1989). Regulatory domain of calcium/calmodulin-dependent protein kinase II. Mechanism of inhibition and regulation by phosphorylation. *J. Biol. Chem.* 264, 4800–4804.

Correa, D., Hesse, E., Seriwatanachai, D., Kiviranta, R., Saito, H., Yamana, K., et al. (2010). Zfp521 is a target gene and key effector of parathyroid hormone-related peptide signaling in growth plate chondrocytes. *Dev. Cell* 19, 533–546. doi: 10.1016/j.devcel.2010.09.008

Ducy, P., and Karsenty, G. (1995). Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.* 15, 1858–1869. doi: 10.1128/MCB.15.4.1858

Erickson, J. R., Joiner, M. L., Guan, X., Kutschke, W., Yang, J., Oddis, C. V., et al. (2008). A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* 133, 462–474. doi: 10.1016/j.cell.2008.02.048

Erlebacher, A., Filvaroff, E. H., Gitelman, S. E., and Derynck, R. (1995). Toward a molecular understanding of skeletal development. *Cell* 80, 371–378. doi: 10.1016/0092-8674(95)90487-5

Geetha-Loganathan, P., Nimmagadda, S., Fu, K., and Richman, J. M. (2014). Avian facial morphogenesis is regulated by c-Jun N-terminal kinase/planar cell polarity (JNK/PCP) wingless-related (WNT) signaling. *J. Biol. Chem.* 289, 24153–24167. doi: 10.1074/jbc.M113.522003

Ginnan, R., Sun, L. Y., Schwarz, J. J., and Singer, H. A. (2012). MEF2 is regulated by CaMKIIdelta2 and a HDAC4-HDAC5 heterodimer in vascular smooth muscle cells. *Biochem. J.* 444, 105–114. doi: 10.1042/BJ20120152

Guo, X., Mak, K. K., Taketo, M. M., and Yang, Y. (2009). The Wnt/beta-catenin pathway interacts differentially with PTHrP signaling to control chondrocyte hypertrophy and final maturation. *PLoS ONE* 4:e6067. doi: 10.1371/journal.pone.0006067

Hampf, M., and Gossen, M. (2006). A protocol for combined Photinus and *Renilla luciferase* quantification compatible with protein assays. *Anal. Biochem.* 356, 94–99. doi: 10.1016/j.ab.2006.04.046

Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997). Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* 386, 296–299. doi: 10.1038/386296a0

Hartmann, C. (2009). Transcriptional networks controlling skeletal development. *Curr. Opin. Genet. Dev.* 19, 437–443. doi: 10.1016/j.gde.2009.09.001

Hartmann, C., and Tabin, C. J. (2000). Dual roles of Wnt signaling during chondrogenesis in the chicken limb. *Development* 127, 3141–3159.

Hill, T. P., Später, D., Taketo, M. M., Birchmeier, W., and Hartmann, C. (2005). Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev. Cell* 8, 727–738. doi: 10.1016/j.devcel.2005.02.013

Hogan, B., Beddington, R., Constantini, F., and Lancy, E. (1994). *Manipulating the Mouse Embryo*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Houben, A., Kostanova-Poliakova, D., Weissenböck, M., Graf, J., Teufel, S., von der Mark, K., et al. (2016). beta-catenin activity in late hypertrophic chondrocytes locally orchestrates osteoblastogenesis and osteoclastogenesis. *Development* 143, 3826–3838. doi: 10.1242/dev.137489

Hudmon, A., and Schulman, H. (2002). Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J.* 364(Pt 3), 593–611. doi: 10.1042/bj20020228

Inada, M., Yasui, T., Nomura, S., Miyake, S., Deguchi, K., Himeno, M., et al. (1999). Maturational disturbance of chondrocytes in Cbfa1-deficient mice. *Dev. Dyn.* 214, 279-290.

Karsenty, G. (2008). Transcriptional control of skeletogenesis. *Annu. Rev. Genomics Hum. Genet.* 9, 183–196. doi: 10.1146/annurev.genom.9.081307.164437

Kato, Y., Kravchenko, V. V., Tapping, R. I., Han, J., Ulevitch, R. J., and Lee, J. D. (1997). BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* 16, 7054–7066. doi: 10.1093/emboj/16.23.7054

Kim, I. S., Otto, F., Zabel, B., and Mundlos, S. (1999). Regulation of chondrocyte differentiation by Cbfa1. *Mech. Dev.* 80, 159–170. doi: 10.1016/S0925-4773(98)00210-X

Kozhemyakina, E., Cohen, T., Yao, T. P., and Lassar, A. B. (2009). Parathyroid hormone-related peptide represses chondrocyte hypertrophy through a protein phosphatase 2A/histone deacetylase 4/MEF2 pathway. *Mol. Cell. Biol.* 29, 5751–5762. doi: 10.1128/MCB.00415-09

Kozhemyakina, E., Lassar, A. B., and Zelzer, E. (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development* 142, 817–831. doi: 10.1242/dev.105536

Kronenberg, H. M. (2003). Developmental regulation of the growth plate. *Nature* 423, 332–336. doi: 10.1038/nature01657

Lantsman, K., and Tombes, R. M. (2005). CaMK-II oligomerization potential determined using CFP/YFP FRET. *Biochim. Biophys. Acta* 1746, 45–54. doi: 10.1016/j.bbamcr.2005.08.005

Lefebvre, V., and Bhattaram, P. (2010). Vertebrate skeletogenesis. *Curr. Top. Dev. Biol.* 90, 291–317. doi: 10.1016/S0070-2153(10)90008-2

Li, Y., Ahrens, M. J., Wu, A., Liu, J., and Dudley, A. T. (2011). Calcium/calmodulin-dependent protein kinase II activity regulates the proliferative potential of growth plate chondrocytes. *Development* 138, 359–370. doi: 10.1242/dev.052324

Li, Y., and Dudley, A. T. (2009). Noncanonical frizzled signaling regulates cell polarity of growth plate chondrocytes. *Development* 136, 1083–1092. doi: 10.1242/dev.023820

Mak, K. K., Kronenberg, H. M., Chuang, P. T., Mackem, S., and Yang, Y. (2008). Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy. *Development* 135, 1947–1956. doi: 10.1242/dev.018044

McLeod, M. J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* 22, 299–301. doi: 10.1002/tera.1420220306

Murtaugh, L. C., Zeng, L., Chyung, J. H., and Lassar, A. B. (2001). The chick transcriptional repressor Nkx3.2 acts downstream of Shh to promote BMP-dependent axial chondrogenesis. *Dev. Cell* 1, 411–422. doi: 10.1016/S1534-5807(01)00039-9

Nakata, K., Ono, K., Miyazaki, J., Olsen, B. R., Muragaki, Y., Adachi, E., et al. (1993). Osteoarthritis associated with mild chondrodysplasia in transgenic mice expressing alpha 1(IX) collagen chains with a central deletion. *Proc. Natl. Acad. Sci. U.S.A.* 90, 2870–2874. doi: 10.1073/pnas.90.7.2870

Naya, F. J., Wu, C., Richardson, J. A., Overbeek, P., and Olson, E. N. (1999). Transcriptional activity of MEF2 during mouse embryogenesis monitored with a MEF2-dependent transgene. *Development* 126, 2045–2052.

Nimmagadda, S., Buchtová, M., Fu, K., Geetha-Loganathan, P., Hosseini-Farahabadi, S., Trachtenberg, A. J., et al. (2015). Identification and functional analysis of novel facial patterning genes in the duplicated beak chicken embryo. *Dev. Biol.* 407, 275–288. doi: 10.1016/j.ydbio.2015.09.007

Quinn, C. O., Rajakumar, R. A., and Agapova, O. A. (2000). Parathyroid hormone induces rat interstitial collagenase mRNA through Ets-1 facilitated by cyclic AMP response element-binding protein and Ca(2^+)/calmodulin-dependent protein kinase II in osteoblastic cells. *J. Mol. Endocrinol.* 25, 73–84. doi: 10.1677/jme.0.0250073

Rosenberg, O. S., Deindl, S., Comolli, L. R., Hoelz, A., Downing, K. H., Nairn, A. C., et al. (2006). Oligomerization states of the association domain and the holoenyzme of Ca2$^+$/CaM kinase II. *FEBS J.* 273, 682–694. doi: 10.1111/j.1742-4658.2005.05088.x

Salter, D. M., Wright, M. O., and Millward-Sadler, S. J. (2004). NMDA receptor expression and roles in human articular chondrocyte mechanotransduction. *Biorheology* 41, 273–281.

Shimazaki, A., Wright, M. O., Elliot, K., Salter, D. M., and Millward-Sadler, S. J. (2006). Calcium/calmodulin-dependent protein kinase II in human articular chondrocytes. *Biorheology* 43, 223–233.

Soderling, T. R. (1996). Structure and regulation of calcium/calmodulin-dependent protein kinases II and IV. *Biochim. Biophys. Acta* 1297, 131–138. doi: 10.1016/S0167-4838(96)00105-7

St-Jacques, B., Hammerschmidt, M., and McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* 13, 2072–2086. doi: 10.1101/gad.13.16.2072

Taschner, M. J., Rafigh, M., Lampert, F., Schnaiter, S., and Hartmann, C. (2008). Ca2$^+$/Calmodulin-dependent kinase II signaling causes skeletal overgrowth and premature chondrocyte maturation. *Dev. Biol.* 317, 132–146. doi: 10.1016/j.ydbio.2008.02.007

Tombes, R. M., Faison, M. O., and Turbeville, J. M. (2003). Organization and evolution of multifunctional Ca(2^+)/CaM-dependent protein kinase genes. *Gene* 322, 17–31. doi: 10.1016/j.gene.2003.08.023

Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., et al. (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* 119, 555–566. doi: 10.1016/j.cell.2004.10.024

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 273, 613–622. doi: 10.1126/science.273.5275.613

Yoshida, C. A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., et al. (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* 18, 952–963. doi: 10.1101/gad.1174704

Zayzafoon, M., Fulzele, K., and McDonald, J. M. (2005). Calmodulin and calmodulin-dependent kinase IIalpha regulate osteoblast differentiation by controlling c-fos expression. *J. Biol. Chem.* 280, 7049–7059. doi: 10.1074/jbc.M412680200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 04 May 2017
doi: 10.3389/fcell.2017.00047

A Novel Role for the BMP Antagonist Noggin in Sensitizing Cells to Non-canonical Wnt-5a/Ror2/ Disheveled Pathway Activation

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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: 30 January 2017
Accepted: 13 April 2017
Published: 04 May 2017

Citation:
Bernatik O, Radaszkiewicz T, Behal M, Dave Z, Witte F, Mahl A, Cernohorsky NH, Krejci P, Stricker S and Bryja V (2017) A Novel Role for the BMP Antagonist Noggin in Sensitizing Cells to Non-canonical Wnt-5a/Ror2/ Disheveled Pathway Activation. Front. Cell Dev. Biol. 5:47. doi: 10.3389/fcell.2017.00047

Mammalian limb development is driven by the integrative input from several signaling pathways; a failure to receive or a misinterpretation of these signals results in skeletal defects. The brachydactylies, a group of overlapping inherited human hand malformation syndromes, are mainly caused by mutations in BMP signaling pathway components. Two closely related forms, Brachydactyly type B2 (BDB2) and BDB1 are caused by mutations in the BMP antagonist Noggin (NOG) and the atypical receptor tyrosine kinase ROR2 that acts as a receptor in the non-canonical Wnt pathway. Genetic analysis of Nog and Ror2 functional interaction via crossing Noggin and Ror2 mutant mice revealed a widening of skeletal elements in compound but not in any of the single mutants, thus indicating genetic interaction. Since ROR2 is a non-canonical Wnt co-receptor specific for Wnt-5a we speculated that this phenotype might be a result of deregulated Wnt-5a signaling activation, which is known to be essential for limb skeletal elements growth and patterning. We show that Noggin potentiates activation of the Wnt-5a-Ror2-Disheveled (Dvl) pathway in mouse embryonic fibroblast (MEF) cells in a Ror2-dependent fashion. Rat chondrosarcoma chondrocytes (RCS), however, are not able to respond to Noggin in this fashion unless growth arrest is induced by FGF2. In summary, our data demonstrate genetic interaction between Noggin and Ror2 and show that Noggin can sensitize cells to Wnt-5a/Ror2-mediated non-canonical Wnt signaling, a feature that in cartilage may depend on the presence of active FGF signaling. These findings indicate an unappreciated function of Noggin that will help to understand BMP and Wnt/PCP signaling pathway interactions.

Keywords: noggin, Wnt5a, non-canonical Wnt pathways, BMP signaling, brachydactyly, Ror2

INTRODUCTION

Limb bud development and the concomitant formation of limb skeletal structures are regulated by the intricate interplay and integration of various signaling pathways, with major roles played by the Shh, BMP, FGF, and Wnt/β-catenin pathways (reviewed for example in, Robert, 2007; Zuniga, 2015). The BMP signaling pathway is of pivotal importance especially for skeletal development. The analysis of inheritable human hand malformation syndromes has been instrumental in understanding the contribution of BMP signaling and other pathways for skeletal development. One example are the brachydactylies, a group of inheritable syndromes that are characterized by shortening or absence of phalanges. Most brachydactyly subtypes are caused by mutations in BMP signaling components or factors that, at different levels, intersect with BMP signaling. Therefore brachydactylies have been interpreted in terms of a molecular disease family (Stricker and Mundlos, 2011). This hypothesis predicts that overlapping phenotypes are likely caused by mutations affecting components that show a close functional interaction within a common signaling network.

Intriguingly, two closely related brachydactyly subtypes, BDB1 and BDB2, are caused by mutations in ROR2 or NOGGIN, respectively (Oldridge et al., 2000; Lehmann et al., 2007). While NOG is well known as a secreted BMP antagonist, ROR2 is an atypical receptor tyrosine kinase that is involved in the inhibition of Wnt/β-catenin signaling (Mikels and Nusse, 2006). In developing digits, Ror2-mediated Wnt/β-catenin inhibition allows BMP-mediated digit outgrowth (Witte et al., 2010). In addition, Ror2 is a Wnt (co)receptor, mainly for Wnt-5a, acting in non-canonical Wnt signaling (Oishi et al., 2003; Schambony and Wedlich, 2007). Recently, activation of the non-canonical Wnt/planar cell polarity (PCP) pathway by Wnt-5a and ROR2 was shown to be critically involved in the regulation of limb skeleton development (Gao et al., 2011; Wang et al., 2011; Ho et al., 2012; Kuss et al., 2014). Moreover, a separate set of mutations in ROR2 causes autosomal recessive Robinow syndrome (RS), which is characterized by diverse malformations including the axial and limb skeleton (Afzal et al., 2000; van Bokhoven et al., 2000). A dominant form of RS is caused by mutations in Wnt/PCP components DVL1, DVL3, and WNT-5A, it is therefore believed that the developmental defects seen in Robinow syndrome are caused by a deregulation of Wnt-5a/Ror2/PCP signaling (Stricker et al., 2017).

The skeletal elements of the limbs are formed by endochondral ossification. In this process a cartilage template is formed that mediates growth of the skeletal element and becomes later replaced by bone. This process is dependent on the formation of stacked columns of proliferating chondrocytes oriented perpendicular to the longitudinal axis of the growing skeletal element (Romereim and Dudley, 2011). Deregulation of PCP signaling in proliferating chondrocytes leads to perturbation of column formation, and to arbitrary chondrocyte orientation that ultimately leads to skeletal malformations typically resulting in a shortening and widening of the skeletal elements (Ahrens et al., 2009; Li and Dudley, 2009; Kuss et al., 2014; Romereim et al., 2014).

Based on the close phenotypic overlap of human brachydactyly-causing mutations in ROR2 and NOG, we hypothesized that NOG may directly interact with the Wnt-5a/Ror2 pathway. We show here a subtle genetic interaction of Noggin with Ror2 during mouse limb development. Mechanistically, we provide evidence that Noggin can sensitize cells to Wnt/PCP pathway activation mediated by ROR2, providing first evidence for a yet uncharacterized level of cross-talk between BMP and Wnt/PCP signaling.

MATERIALS AND METHODS

Mouse Lines and Phenotypical Analysis

$Ror2^{+/-}$ (Takeuchi et al., 2000) and $Nog^{+/-}$ (McMahon et al., 1998) were maintained as heterozygous lines and intercrossed to yield compound mutants. Timed matings were set up and embryos were collected at E18.5. Skeletal preparations were performed as described previously (Mundlos, 2000). All animal procedures were carried out in accordance with European Union and German law. Animals were maintained in the SPF mouse facility of the Max Planck Institute for Molecular Genetics, Berlin under license from the Landesamt für Gesundheit und Soziales (LAGeSo) under license numbers ZH120 and G0346/13.

Cell Culture and Treatments

$Ror1^{-/-}$ $Ror2^{-/-}$ mouse embryonic fibroblasts (MEF) were derived from Ror1 $^{flox/flox}$ Ror2 $^{flox/flox}$ MEF cells as described previously (Ho et al., 2012). MEF and RCS cells were propagated in DMEM, 10% FCS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 units/ml streptomycin. RCS cells were seeded in 24-well plates, grown for 24 h and treated as indicated. Following reagents: Wnt-5a (R&D systems, 645-WN-010), Noggin (R&D Systems, 1967-NG-025), FGF2 (5 ng/ml, R&D Systems) and Wnt-C59 5 μM (Tocris Bioscience, 5148) were used for treatment. Wnt-5a conditioned media was produced from L Wnt-5a cells (ATCC CRL-2814) according to ATCC instructions. RCS cells intended for WB analysis were treated by FGF2 for 48 h, then were treated by the porcupine inhibitor Wnt-C59 (to reduce background autocrine Wnt activity), Noggin and Wnt-5a in indicated doses for additional 24 h. Total time of FGF2 treatment was 72 h.

Western Blotting

Lysates for western blotting were prepared as follows: Growth medium was removed and cells were directly lysed in 100 mM Tris/HCl (pH 6.8), 20% glycerol, 1% SDS, 0.01% bromophenol blue and 1% 2-mercaptoethanol. Western blotting was performed according to manufacturer's instructions with minor adjustments [SDS-PAGE run on 150 V, transfer onto PVDF membrane 1 h on 100 V, both steps on ice (BIO-RAD)]. Antibodies were from Santa Cruz Biotechnologies: anti-Dvl2 (dephospho-Dvl2)–sc8026, anti-beta-Actin–sc1615-R, anti-Dvl3 sc8027 and from Cell Signaling Technologies: anti-Dvl2–CS3224. Anti-Ror2 antibody was a gift from Henry Ho (UC Davis) (Ho et al., 2012). Phosphorylation status of Dvl2 and Dvl3 was quantified

by densitometric analysis of Western Blot in three independent replicates using Fiji distribution of ImageJ software as described (Bernatik et al., 2014). For pDvl/Dvl rations the peak area for the upper band representing P-Dvl was divided by the peak area of the lower band (Dvl). Data was analyzed by paired *t*-test (GraphPad Prism).

Dual Luciferase Assay

RCS cells were transfected using pRLtkLuc and Super8X TopFlash plasmid. 9μg Super8X TopFlash and 3 μg pRLtkLuc plasmid were mixed with 38.4 μl of Fugene6 (E2691, Promega) in 1200 μl of DMEM. Cells were treated by 0.3% collagenase type II (GIBCO, cat.no.17101015) before transfection, 50 μl of transfection mixture and 500 μl of collagenase treated RCS cells in DMEM were used per 1 well of 24 well plate. Transfection was carried out overnight, cells were treated according to the experimental scheme for 20 h, and samples were processed by Dual-Luciferase® Reporter Assay System according to the manufacturer instructions (Promega, E1960).

RESULTS

Noggin Genetically Interacts with Ror2

To get a first indication whether Ror2 and Noggin might functionally interact we generated compound mutants for Ror2 and Noggin. $Ror2^{+/-}$ mice (Takeuchi et al., 2000) were crossed to $Noggin^{+/-}$ mice (Brunet et al., 1998; McMahon et al., 1998). Heterozygous inactivation of either Ror2 or Noggin does not result in any skeletal alteration (**Figure 1A**). In $Ror2^{+/-}$;$Nog^{+/-}$compound heterozygotes the overall appearance of the limb skeleton was normal; however the skeletal elements of the stylopod (the humerus) and the zeugopod (radius and ulna) showed a consistent small lateral expansion (**Figure 1A**, width of skeletal elements in wild type and single mutants indicated in yellow, width in compound mutant indicated in orange). All skeletal elements showed a tendency toward widening at both metaphyseal sides, however statistical significance was only reached for the distal humerus and radius, respectively. This feature was not seen in single heterozygotes, indicating genetic interaction between *Nog* and *Ror2*.

$Ror2^{-/-}$ mice are a model for RRS, recapitulating several of its features including mesomelic limb shortening as well as mild brachydactyly (Schwabe et al., 2004). $Ror2^{-/-}$ mice have shortened digits, however all phalanges (two in the thumb/digit 1, three in digits 2–5) as well as the interphalangeal joints separating the phalanges are present (Takeuchi et al., 2000; Schwabe et al., 2004; Schwarzer et al., 2009) (**Figure 1B**). Noggin heterozygous mice have phenotypically normal digits. When one allele of Noggin was removed on the $Ror2^{-/-}$ background, shortening of phalanges was further increased. In digit 3 the appearance of 3 individual phalanges, which were smaller than those in the $Ror2^{-/-}$, was preserved. In digits 2 and 5 loss of one Noggin allele on the $Ror2^{-/-}$ background led to loss of an individual phalanx 2, concomitant with a longer phalanx 3, indicating failure of distal joint formation. Distal joint fusion is also a feature seen sometimes in BDB1 (ROR2 mutation) and frequently in BDB2 (NOG mutation). In addition, joint fusions are the hallmark of proximal symphalangism 1A (SYM1A) and multiple synostosis syndrome (SYSN1), two conditions caused by a different set of NOG mutations (Stricker and Mundlos, 2011). Altogether the compound mutants support the notion of a genetic and functional interaction of Ror2 and Nog in skeletal development.

Noggin Potentiates Wnt/PCP Signaling in a Ror2-Dependent Manner

In digit formation, Ror2 acts in part via inhibition of β-catenin signaling leading to derepression of BMP/SMAD signaling in a structure called phalanx-forming region (Witte et al., 2010). Evidence however has accumulated that in addition or in parallel to this function Ror2 and its paralog Ror1 are both required for Wnt-5a/PCP signaling activation during digit development (Gao et al., 2011; Ho et al., 2012). Our genetic interaction experiments cannot distinguish the origin of the interaction seen, i.e., whether it originated from Nog function in the BMP pathway, or a yet uncharacterized role in the Wnt-5a/PCP pathway. Noggin thus might not only influence activity of BMP, but also of Wnt-5a-Ror2 pathway. To test if Noggin is able to activate Ror2 we treated mouse embryonal fibroblasts (MEF) with increasing doses of Noggin. The activation of endogenous Ror2 can be monitored as a phosphorylation-dependent mobility shift on Western blotting (Oishi et al., 2003). As we show in **Figure 2A**, even in the highest concentrations used (1,500 ng/ml) Noggin did not induce phosphorylation of Ror2 and was unable to promote phosphorylation of Ror2 induced by its cognate ligand Wnt-5a. This suggests that at the receptor level Noggin is unable to act either directly as a ligand for Ror2, or indirectly.

In the next step we tested if Noggin can promote any of the Ror2-downstream events. A robust readout of non-canonical Wnt pathways activation is the Wnt-5a-induced phosphorylation of Disheveled (Dvl) 2, an event dependent on the Ror1 and Ror2 receptors (Ho et al., 2012). We took advantage of an anti-Dvl2 antibody that recognizes only the inactive, dephosphorylated form of Dvl2 in MEF cells (Gonzalez-Sancho et al., 2013). Disappearance of non-phosphorylated Dvl2 currently represents one of the most sensitive tools for visualization of Dvl2 phosphorylation and hence Wnt/PCP pathway activation. When we treated MEF cells with increasing doses of Wnt-5a, the non-phospho Dvl2 signal disappeared (**Figure 2Bi**), indicative of activated Wnt-5a-Ror-Dvl signaling. No such phenotype was observed when cells were treated by Noggin, confirming our previous observation that Noggin itself is not able to activate signaling via Ror2 (**Figure 2Bii**). However, when cells were treated with 100 ng/ml of Noggin, we could clearly observe stronger effects of Wnt-5a on Dvl2 activation (compare **Figure 2Bi** vs. **Figure 2Biii**). This indicates that Noggin can sensitize MEF cells to Wnt-5a/Ror2 signaling. To confirm this observation, we treated cells with 25 ng/ml of Wnt-5a, which is a suboptimal dose unable to trigger Dvl2 activation (**Figure 2Bi**). When cells pre-treated by 25 ng/ml of Wnt-5a were supplemented with increasing doses of Noggin, activation of Dvl2 was observed in a dose dependent manner (**Figure 2Biv**),

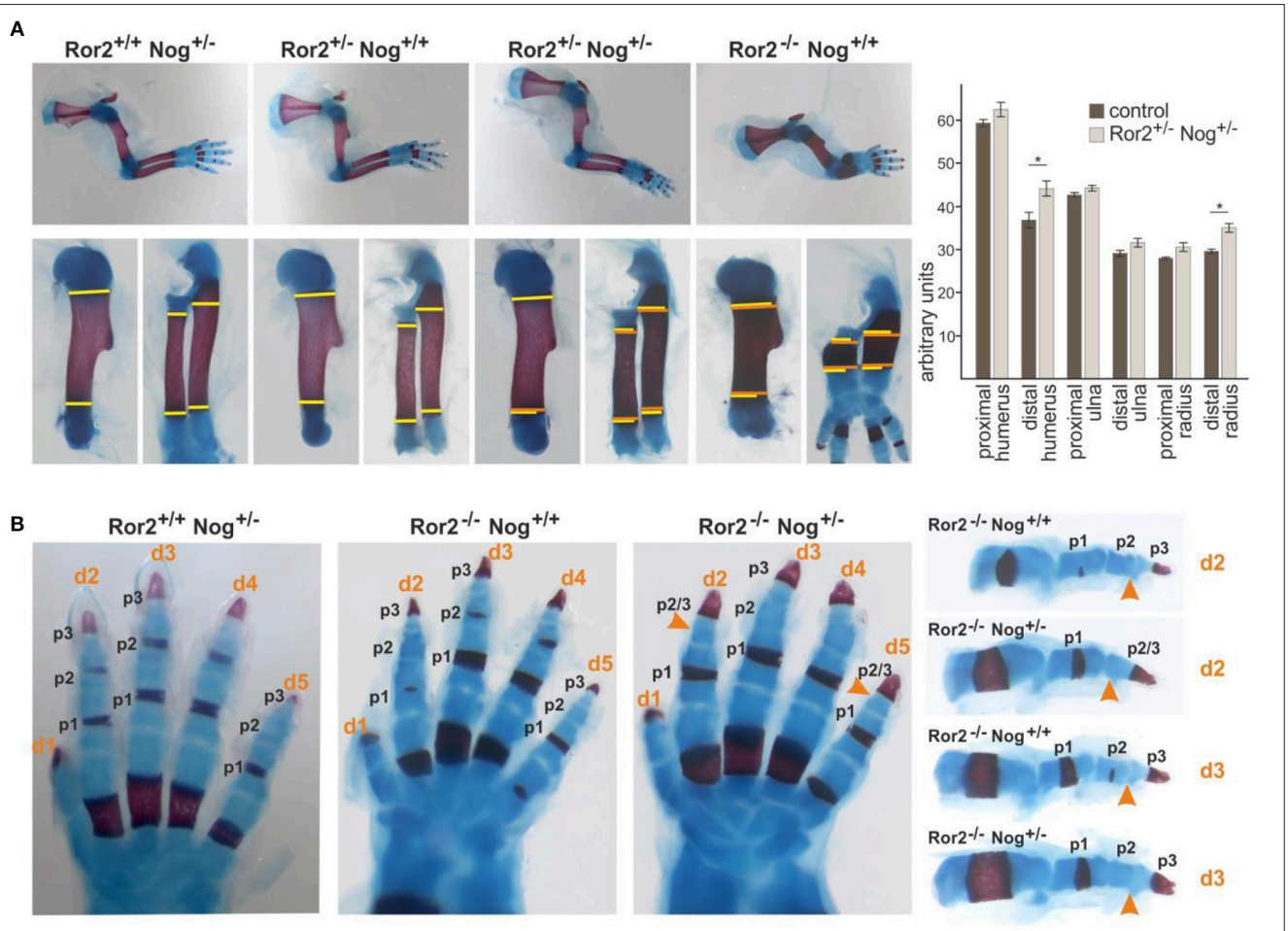

FIGURE 1 | Noggin genetically interacts with Ror2. Skeletal preparations of E18.5 embryos of the indicated allelic combinations are shown. Cartilage stains blue, bone stains red. **(A)** Top panel: Limbs of compound Ror2 and Noggin heterozygous mutants have a normal appearance. Ror2$^{-/-}$ skeletal elements are visibly shortened and enlarged. Bottom panel: magnifications of humerus and radius/ulna. The width of the wild type or single heterozygous skeletal elements is indicated by a yellow line on either side of the ossification center. Width of the double heterozygous or Ror2$^{-/-}$ skeletal elements is indicated by orange line for comparison. A quantification of skeletal element width is shown right; significant effects were observed for the distal humerus and distal radius ($p < 0,05$; student's *t*-test). **(B)** Digit development in compound mutants. Ror2$^{-/-}$ digits are shortened, but individual phalanges (p1, p2, and p3) are present, separated by synovial joints. In Ror2$^{-/-}$;Nog$^{+/-}$ animals, the medial phalange (p2) shows additional shortening, which in digits 2 and 5 leads to distal symphalangism of p2 and p3.

indicating that presence of Noggin can reveal biological activity of previously sub-threshold Wnt-5a concentrations.

All these data suggest that Noggin, despite its inability to activate Ror2 on its own, can efficiently potentiate the Wnt-5a-Ror2 signaling axis and sensitize cells to low amounts of Wnt-5a. Ror2 can have redundant function with closely related Ror1 (Ho et al., 2012) that can also bind Wnt-5a. To confirm that the effects of Noggin are indeed dependent on Ror1/Ror2, Ror1$^{-/-}$ Ror2$^{-/-}$ double knockout MEF cells were isolated from conditional Ror1/Ror2 knockout mice (as described in Ho et al., 2012). Individual clones were tested by Western blotting (**Figure 2C**) and one of the Ror1/Ror2 double negative clones (#13) was further used for functional analysis. When Ror1/Ror2-deficient MEF cells were treated with 30 ng/ml of Wnt-5a and 500 ng/ml of Noggin simultaneously, no shift of Dvl2 mobility (upper panels) or effects on non-phospho Dvl2 (middle panel) was observed, in contrast to wt MEF where Dvl2 was activated by the combination of Wnt-5a (30 ng/ml) and Noggin (500 ng/ml) (**Figure 2D**). This data show that Noggin is able to potentiate the activation of the Wnt-5a-Ror2 signaling circuit and demonstrate that the observed Noggin/Wnt-5a synergism toward Dvl2 is dependent on Ror1/Ror2.

FGF2-Induced Chondrocyte Growth Arrest Enables Noggin-Mediated Wnt/PCP Potentiation in RCS Cells

The genetic interaction between Ror2 and Noggin observed in mice as well as the skeletal involvement in human syndromes characterized by NOG and ROR2 mutations pointed toward the importance of a functional Noggin-Ror2 interaction for skeletal development. To test the Noggin-Ror2 synergy in a model system that is more relevant to skeletal development we decided to use the rat chondrosarcoma (RCS) cell line.

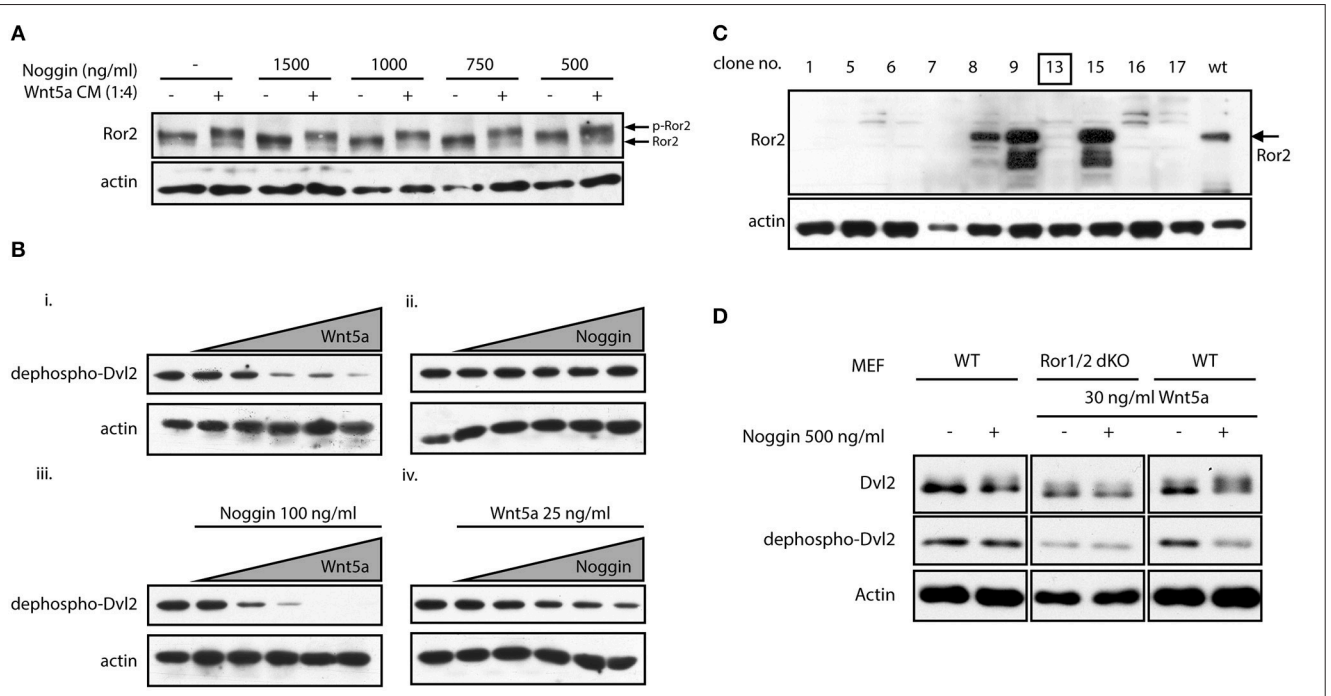

FIGURE 2 | Noggin potentiates Wnt/PCP signaling. (A) MEF cells were treated by Wnt-5a conditioned medium (CM) and stimulated by increasing doses of Noggin protein. Activation of Ror2 was analyzed as a phosphorylation-dependent shift by Western blotting. Noggin alone, in contrast to Wnt-5a CM, is not able to trigger activation of Ror2. **(B)** MEF cells were treated with increasing doses of Wnt-5a (0, 25, 50, 100, 150, and 200 ng/ml) and Noggin (0, 25, 50, 100, 150, and 200 ng/ml) for 2 h. The activation of Wnt signaling was assessed by Western blotting as a decrease in the signal of dephospho-Dvl2. Wnt-5a could cause phosphorylation of Dvl2 visible as a disappearance of dephospho-Dvl2 signal **(i)**, whereas Noggin is inactive in the same assay **(ii)**. Interestingly, pre-treatment of MEF cells by Noggin (100 ng/ml) enhanced the effect of Wnt-5a **(iii)**. On the other hand, Noggin, in a dose-dependent manner, potentiated the response to suboptimal doses of Wnt-5a (25 ng/ml), which are otherwise ineffective—see lane 2 in panel "i" **(iv)**. Actin is used as a loading control. **(C)** *Ror1*$^{flox/flox}$; *Ror2*$^{flox/flox}$ MEF cells were treated by tamoxifen and *Ror1*$^{-/-}$; *Ror2* $^{-/-}$ isogenic MEF line was isolated by serial dilutions method. The presence of Ror2 was tested by Western blotting and the clone no. 13 used for further studies is indicated. **(D)** MEF wt and MEF *Ror1*$^{-/-}$; *Ror2*$^{-/-}$ (Ror1/2 dKO) cells were treated by combinations of Noggin and Wnt-5a as indicated. Noggin itself cannot stimulate activation of Dvl2—visible as a phosphorylation-dependent shift of Dvl2 (upper blots) or decrease in dephospho-Dvl2 (middle blots) signal. Noggin, however, increases activity of suboptimal dose of Wnt-5a (30 ng/ml), an effect that is lost in *Ror1*$^{-/-}$ *Ror2*$^{-/-}$ MEF cells.

RCS chondrocytes maintain a fully differentiated proliferating chondrocyte phenotype in culture, manifested by abundant production of cartilaginous extracellular matrix rich in sulfated proteoglycans and collagen type 2, but not collagen type 10 characteristic for hypertrophic chondrocytes (Mukhopadhyay et al., 1995). Moreover, RCS chondrocytes faithfully recapitulate FGF-receptor 3 (FGFR3) signaling in the growth plate cartilage. Many essential features of FGFR3 signaling in the growth plate cartilage, such as the FGF-mediated chondrocyte growth-arrest have been unraveled using the RCS chondrocyte model system (Aikawa et al., 2001; Dailey et al., 2003; Krejci et al., 2005).

To define this experimental system, we first investigated whether the FGF-induced growth arrest in RCS cells is influenced by addition of Noggin and Wnt-5a. Noggin, Wnt-5a and/or their combination did not induce a growth arrest by themselves, and also did not modulate the FGF-induced growth arrest of RCS cells (**Figure 3A**). We also wanted to exclude that any possible observations in RCS cells are caused by modulation of canonical Wnt pathway that was shown to oppose Wnt/PCP pathway in chondrogenesis. Since it was shown that RCS cells are responsive to canonical Wnt ligands, e.g., Wnt3a (Krejci et al., 2012), we tested whether Noggin and Wnt-5a treatment alters the canonical Wnt pathway in RCS cells using TopFlash reporter assay. These results (**Figure 3B**) showed that Noggin and Wnt-5a could not activate or inhibit the canonical Wnt pathway even though RCS cells responded well to canonical Wnt ligands such as Wnt-3a (**Figure 3B**). We conclude that combined treatment of RCS cells with Noggin/Wnt-5a does not influence FGF2 induced growth arrest or the canonical Wnt signaling pathway in RCS cells.

Finally, we analyzed whether RCS cells respond to combined Noggin/Wnt-5a treatment similarly to MEF cells. As we could not detect any signal by using the dephospho-Dvl2 antibody used in MEF cells (not shown), we have used an alternative readout—electrophoretic mobility shift of Dvl induced by Wnt-5a. Such mobility shift indeed represents a phosphorylation and can be effectively abrogated by alkaline phosphatase (AP) treatment (**Figure 3C**). Using this readout we next tested whether Noggin could potentiate the response to Wnt-5a in RCS cells similarly as was observed in MEF cells. When RCS cells were treated by combination of Wnt-5a and Noggin, no potentiation of Wnt-5a-Ror2 signaling was observed (**Figure 3D**, quantified in **Figure 3E**), and only the highest dose of Wnt-5a triggered

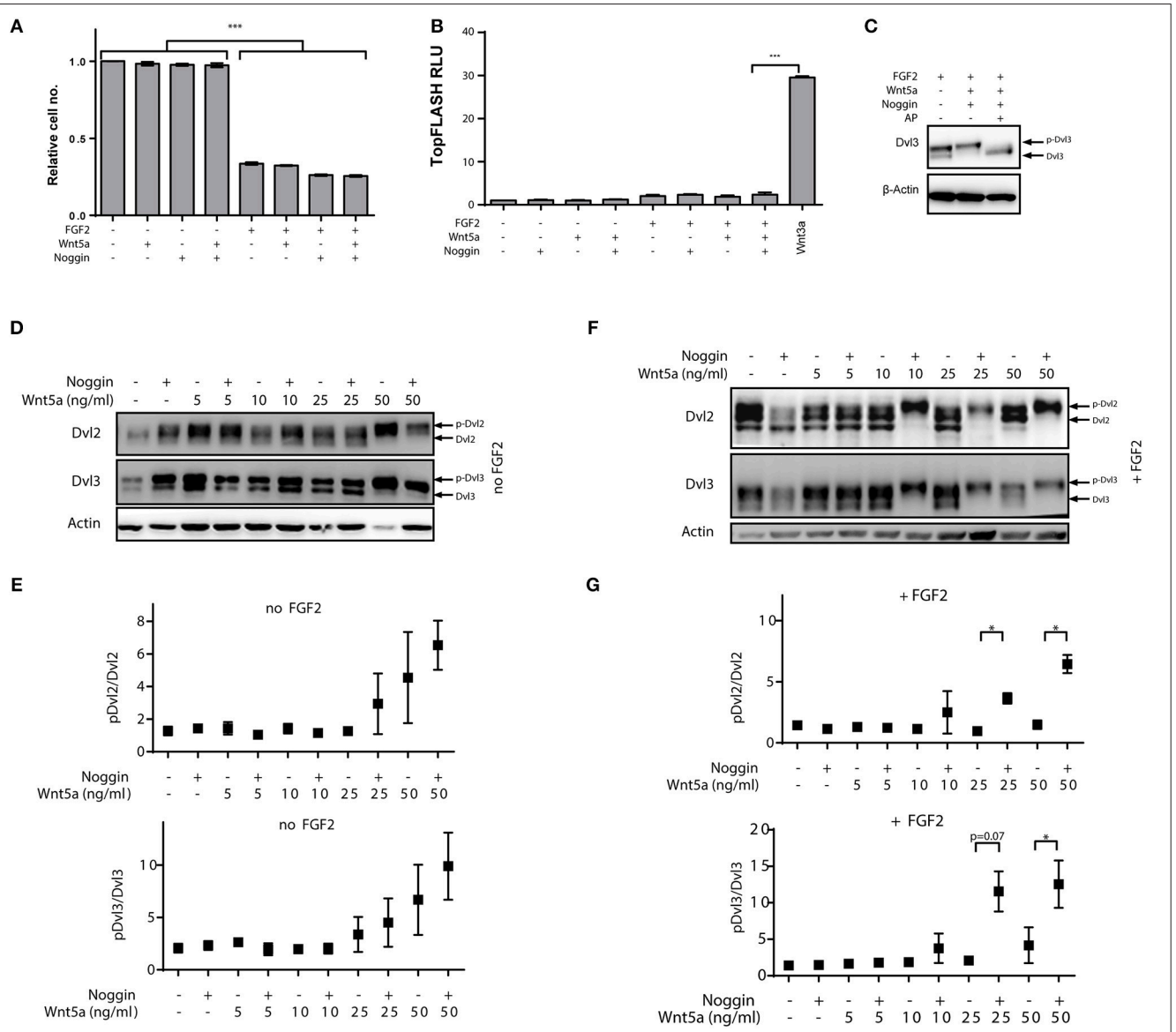

FIGURE 3 | Positive effects of Noggin on Wnt/PCP activation in chondrocytes is induced by co-stimulation with FGF2. RCS cells were treated by FGF2 to induce growth arrest, and then treated by the porcupine inhibitor Wnt-C59 (5 μM) to reduce the autocrine Wnt activity and by Noggin and Wnt-5a as indicated. Timepoints are specified in Materials and Methods section. **(A)** Wnt-5a, Noggin and their combination does not alleviate growth arrest of RCS cells induced by FGF2 (72 h), graph shows average and SD from two independent experiments, ***$p < 0.001$ [One-way ANOVA (ANalysis Of VAriance) with *post-hoc* Tukey test]. **(B)** Treatment of RCS cells by FGF2, Noggin and Wnt-5a does not activate canonical Wnt pathway analyzed by TopFlash reporter system. Treatment with Wnt3a was used as a positive control. RLU—relative light units, graph shows average and SD from two independent experiments, ***$p < 0.001$ [One-way ANOVA (ANalysis Of VAriance) with *post-hoc* Tukey test]. **(C)** Alkaline phosphatase (AP) treatment can remove the electrophoretic mobility shift of Dvl3 induced by FGF2/Noggin/Wnt-5a treatment, which suggests that the mobility changes (used in **D–G**) are caused by phosphorylation. **(D)** Wnt-5a can activate downstream signaling—visible as phosphorylation-dependent shift (p-Dvl) of Dvl2 and Dvl3—at 50 ng/ml and this effect is not positively modulated by the addition of Noggin (125 ng/ml). **(E)** Quantification of p-Dvl/Dvl ratios for Dvl2 and Dvl3 from three independent experiments. **(F)** Similar experiment as in **(D)** but RCS cells were pre-treated also by FGF2 (5 ng/ml) for total 72 h to induce FGFR3-mediated growth arrest. Under these conditions 24 h treatment by C59, Noggin (125 ng/ml) and Wnt-5a can dramatically induce the Wnt-5a-induced activation of Dvl2 and Dvl3. **(G)** Quantification of three independent experiments. *$p < 0.01$ (paired *t*-test).

phosphorylation of Dvl2 and Dvl3. However, when RCS cells were pre-treated with FGF2 for 2 days in order to induce growth arrest (Krejci et al., 2010), Noggin dramatically improved the response of RCS cells to low doses of Wnt-5a (**Figure 3F**, quantified in **Figure 3G**). Importantly, acute treatment of RCS cells with FGF2, Noggin and Wnt-5a was unable to induce such "sensitization" (data not shown). These data thus argue that the synergism between Noggin and Wnt-5a-Ror2 is not a proximal effect of FGF2-induced signaling or an inhibition of the canonical branch of Wnt signaling but is rather induced by cell changes caused by prolonged FGF2 treatment and cell cycle arrest.

DISCUSSION

Signaling pathways do not operate as standalone units but functionally cooperate and interact. Inspired by the phenotypic resemblance of BDB1 and BDB2, inheritable syndromes caused by mutations in ROR2 or NOGGIN, respectively, we decided to study how Noggin, an inhibitor of BMP pathway, and non-canonical Wnt signaling, driven by Ror2 receptor, can interact. We could show that Noggin increased biological activity of Wnt-5a and rendered cells sensitive to Wnt-5a concentrations otherwise not causing cellular responses. This function was dependent on the presence of Ror2, but Noggin did not elicit a signal on its own via Ror2.

Our study does not elucidate the molecular mechanism behind this interaction. One mechanism may involve BMP receptor type 1 b (Bmpr1b), which is mutated in BDA2 (Lehmann et al., 2003). *In vitro*, Ror2 and Bmpr1b were shown to interact and Ror2 is phosphorylated by Bmpr1b (Sammar et al., 2004, 2009). The functional consequence of this phosphorylation remains unclear but one can speculate that the effects of Bmpr1b on Ror2 are controlled by BMP ligands, whose active concentration is controlled by Noggin. Another possibility, which we were, however, not able to prove (data not shown) can be formation of Noggin-Wnt-5a-Ror2 ternary complex with the increased signaling capacity in comparison to Wnt-5a-Ror2 only. As another alternative, Noggin can, via regulation of BMP pathway, control signaling competence or cell surface amount of Ror2—here a possible point of crosstalk can be represented by Smurf family E3-ligases, which were reported to control both BMP pathway (negatively) as well as Wnt/PCP pathway (positively) (Narimatsu et al., 2009).

The importance of the BMP pathway and its tight regulation by antagonists for digit development is underscored by the fact that the majority of human brachydactylies are caused by mutations in different members of this signaling network (reviewed in Stricker and Mundlos, 2011). A necessity for integration of BMP and Wnt/β-catenin pathways has been reported for numerous developmental processes (Itasaki and Hoppler, 2010). For example, in digit outgrowth, BMP/SMAD signaling is fine-tuned by inhibition from the Wnt/β-catenin pathway, which itself is kept in check by Ror2 (Witte et al., 2010). Non-canonical (or alternative) Wnt pathways regulate entirely different aspects of tissue development compared to the Wnt/β-catenin pathway, but are connected with the BMP pathway as well, albeit the connection has not been studied to the same depth (Narimatsu et al., 2009; Schille et al., 2016). In developing limbs, Wnt/PCP signaling was involved in both digit shaping and outgrowth (Gao et al., 2011; Wang et al., 2011; Ho et al., 2012). Altogether this substantiates that both BMP and non-canonical Wnt pathways are required and act in concert during the establishment of the limb skeleton. Ror2 appears to be a pivotal intersection point between these two pathways.

Our work on RCS chondrocytes, a cell model for chondrocyte growth and differentiation that to some extent recapitulate the behavior of developing limb growth plate cartilage (Krejci et al., 2012) showed that Noggin could potentiate Wnt-5a-Ror2 pathway activity much more effectively when growth arrest was induced by FGF2 stimulation. It was previously shown in RCS chondrocytes that the FGF pathway can stimulate phosphorylation of LRP6, a co-receptor of the Wnt/β-catenin pathway (Krejci et al., 2012; Buchtova et al., 2015). We speculated that FGF signaling might be involved in activation of Wnt-5a-Ror2 in RCS cells, as it is known that Wnt/β-catenin and non-canonical Wnt pathways receptors can be activated by common mechanisms (Bryja et al., 2009; Grumolato et al., 2010). However, Wnt/β-catenin is likely not involved in the Noggin/Wnt-5a/Ror2 crosstalk in RCS cells because no differences in the activity analyzed by the TopFlash reporter system were observed.

Where can such FGF-dependent Noggin-induced activation of Wnt-5a-Ror2 signaling pathway in chondrocytes take place *in vivo*? In limb cartilage development, Wnt/PCP signaling appears to be involved at two steps: during condensation of cartilage elements, especially the digits (Gao et al., 2011; Wang et al., 2011; Ho et al., 2012), and for establishing cartilage growth plate morphology (Ahrens et al., 2009; Li and Dudley, 2009; Kuss et al., 2014; Romereim et al., 2014). In the first scenario, Wnt-5a is required for digit formation, and mice deficient for Wnt-5a form rudimentary digits (Yamaguchi et al., 1999). The Wnt-5a null phenotype is recapitulated by either Ror1/Ror2 double null mutants (Ho et al., 2012) or Ror2/Vangl2 double null mutants (Gao et al., 2011), clearly establishing that a Wnt-5a/Ror2/PCP pathway is necessary for digit formation. Noggin is expressed in forming cartilage condensations (Brunet et al., 1998) and could hence facilitate this process. During digit outgrowth, FGFs are expressed in the apical ectodermal ridge (AER). FGF signaling from the AER is thought to keep distal mesenchymal cells proliferating and undifferentiated (ten Berge et al., 2008). *In vitro*, FGFs inhibit chondrogenesis (Buchtova et al., 2015), but on the other hand application of FGF beads can induce ectopic digit formation *in vivo* (Montero et al., 2001). One possibility is that FGF signaling that acts at a distance from the AER on prechondrogenic cells provides competence for Noggin activity toward the Wnt-5a/Ror2/PCP pathway, and is thus enforcing PCP signaling in cells undergoing chondrogenic differentiation. In the growth plate, both Wnt-5a and Ror2 are essential for cellular polarity (Yang et al., 2003; Schwabe et al., 2004), and Wnt-5a acts via a PCP pathway (Gao et al., 2011; Kuss et al., 2014). Noggin is expressed throughout the growth plate (Brunet et al., 1998), and FGF signaling, which is a major regulator of growth plate chondrocyte proliferation, is active here as well (Horton et al., 2007).

In summary our data pinpoint a novel, yet unappreciated role for Noggin in sensitizing cells to Wnt-5a. The cellular mechanism by which Noggin accomplishes this effect on the Wnt-5a-Ror2 pathway remains to be elucidated.

AUTHOR CONTRIBUTIONS

PK, SS, and VB designed research; MB, TR, OB, ZD, FW, AM, NC, and PK performed research; all authors analyzed data; and OB, SS, and VB wrote the paper.

ACKNOWLEDGMENTS

This work was supported by the Czech Science Foundation (15-21789S, 17-16680S, 17-09525S). TR and ZD are supported by the Marie Curie ITN WntsApp. PK was supported by Ministry of Education, Youth and Sports of the Czech Republic (KONTAKT II LH15231) and Ministry of Health of the Czech Republic (15-33232A, 15-34405A). This work was supported by the Deutsche Forschungsgemeinschaft (DFG SFB 577).

REFERENCES

Afzal, A. R., Rajab, A., Fenske, C. D., Oldridge, M., Elanko, N., Ternes-Pereira, E., et al. (2000). Recessive robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. *Nat. Genet.* 25, 419–422. doi: 10.1038/78107

Ahrens, M. J., Li, Y., Jiang, H., and Dudley, A. T. (2009). Convergent extension movements in growth plate chondrocytes require gpi-anchored cell surface proteins. *Development* 136, 3463–3474. doi: 10.1242/dev.040592

Aikawa, T., Segre, G. V., and Lee, K. (2001). Fibroblast growth factor inhibits chondrocytic growth through induction of p21 and subsequent inactivation of cyclin E-Cdk2. *J. Biol. Chem.* 276, 29347–29352. doi: 10.1074/jbc.M101859200

Bernatik, O., Sedova, K., Schille, C., Ganji, R. S., Cervenka, I., Trantirek, L., et al. (2014). Functional analysis of dishevelled-3 phosphorylation identifies distinct mechanisms driven by casein kinase 1 and frizzled5. *J. Biol. Chem.* 289, 23520–23533. doi: 10.1074/jbc.M114.590638

Brunet, L. J., McMahon, J. A., McMahon, A. P., and Harland, R. M. (1998). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 280, 1455–1457. doi: 10.1126/science.280.5368.1455

Bryja, V., Andersson, E. R., Schambony, A., Esner, M., Bryjova, L., Biris, K. K., et al. (2009). The extracellular domain of Lrp5/6 inhibits noncanonical Wnt signaling *in vivo*. *Mol. Biol. Cell* 20, 924–936. doi: 10.1091/mbc.E08-07-0711

Buchtova, M., Oralova, V., Aklian, A., Masek, J., Vesela, I., Ouyang, Z., et al. (2015). Fibroblast growth factor and canonical WNT/beta-catenin signaling cooperate in suppression of chondrocyte differentiation in experimental models of FGFR signaling in cartilage. *Biochim. Biophys. Acta* 1852, 839–850. doi: 10.1016/j.bbadis.2014.12.020

Dailey, L., Laplantine, E., Priore, R., and Basilico, C. (2003). A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. *J. Cell Biol.* 161, 1053–1066. doi: 10.1083/jcb.200302075

Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M. A., et al. (2011). Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. *Dev. Cell* 20, 163–176. doi: 10.1016/j.devcel.2011.01.001

Gonzalez-Sancho, J. M., Greer, Y. E., Abrahams, C. L., Takigawa, Y., Baljinnyam, B., Lee, K. H., et al. (2013). Functional consequences of Wnt-induced dishevelled 2 phosphorylation in canonical and noncanonical Wnt signaling. *J. Biol. Chem.* 288, 9428–9437. doi: 10.1074/jbc.M112.448480

Grumolato, L., Liu, G., Mong, P., Mudbhary, R., Biswas, R., Arroyave, R., et al. (2010). Canonical and noncanonical Wnts use a common mechanism to activate completely unrelated coreceptors. *Genes Dev.* 24, 2517–2530. doi: 10.1101/gad.1957710

Ho, H. Y., Susman, M. W., Bikoff, J. B., Ryu, Y. K., Jonas, A. M., Hu, L., et al. (2012). Wnt-5a-Ror-Dishevelled signaling constitutes a core developmental pathway that controls tissue morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 4044–4051. doi: 10.1073/pnas.1200421109

Horton, W. A., Hall, J. G., and Hecht, J. T. (2007). Achondroplasia. *Lancet* 370, 162–172. doi: 10.1016/S0140-6736(07)61090-3

Itasaki, N., and Hoppler, S. (2010). Crosstalk between Wnt and bone morphogenic protein signaling: a turbulent relationship. *Dev. Dyn.* 239, 16–33. doi: 10.1002/dvdy.22009

Krejci, P., Aklian, A., Kaucka, M., Sevcikova, E., Prochazkova, J., Masek, J. K., et al. (2012). Receptor tyrosine kinases activate canonical WNT/beta-catenin signaling via MAP Kinase/LRP6 pathway and direct beta-catenin phosphorylation. *PLoS ONE* 7:e35826. doi: 10.1371/journal.pone.0035826

Krejci, P., Masri, B., Fontaine, V., Mekikian, P. B., Weis, M., Prats, H., et al. (2005). Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis. *J. Cell Sci.* 118, 5089–5100. doi: 10.1242/jcs.02618

Krejci, P., Murakami, S., Prochazkova, J., Trantirek, L., Chlebova, K., Ouyang, Z., et al. (2010). NF449 is a novel inhibitor of fibroblast growth factor receptor 3 (FGFR3) signaling active in chondrocytes and multiple myeloma cells. *J. Biol. Chem.* 285, 20644–20653. doi: 10.1074/jbc.M109.083626

Kuss, P., Kraft, K., Stumm, J., Ibrahim, D., Vallecillo-Garcia, P., Mundlos, S., et al. (2014). Regulation of cell polarity in the cartilage growth plate and perichondrium of metacarpal elements by HOXD13 and WNT-5A. *Dev. Biol.* 385, 83–93. doi: 10.1016/j.ydbio.2013.10.013

Lehmann, K., Seemann, P., Silan, F., Goecke, T. O., Irgang, S., Kjaer, K. W., et al. (2007). A new subtype of brachydactyly type B caused by point mutations in the bone morphogenetic protein antagonist NOGGIN. *Am. J. Hum. Genet.* 81, 388–396. doi: 10.1086/519697

Lehmann, K., Seemann, P., Stricker, S., Sammar, M., Meyer, B., Suring, K., et al. (2003). Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12277–12282. doi: 10.1073/pnas.2133476100

Li, Y., and Dudley, A. T. (2009). Noncanonical frizzled signaling regulates cell polarity of growth plate chondrocytes. *Development* 136, 1083–1092. doi: 10.1242/dev.023820

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C. M., Harland, R. M., and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* 12, 1438–1452. doi: 10.1101/gad.12.10.1438

Mikels, A. J., and Nusse, R. (2006). Purified Wnt-5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol.* 4:e115. doi: 10.1371/journal.pbio.0040115

Montero, J. A., Ganan, Y., Macias, D., Rodriguez-Leon, J., Sanz-Ezquerro, J. J., Merino, R., et al. (2001). Role of FGFs in the control of programmed cell death during limb development. *Development* 128, 2075–2084.

Mukhopadhyay, K., Lefebvre, V., Zhou, G., Garofalo, S., Kimura, J. H., and de Crombrugghe, B. (1995). Use of a new rat chondrosarcoma cell line to delineate a 119-base pair chondrocyte-specific enhancer element and to define active promoter segments in the mouse pro-alpha 1(II) collagen gene. *J. Biol. Chem.* 270, 27711–27719. doi: 10.1074/jbc.270.46.27711

Mundlos, S. (2000). Skeletal morphogenesis. *Methods Mol. Biol.* 136, 61–70. doi: 10.1385/1-59259-065-9:61

Narimatsu, M., Bose, R., Pye, M., Zhang, L., Miller, B., Ching, P., et al. (2009). Regulation of planar cell polarity by Smurf ubiquitin ligases. *Cell* 137, 295–307. doi: 10.1016/j.cell.2009.02.025

Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., et al. (2003). The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt-5a/JNK signalling pathway. *Genes Cells* 8, 645–654. doi: 10.1046/j.1365-2443.2003.00662.x

Oldridge, M., Fortuna, A. M., Maringa, M., Propping, P., Mansour, S., Pollitt, C., et al. (2000). Dominant mutations in ROR2, encoding an orphan receptor tyrosine kinase, cause brachydactyly type B. *Nat. Genet.* 24, 275–278. doi: 10.1038/73495

Robert, B. (2007). Bone morphogenetic protein signaling in limb outgrowth and patterning. *Dev. Growth Differ.* 49, 455–468. doi: 10.1111/j.1440-169X.2007.00946.x

Romereim, S. M., Conoan, N. H., Chen, B., and Dudley, A. T. (2014). A dynamic cell adhesion surface regulates tissue architecture in growth plate cartilage. *Development* 141, 2085–2095. doi: 10.1242/dev.105452

Romereim, S. M., and Dudley, A. T. (2011). Cell polarity: the missing link in skeletal morphogenesis? *Organogenesis* 7, 217–228. doi: 10.4161/org.7.3.18583

Sammar, M., Sieber, C., and Knaus, P. (2009). Biochemical and functional characterization of the Ror2/BRIb receptor complex. *Biochem. Biophys. Res. Commun.* 381, 1–6. doi: 10.1016/j.bbrc.2008.12.162

Sammar, M., Stricker, S., Schwabe, G. C., Sieber, C., Hartung, A., Hanke, M., et al. (2004). Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. *Genes Cells* 9, 1227–1238. doi: 10.1111/j.1365-2443.2004.00799.x

Schambony, A., and Wedlich, D. (2007). Wnt-5A/Ror2 regulate expression of XPAPC through an alternative noncanonical signaling pathway. *Dev. Cell* 12, 779–792. doi: 10.1016/j.devcel.2007.02.016

Schille, C., Bayerlova, M., Bleckmann, A., and Schambony, A. (2016). Ror2 signaling is required for local upregulation of GDF6 and activation of BMP signaling at the neural plate border. *Development* 143, 3182–3194. doi: 10.1242/dev.135426

Schwabe, G. C., Trepczik, B., Suring, K., Brieske, N., Tucker, A. S., Sharpe, P. T., et al. (2004). Ror2 knockout mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome. *Dev. Dyn.* 229, 400–410. doi: 10.1002/dvdy.10466

Schwarzer, W., Witte, F., Rajab, A., Mundlos, S., and Stricker, S. (2009). A gradient of ROR2 protein stability and membrane localization confers brachydactyly type B or Robinow syndrome phenotypes. *Hum. Mol. Genet.* 18, 4013–4021. doi: 10.1093/hmg/ddp345

Stricker, S., and Mundlos, S. (2011). Mechanisms of digit formation: human malformation syndromes tell the story. *Dev. Dyn.* 240, 990–1004. doi: 10.1002/dvdy.22565

Stricker, S., Rauschenberger, V., and Schambony, A. (2017). ROR-Family Receptor Tyrosine Kinases. *Curr. Top. Dev. Biol.* 123, 105–142. doi: 10.1016/bs.ctdb.2016.09.003

Takeuchi, S., Takeda, K., Oishi, I., Nomi, M., Ikeya, M., Itoh, K., et al. (2000). Mouse Ror2 receptor tyrosine kinase is required for the heart development and limb formation. *Genes Cells* 5, 71–78. doi: 10.1046/j.1365-2443.2000.00300.x

ten Berge, D., Brugmann, S. A., Helms, J. A., and Nusse, R. (2008). Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. *Development* 135, 3247–3257. doi: 10.1242/dev.023176

van Bokhoven, H., Celli, J., Kayserili, H., van Beusekom, E., Balci, S., Brussel, W., et al. (2000). Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. *Nat. Genet.* 25, 423–426. doi: 10.1038/78113

Wang, B., Sinha, T., Jiao, K., Serra, R., and Wang, J. (2011). Disruption of PCP signaling causes limb morphogenesis and skeletal defects and may underlie Robinow syndrome and brachydactyly type B. *Hum. Mol. Genet.* 20, 271–285. doi: 10.1093/hmg/ddq462

Witte, F., Chan, D., Economides, A. N., Mundlos, S., and Stricker, S. (2010). Receptor tyrosine kinase-like orphan receptor 2 (ROR2) and Indian hedgehog regulate digit outgrowth mediated by the phalanx-forming region. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14211–14216. doi: 10.1073/pnas.1009314107

Yamaguchi, T. P., Bradley, A., McMahon, A. P., and Jones, S. (1999). A Wnt-5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* 126, 1211–1223.

Yang, Y., Topol, L., Lee, H., and Wu, J. (2003). Wnt-5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. *Development* 130, 1003–1015. doi: 10.1242/dev.00324

Zuniga, A. (2015). Next generation limb development and evolution: old questions, new perspectives. *Development* 142, 3810–3820. doi: 10.1242/dev.125757

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 23 March 2017
doi: 10.3389/fcell.2017.00026

Mechanical Control of Myotendinous Junction Formation and Tendon Differentiation during Development

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The development of the musculoskeletal system is a great model to study the interplay between chemical and mechanical inter-tissue signaling in cell adhesion, tissue morphogenesis and differentiation. In both vertebrates and invertebrates (e.g., *Drosophila melanogaster*) the formation of muscle-tendon interaction generates mechanical forces which are required for myotendinous junction maturation and tissue differentiation. In addition, these forces must be withstood by muscles and tendons in order to prevent detachment from each other, deformation or even losing their integrity. Extracellular matrix remodeling at the myotendinous junction is key to resist mechanical load generated by muscle contraction. Recent evidences in vertebrates indicate that mechanical forces generated during junction formation regulate chemical signaling leading to extracellular matrix remodeling, however, the mechanotransduction mechanisms associated to this response remains elusive. In addition to extracellular matrix remodeling, the ability of *Drosophila* tendon-cells to bear mechanical load depends on rearrangement of tendon cell cytoskeleton, thus studying the molecular mechanisms involved in this process is critical to understand the contribution of mechanical forces to the development of the musculoskeletal system. Here, we review recent findings regarding the role of chemical and mechanical signaling in myotendinous junction formation and tendon differentiation, and discuss molecular mechanisms of mechanotransduction that may allow tendon cells to withstand mechanical load during development of the musculoskeletal system.

Keywords: tendon cells, myotendinous junction, mechanical forces, morphogenesis, mechanoresponse

OPEN ACCESS

Edited by:
Juan Jose Sanz-Ezquerro, Consejo Superior de Investigaciones Científicas (CSIC), Spain

Reviewed by:
Peter Bräunig, RWTH Aachen University, Germany
Krzysztof Jagla, Institut National de la Santé et de la Recherche Médicale (INSERM), France
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *30 December 2016*
Accepted: *07 March 2017*
Published: *23 March 2017*

Citation:
Valdivia M, Vega-Macaya F and Olguín P (2017) Mechanical Control of Myotendinous Junction Formation and Tendon Differentiation during Development. Front. Cell Dev. Biol. 5:26. doi: 10.3389/fcell.2017.00026

INTRODUCTION

Living cells and tissues are in a constant state of isometric tension allowing them to respond to mechanical cues (Ingber, 1997; Wang et al., 2001; Mammoto and Ingber, 2010). During embryogenesis, mechanical stress is generated within the tissue and by its interaction with external factors and/or other tissues. Shear stress generated by blood flow modulates blood vessels morphogenesis, regulates the fate acquisition of arteries and veins, and is required for the development of the hematopoietic system (le Noble et al., 2004; Adamo et al., 2009; North et al., 2009). In addition, hemodynamic forces are required for heart morphogenesis. Disturbing blood flow at either the inflow or outflow tracts of the zebrafish heart results in several defects including abnormal formation of third chamber and heart looping (Hove et al., 2003). Furthermore, mechanotransduction mechanisms and its role in development are evolutionary conserved across

species. In zebrafish and *Drosophila,* mechanical cues generated during gastrulation (epiboly in zebrafish, and mesoderm invagination in flies) induce β-Catenin release from E-Cadherin based junctions, and translocation to the nucleus of mesodermal cells, where it promotes gene expression changes and cell specification (Farge, 2003; Desprat et al., 2008; Brunet et al., 2013).

The development of muscle-tendon attachment is a great model to study the role of chemical and mechanical signaling between tissues in morphogenesis and differentiation (Schweitzer et al., 2010; Subramanian and Schilling, 2015). During embryogenesis, tendon cells attach to the developing muscle through the Extracellular Matrix (ECM) forming a specialized junction called Myotendinous Junction (MTJ) (Schweitzer et al., 2010; Subramanian and Schilling, 2015). MTJ development relays mainly on the interaction of Integrins and ECM molecules secreted by tendons and muscles, although, other proteins, like Dystroglycan and Kon-tiki (Kon) also contribute to the formation of the MTJ. While Dystroglycan participates on muscle binding to the ECM, Kon controls muscle guidance and attachment to muscle attachment sites (Pérez-Moreno et al., 2014; Weitkunat et al., 2014; Maartens and Brown, 2015; Subramanian and Schilling, 2015). Strain generated by the contraction of the developing muscles contributes to MTJ maturation and muscle and tendon differentiation (Weitkunat et al., 2014; Havis et al., 2016). Here we will review recent evidences regarding the role of mechanical signaling in tendon differentiation and MTJ formation in vertebrates and *Drosophila.* Additionally, we will discuss the mechanisms of mechanoresponse that may allow tendon cells to sense and respond to mechanical load during development of the muscle-tendon interaction.

THE ROLE OF MECHANICAL AND CHEMICAL SIGNALING IN VERTEBRATE TENDON DIFFERENTIATION

Mechanical control of tendon differentiation and remodeling has been widely studied in vertebrates (reviewed in Shwartz et al., 2013). Tendons are formed by ECM, composed principally by strong collagens fibril arrays, and a type of fibroblast termed tenocyte (Subramanian and Schilling, 2015). In response to mechanical forces, tenocytes secrete collagens and proteoglycans, modifying ECM composition and elastic properties (Chen X. et al., 2012; Li et al., 2015). These changes confer tendons with the ability to resist mechanical load generated during muscle contraction and to form functional attachments to bones (Evans and Barbenel, 1975; Kjaer and Kjær, 2004; Maeda et al., 2011; Schwartz et al., 2013; Havis et al., 2016). How force is sensed by tenocytes and transduced into a cellular response? Recent studies on the development of the MTJ shed lights into this problem. In chicks and mice, the morphogenesis of the limb MTJ is divided in two phases (Subramanian and Schilling, 2015). The first phase is independent of muscle derived signals (Pryce et al., 2009). Here, the initial expression of Scleraxis (Scx), a tendon-specific bHLH transcription factor that promotes tendon differentiation and tenocyte specification (Alberton et al., 2012; Chen L. et al., 2012; Li et al., 2015), is stimulated by Fibroblast Growth Factor (FGF) and Transforming Growth Factor-beta (TGFβ) through MAPK/ERK and SMAD2/3 signaling pathways, respectively (Schweitzer et al., 2001; Havis et al., 2014; **Figure 1A**). Scx mutant mice display disrupted tenocyte differentiation leading to disorganized ECM, however, tenocyte precursor cells are still specified, indicating that other genes are required for early specification (Murchison et al., 2007). During the second phase of tendon differentiation, the interaction with the developing myofiber is mandatory to maintain the expression levels of Scx and other tendon markers (Havis et al., 2016). Pharmacological inhibition of muscle contraction disturbs tendon differentiation, even in presence of FGF and TGFβ, diminishing the levels of Scx. Moreover, force exerted by muscles on tendons is required for the activation of FGF and TGFβ at the muscle-tendon interface, maintaining the expression levels of Scx, leading to tendon terminal differentiation (Maeda et al., 2011; Havis et al., 2016).

TGFβ-ligands are secreted bound to TGFβ-binding proteins which form a complex with the large latency complex (LLC) in the ECM, capturing TGFβ and precluding its binding to TGFβ-receptors (Wipff et al., 2007; Maeda et al., 2011; **Figure 1A**). Shearing forces generated during muscle contraction may stimulate TGFβ release from the LLC through its degradation by proteases, allowing its binding to the receptor (**Figure 1A**). Moreover, it may promote the activation of Integrin signaling through the binding of the RGD motifs present on the latency TGFβ binding proteins associated to LLC (Munger and Sheppard, 2011; Subramanian and Schilling, 2015; **Figure 1A**). TGFβ signaling maintains Scx expression under normal muscular-load regime in mice (Maeda et al., 2010, 2011) and in response to mechanical stress promotes expression of Integrins (Popov et al., 2015). Thus, different mechanotransduction mechanisms appear to function at the ECM levels, activating either TGFβ or Integrin signaling. In vertebrates, recent evidence have shown that mechanical forces appears to be required for muscle development. Mechanical force driven by muscle contraction is necessary to maintain the pool of muscle progenitors during chick fetal myogenesis (de Lima et al., 2016), and *in vitro* studies suggest that strain drives mesenchymal stem cells differentiation into myoblasts (Lisio et al., 2014; Lemke and Schnorrer, in press).

THE ROLE OF MECHANICAL SIGNALING IN *DROSOPHILA* MYOTENDINOUS JUNCTION FORMATION AND TENDON DIFFERENTIATION

In contrast to vertebrates, *Drosophila* displays an exoskeleton instead of an internal skeleton and its connection with muscles relays on epithelial cells of ectodermal origin called tendon cells, which are analogs to vertebrate tendons (Fernandes et al., 1996; **Figure 1B**). Similar to vertebrates, signals emanated from tendon cells are required for MTJ formation, both during embryogenesis and metamorphosis (Costello and Wyman, 1986; Fernandes et al., 1991; Wayburn and Volk, 2009; Ordan

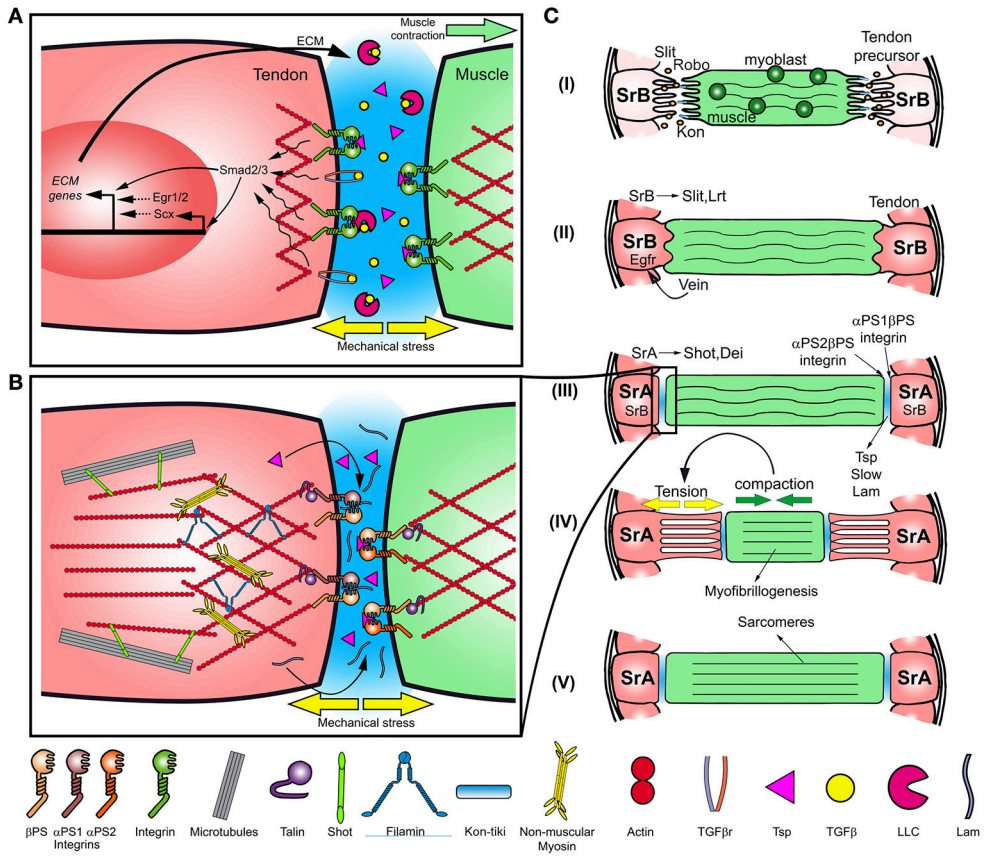

FIGURE 1 | Myotendinous junction formation in vertebrates and *Drosophila*. **(A)** Scheme of vertebrate myotendinous junction formation. Mechanical stress on the ECM may cause the release of the secreted TGFβ from the large latent complex (LLC) and activation of the receptor. In addition, TGFβ bound to LLC activates Integrin receptors. Smad2/3 along with Integrin signaling, activate Scx and Egr1/2, inducing the expression and deposition of ECM proteins. **(B)** Scheme of the myotendinous junction in *Drosophila*. In tendon cells, the link between Integrin and the actin cytoskeleton is mediated by Talin and the three-dimensional organization of the actin cytoskeleton is modulated by cross-linkers and motor proteins, such as and Filamin and Myosin. **(C)** Scheme of myotendinous system development in *Drosophila*. (I) The developing myotube migrates toward the tendon precursor cells (specified by SrB) directed by the Slit-Robo signaling and Kon-tiki, while myoblasts fuse with the myofiber. (II) After recognition tendon and myotube extensions interdigitate, in addition Vein is secreted promoting SrB expression. (III) ECM components, as Thrombospondin (Tsp) and Laminin (Lam), are secreted to the MTJ. In tendon cells, SrA is expressed and SrB expression diminishes. (IV) Myotube compacts generating mechanical stress on the system triggering myofibrillogenesis. (V) Sarcomeres are formed, and muscle elongate back toward tendon cells.

et al., 2015). In order to resist mechanical load, tendon cells modify their elastic properties deploying an array of polarized microtubules and actin filaments that stretch along their apical-basal axis, from the exoskeleton attachment site to the MTJ (Subramanian et al., 2003; Alves-Silva et al., 2008).

The development of the interaction between the Indirect Flight Muscles (IFMs) and the tendon cells of the dorsal thorax (notum) is an interesting model to study the role of mechanical signaling in tissue morphogenesis and cell differentiation (Olguín et al., 2011; Weitkunat et al., 2014). The notum develops from a monolayer epithelium, from which a subset of epithelial cells differentiates as analogs to vertebrate tendons, serving as bridges between the flight muscles and the exoskeleton (Fernandes et al., 1991; Weitkunat et al., 2014). At early stages of tendon differentiation, tendon precursors are specified by the activity of the isoform B of the Stripe transcription factor (SrB), which is required and sufficient to specify tendon cells (Volk and VijayRaghavan, 1994; Frommer et al., 1996; Becker et al., 1997; **Figure 1C**). The *stripe* homologous in vertebrates, Egr1 and Egr2, are required for tendon terminal differentiation, specifically to promote the expression of ECM proteins (Frommer et al., 1996; Lejard et al., 2011; Guerquin et al., 2013), however, as Scx, they are not strictly required for tendon specification (Lejard et al., 2011; Guerquin et al., 2013). Once specified, embryonic tendon cells provide initial attracting cues to the myotube and secrete Slit, a ligand that binds Robo receptor, which is expressed at the tips of myotubes (**Figure 1C**; Kramer et al., 2001; Ordan et al., 2015). Whether Slit acts as a chemoattractant in this context, remains to be elucidated. During this first stage of myotendinous system development, myotubes extend bipolar extensions that migrate toward their tendon targets, conversely, tendon cells extend processes that interact with

the myotube extension tips (**Figure 1C**; Vega-Macaya et al., 2016). Muscle migration requires the accumulation of Kon, a single pass transmembrane protein, on the muscle leading ends (**Figure 1C**; Estrada et al., 2007; Schnorrer et al., 2007). Loss of function of Kon in the ventral longitudinal muscles causes abnormal projection of filopodia, altering the myotube migration pattern (Schnorrer et al., 2007). Following, in a second stage, myotubes secrete Vein, a short range signaling molecule that binds to the epidermal growth factor receptor (EGFR) expressed in tendon cells, promoting SrB expression (Yarnitzky et al., 1997; **Figure 1C**). High levels of SrB induce Slit secretion and Leucine Rich repeat Transmembrane protein (LRT) expression, which bind to Robo and are both required for muscle migration arrest (**Figure 1C**; Wayburn and Volk, 2009; Ordan and Volk, 2015, 2016). Slit acts as a short range repellent signal that arrests muscle migration. This mechanism depends on Slit cleavage by Amontillado, a Pheromone Convertase 2 homolog, sequestering Slit on the tendon cell membrane, stopping muscle migration (Ordan et al., 2015; Ordan and Volk, 2016). In a third stage, the MTJ starts forming mainly through the association of Integrin with ECM proteins secreted by tendon and myotube (Chanana et al., 2007; Subramanian et al., 2007; Gilsohn and Volk, 2010; **Figures 1B,C**). The muscle-specific αPS2βPS Integrin binds to Thrombospondin (Tsp) and its regulator Slow, conversely, Laminin (Lam) associates with the tendon-specific αPS1βPS Integrin (Gotwals et al., 1994; Martin et al., 1999). The induction of SrA isoform and the decrease of SrB expression levels is essential to promote the expression of tendon specific differentiation genes such as *Delilah* (Dei), a transcription factor that promotes βPS expression, and *shortstop/kakapo* (Shot), a plakin that connects the actin cytoskeleton to microtubules, regulating the elastic properties of tendon cells (Subramanian et al., 2003; Schweitzer et al., 2010). Thus, during this stage EGFR and Integrin signaling promotes junction formation and terminal differentiation of tendon cells.

During metamorphosis, developing tendons and muscles express the same combinations of Integrin subunits and secrete extracellular matrix components such as Tsp, forming stable hemiadherent junctions (Subramanian et al., 2007; Gilsohn and Volk, 2010; Weitkunat et al., 2014). Following, IFMs compaction, driven by Myosin Heavy Chain (MHC) motor activity, generates mechanical strain at the MTJ (Weitkunat et al., 2014; **Figure 1C**). In addition, the overlying notum epithelium migrates toward anterior through a still unknown mechanism, which may contribute to the mechanical strain generated between these tissues (Bosveld et al., 2012). Recently, it has been shown that mechanical strain at the MTJ is required for myofibrillogenesis, indicating that mechanical signaling is also required for muscle morphogenesis (Weitkunat et al., 2014). In response to muscle compaction, tendon extensions attached to the myotube elongate (Weitkunat et al., 2014; **Figure 1C**). During this process, MTJ must be able to withstand mechanical load, and tendon cells might regulate its elastic properties in order to maintain its integrity and shape.

MEMBRANE MECHANORECEPTORS AND MECHANICAL SIGNALING AT THE MYOTENDINOUS SYSTEM

At focal adhesions, the Integrin signaling pathway might be triggered in response to deformation or changes in the rigidity of the ECM (outside-in activation) (Takagi et al., 2003; Campbell and Humphries, 2011). In absence of external forces, Integrins remain in a restings state, associated with Filamin (**Figure 2A**). Mechanical stimuli may cause the opening of the extracellular domains of the Integrin heterodimer, which is transmitted to its cytoplasmic portion where it could recruit the actin binding protein Talin, although it is not the most characterized mechanism of Integrin signaling (Nieves et al., 2010; **Figure 2B**). The activation of Integrins also results in the recruitment of several other proteins, like Src kinases, promoting cell proliferation and migration (Arias-Salgado et al., 2003). Importantly, Src activates Rho signaling pathway, which through Rho-kinase (ROCK) induces the phosphorylation of the myosin regulatory light chain (MRLC) and the contraction of the acto-myosin network, building up tension at the focal adhesions (Arthur et al., 2000; Arias-Salgado et al., 2003). The Integrin signaling cascade may be activated also by an inside-out mechanism (Otoole et al., 1994; Vinogradova et al., 2002). There is evidence that certain proteins, like Talin, are able to respond to mechanical deformation (Lee et al., 2007; del Rio et al., 2009). *In vitro* studies have shown that Talin has cryptic vinculin interacting domains that are exposed by deformation (Lee et al., 2007; del Rio et al., 2009). Stretching of the actin cytoskeleton may be directly transmitted to Talin, releasing its Vinculin binding site, triggering the recruitment of Talin and Vinculin toward Integrins, promoting adhesion.

Integrin-ECM interaction plays an important role in the formation of the vertebrate and *Drosophila* MTJ (Brown, 2000; **Figure 1A**). *Drosophila* mutant embryos for either βPS Integrin or *tsp* show detachment of developing muscle fibers from tendons, due to the loss of the αPS2βPS-Tsp interaction (Chanana et al., 2007; Subramanian et al., 2007; **Figure 1B**). Moreover, Talin mutants display similar defects suggesting that Talin-related signaling is required for functional MTJ formation (Brown et al., 2002).

Similar to Focal adhesion, activation of Rho signaling downstream of Integrins appears to be indispensable for MTJ formation (reviewed in Geiger and Bershadsky, 2002). We have recently shown that *Drosophila* Rho-kinase (DRok) loss of function in tendon cells results in diminished phosphorylation of MRLC and abnormal βPS localization and Tsp accumulation at the MTJ, suggesting that DRok could be part of the inside-out mechanism of Integrin activation (Vega-Macaya et al., 2016). Interestingly, ROCK activity appears to be required for stretch-induced tenocyte differentiation from human Mesenchymal Stem Cells (hMSCs) (Xu et al., 2012). Stretching of hMSCs elicited enhanced expression of Scx, Collagen I and II, among other tendon specific genes. The addition of a ROCK inhibitor results in an attenuated expression of these genes (Xu et al., 2012). Whether DRok activity contributes to tendon

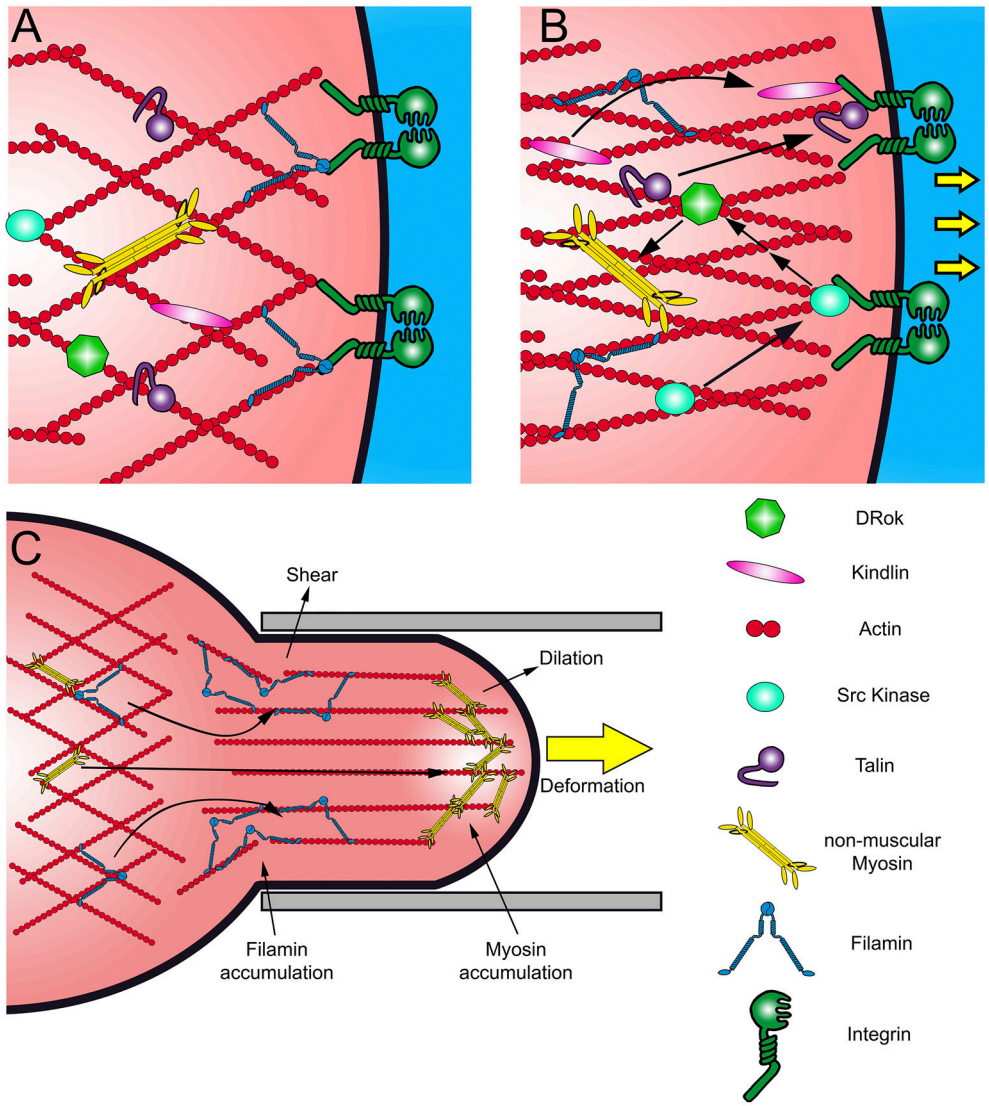

FIGURE 2 | (A,B) Scheme of canonical cell response to mechanical stimuli. Mechanical stress results in Integrin activation and the recruitment of Kindlin and Talin, rearranging the actin network. The recruitment of Src kinase activates several pathways in response to the stress, like the Rho-ROCK pathway. **(C)** Scheme of a cell aspirated by micropipette and the redistribution of Myosin II and Filamin. These proteins accumulate as an immediate response to different types of mechanical stimuli. Filamins accumulate in response to shear stress and Myosin II in response to dilation stress.

cell differentiation in response to mechanical forces, through regulation of gene expression in *Drosophila* remains to be explored. In addition to its role in MTJ maturation, DRok regulates the orientation of tendon extensions toward IFMs during recognition stage, enabling the correct attachment to the muscle fibers (Vega-Macaya et al., 2016; **Figure 1C**). *DRok* mutant tendon cells display miss-oriented tendon extensions, resulting in irregular attachments to the muscle fiber. Tendon extensions appears to be unable to resist the pulling forces generated by IFMs compaction, resulting in muscle detachment and death (Vega-Macaya et al., 2016). How DRok regulates tendon recognition of the myotube ends remains to be elucidated.

In conclusion, the membrane mechanoreceptor model explains how forces are sensed and transduced at the MTJ, but how tension exerted by muscle compaction is withstood by the whole tendon cell is still unclear.

ACTIN CROSSLINKERS AS INTRACELLULAR MECHANOSENSORS AND REGULATORS OF THE ACTIN NETWORK

In vitro and *in vivo* experiments show that mechanical perturbation of cell shape causes a redistribution of actin

crosslinkers and a rearrangement of the actin network (Gardel et al., 2004; Chaudhuri et al., 2007; Luo et al., 2013). Studies in *Drosophila* epithelial cells, *Dyctiostelium discoideum* and mammalian cells have demonstrated that mechanical deformation of the plasma membrane results in accumulation of crosslinking and motor proteins such as Filamin and myosin, respectively, to the perturbation site in distinctive ways (Fernandez-Gonzalez et al., 2009; Luo et al., 2013; Schiffhauer et al., 2016; **Figure 2C**). Myosin is recruited to regions under dilation stress, counteracting cell deformation by contraction of the acto-myosin filaments (**Figure 2C**). On the other hand, Filamin is recruited to sites subjected to shear stress (Luo et al., 2013; Schiffhauer et al., 2016; **Figure 2C**).

In contrast to Myosin, Filamin does not act as a contractile unit; instead, it enhances elasticity of the actin network to allow cell shape adaptation and remodeling (Luo et al., 2013; Schiffhauer et al., 2016). Filamin is a large actin-binding protein that works as a dimer (Noegel et al., 2004). Each Filamin monomer binds to one actin filament forming orthogonal and elastic actin networks by dimerization via their C-terminal immunoglobulin-like domains (Tseng et al., 2004; Pudas et al., 2005; **Figures 2A–C**). Both, Jitterbug, one of the two Filamins present in *Drosophila,* and non-muscle Myosin II (MyoII) are required to maintain the shape and polarity of tendon cells and partially co-distribute with actin filaments and Shot (Olguín et al., 2011). Interestingly, Shot loss of function display similar epithelial deformation phenotypes to Jbug (Olguín et al., 2011), suggesting that both microtubule and actin arrays that stretched along the apical-basal axis of tendon cells are required to withstand mechanical load.

At the signal-transduction level, Filamin acts as a scaffold for other actin regulatory proteins (Popowicz et al., 2006). In monocytes, Filamin recruits the small GTPases of the Rho family, their effectors and regulators (Leung et al., 2010). In migrating mammalian cells, Filamin recruits ROCK (Ueda et al., 2003), which may promote acto-myosin network contraction and stabilization by activation of the myosin regulatory light chain, α-Adducin and LIMK (Maekawa et al., 1999; Zhang et al., 2003). During cell migration, Filamin also interacts with the Integrin beta subunit, keeping it in a resting state, preventing focal adhesion formation (Liu et al., 2015; **Figure 2A**). A proposed mechanism is that after Integrin interaction with a stiffer ECM, Filamin dissociates from Integrin cytoplasmic domain leading to Talin and Vinculin recruitment in its place, reinforcing adhesion (Nieves et al., 2010; **Figure 2B**).

Based on these evidences, Filamin could play a dual role in tendon cell mechanoresponse during MTJ formation: as a molecular scaffold for actin regulators at the MTJ, and as regulator of tendon cell elastic properties at specific cellular regions. Moreover, Filamin redistribution may regulate its role as a scaffold at the MTJ.

CONCLUDING REMARKS

The ability of cells and tissues to respond to mechanical stress during development is crucial to shape organs and the whole individual. The combination of molecular tools that allows to measure in developing animals, mechanical stress across developmental fields, dynamic signaling pathway activity and cytoskeleton organization will be key to unveil the interplay between mechanical and chemical signaling during embryogenesis, including the formation of the musculoskeletal system.

AUTHOR CONTRIBUTION

MV and FV contributed equally to this work. MV wrote sections of the manuscript, then contributed to its editing and final formatting. FV made **Figures 1**, **2**. PO contributed to writing, editing materials written by MV and FV, and final integration of the various sections.

FUNDING

This work was supported by DRiDANS, PIA ACT-1401 and Biomedical Neuroscience Institute, Iniciativa Científica Milenio, ICM P09015F.

ACKNOWLEDGMENTS

We thank Gonzalo Olivares for advice and discussion during the preparation of the manuscript.

REFERENCES

Adamo, L., Naveiras, O., Wenzel, P. L., McKinney-Freeman, S., Mack, P. J., Gracia-Sancho, J., et al. (2009). Biomechanical forces promote embryonic haematopoiesis. *Nature* 459, 1131–1135. doi: 10.1038/nature08073

Alberton, P., Popov, C., Prägert, M., Kohler, J., Shukunami, C., Schieker, M., et al. (2012). Conversion of human bone marrow-derived mesenchymal stem cells into tendon progenitor cells by ectopic expression of scleraxis. *Stem Cells Dev.* 21, 846–858. doi: 10.1089/scd.2011.0150

Alves-Silva, J., Hahn, I., Huber, O., Mende, M., Reissaus, A., and Prokop, A. (2008). Prominent actin fiber arrays in *Drosophila* tendon cells represent architectural elements different from stress fibers. *Mol. Biol. Cell* 19, 4287–4297. doi: 10.1091/mbc.E08-02-0182

Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H., and Shattil, S. J. (2003). Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13298–13302. doi: 10.1073/pnas.2336149100

Arthur, W. T., Petch, L. A., and Burridge, K. (2000). Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. *Curr. Biol.* 10, 719–722. doi: 10.1016/S0960-9822(00)00537-6

Becker, S., Pasca, G., Strumpf, D., Min, L., and Volk, T. (1997). Reciprocal signaling between *Drosophila* epidermal muscle attachment cells and their corresponding muscles. *Development* 124, 2615–2622.

Bosveld, F., Bonnet, I., Guirao, B., Tlili, S., Wang, Z., Petilatot, A., et al. (2012). Mechanical control of morphogenesis by Fat/Dachsous/Four-jointed planar cell polarity pathway. *Science* 336, 724–727. doi: 10.1126/science.1221071

Brown, N. (2000). Cell-cell adhesion via the ECM: integrin genetics in fly and worm. *Matrix Biol.* 19, 191–201. doi: 10.1016/S0945-053X(00)00064-0

Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A. H., et al. (2002). Talin is essential for integrin function in *Drosophila*. *Dev. Cell* 3, 569–579. doi: 10.1016/S1534-5807(02)00290-3

Brunet, T., Bouclet, A., Ahmadi, P., Mitrossilis, D., Driquez, B., Brunet, A.-C., et al. (2013). Evolutionary conservation of early mesoderm specification by mechanotransduction in Bilateria. *Nat. Commun.* 4:2821. doi: 10.1038/ncomms3821

Campbell, I. D., and Humphries, M. J. (2011). Integrin structure, activation, and interactions. *Cold Spring Harb. Perspect. Biol.* 3, 1–15. doi: 10.1101/cshperspect.a004994

Chanana, B., Graf, R., Koledachkina, T., Pflanz, R., and Vorbrüggen, G. (2007). aPS2 integrin-mediated muscle attachment in *Drosophila* requires the ECM protein Thrombospondin. *Mech. Dev.* 124, 463–475. doi: 10.1016/j.mod.2007.03.005

Chaudhuri, O., Parekh, S. H., and Fletcher, D. A. (2007). Reversible stress softening of actin networks. *Nature* 445, 295–298. doi: 10.1038/nature05459

Chen, L., Liu, R., Liu, Z.-P., Li, M., and Aihara, K. (2012). Detecting early-warning signals for sudden deterioration of complex diseases by dynamical network biomarkers. *Sci. Rep.* 2, 18–20. doi: 10.1038/srep00342

Chen, X., Yin, Z., Chen, J., Shen, W., Liu, H., Tang, Q., et al. (2012). Force and scleraxis synergistically promote the commitment of human ES cells derived MSCs to tenocytes. *Sci. Rep.* 2:977. doi: 10.1038/srep00977

Costello, W. J., and Wyman, R. J. (1986). Development of an indirect flight muscle in a muscle-specific mutant of *Drosophila melanogaster*. *Dev. Biol.* 118, 247–258. doi: 10.1016/0012-1606(86)90092-8

de Lima, J. E., Bonnin, M. A., Birchmeier, C., and Duprez, D. (2016). Muscle contraction is required to maintain the pool of muscle progenitors via YAP and NOTCH during fetal myogenesis. *eLife* 5, 1–25. doi: 10.7554/eLife.15593

del Rio, A., Perez-jimenez, R., Liu, R., Roca-cusachs, P., Fernandez, J. M., and Sheetz, M. P. (2009). Stretching single talin rod molecules activates vinculin binding. *Science* 323, 638–641. doi: 10.1126/science.1162912

Desprat, N., Supatto, W., Pouille, P. A., Beaurepaire, E., and Farge, E. (2008). Tissue deformation modulates twist expression to determine anterior midgut differentiation in *Drosophila* embryos. *Dev. Cell* 15, 470–477. doi: 10.1016/j.devcel.2008.07.009

Estrada, B., Gisselbrecht, S. S., and Michelson, A. M. (2007). The transmembrane protein Perdido interacts with Grip and integrins to mediate myotube projection and attachment in the *Drosophila* embryo. *Development* 134, 4469–4478. doi: 10.1242/dev.014027

Evans, J. H., and Barbenel, J. C. (1975). Structural and mechanical properties of tendon related to function. *Equine Vet. J.* 7, 1–8. doi: 10.1111/j.2042-3306.1975.tb03221.x

Farge, E. (2003). Mechanical induction of Twist in the *Drosophila* foregut/stomodeal primordium. *Curr. Biol.* 13, 1365–1377. doi: 10.1016/S0960-9822(03)00576-1

Fernandes, J., Bate, M., and Vijayraghavan, K. (1991). Development of the indirect flight muscles of *Drosophila*. *Development* 113, 67–77.

Fernandes, J. J., Celniker, S. E., and VijayRaghavan, K. (1996). Development of the indirect flight muscle attachment sites in *Drosophila*: role of the PS integrins and the stripe gene. *Dev. Biol.* 176, 166–184. doi: 10.1006/dbio.1996.0125

Fernandez-Gonzalez, R., de Simoes, S, M., Röper, J. C., Eaton, S., and Zallen, J. A. (2009). Myosin II dynamics are regulated by tension in intercalating cells. *Dev. Cell* 17, 736–743. doi: 10.1016/j.devcel.2009.09.003

Frommer, G., Vorbruggen, G., Pasca, G., Jackle, H., and Volk, T. (1996). Epidermal egr-like zinc finger protein of *Drosophila* participates in myotube guidance. *EMBO J.* 15, 1642–1649.

Gardel, M. L., Shin, J. H., MacKintosh, F. C., Mahadevan, L., Matsudaira, P., and Weitz, D. A. (2004). Elastic behavior of cross-linked and bundled actin networks. *Science* 304, 1301–1305. doi: 10.1126/science.1095087

Geiger, B., and Bershadsky, A. (2002). Exploring the neighborhood: adhesion-coupled cell mechanosensors. *Cell* 110, 139–142. doi: 10.1016/S0092-8674(02)00831-0

Gilsohn, E., and Volk, T. (2010). Slowdown promotes muscle integrity by modulating integrin-mediated adhesion at the myotendinous junction. *Development* 137, 785–794. doi: 10.1242/dev.043703

Gotwals, P. J., Fesslert, L. I., Wehrlii, M., and Hynes, R. (1994). *Drosophila* PS1 integrin is a laminin receptor and differs in ligand specificity from PS2. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11447–11451. doi: 10.1073/pnas.91.24.11447

Guerquin, M. J., Charvet, B., Nourissat, G., Havis, E., Ronsin, O., Bonnin, M. A., et al. (2013). Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. *J. Clin. Invest.* 123, 3564–3576. doi: 10.1172/JCI67521

Havis, E., Bonnin, M., de Lima, J., Charvet, B., Milet, C., and Duprez, D. (2016). TGFβ and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development. *Development* 143, 3839–3851. doi: 10.1242/dev.136242

Havis, E., Bonnin, M.-A., Olivera-Martinez, I., Nazaret, N., Ruggiu, M., Weibel, J., et al. (2014). Transcriptomic analysis of mouse limb tendon cells during development. *Development* 141, 3683–3696. doi: 10.1242/dev.108654

Hove, J. R., Koster, R. W., Forouhar, A. S., Acevedo-Bolton, G., Fraser, S. E., and Gharib, M. (2003). Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. *Nature* 421, 172–177. doi: 10.1038/nature01282

Ingber, D. E. (1997). Tensegrity: the architectural basis of cellular mechanotransduction. *Annu. Rev. Physiol.* 59, 575–599. doi: 10.1146/annurev.physiol.59.1.575

Kjaer, M., and Kjær, M. (2004). Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol. Rev.* 84, 649–698. doi: 10.1152/physrev.00031.2003

Kramer, S. G., Kidd, T., Simpson, J. H., and Goodman, C. S. (2001). Switching repulsion to attraction : changing responses to slit during transition in mesoderm migration. *Science* 292, 737–741. doi: 10.1126/science.1058766

Lee, S. E., Kamm, R. D., and Mofrad, M. R. K. (2007). Force-induced activation of Talin and its possible role in focal adhesion mechanotransduction. *J. Biomech.* 40, 2096–2106. doi: 10.1016/j.jbiomech.2007.04.006

Lejard, V., Blais, F., Guerquin, M. J., Bonnet, A., Bonnin, M. A., Havis, E., et al. (2011). EGR1 and EGR2 involvement in vertebrate tendon differentiation. *J. Biol. Chem.* 286, 5855–5867. doi: 10.1074/jbc.M110.153106

Lemke, S. B., and Schnorrer, F. (in press). Mechanisms of development mechanical forces during muscle development. *Mech. Dev.* doi: 10.1016/j.mod.2016.11.003

le Noble, F., Moyon, D., Pardanaud, L., Yuan, L., Djonov, V., Matthijsen, R., et al. (2004). Flow regulates arterial-venous differentiation in the chick embryo yolk sac. *Development* 131, 361–375. doi: 10.1242/dev.00929

Leung, R., Wang, Y., Cuddy, K., Sun, C., Magalhaes, J., Grynpas, M., et al. (2010). Filamin a regulates monocyte migration through rho small gtpases during osteoclastogenesis. *J. Bone Miner. Res.* 25, 1077–1091. doi: 10.1359/jbmr.091114

Li, Y., Ramcharan, M., Zhou, Z., Leong, D. J., Akinbiyi, T., Majeska, R. J., et al. (2015). The role of scleraxis in fate determination of mesenchymal stem cells for tenocyte differentiation. *Sci. Rep.* 5:13149. doi: 10.1038/srep13149

Lisio, M., De Jensen, T., Sukiennik, R. A., Huntsman, H. D., and Boppart, M. D. (2014). Substrate and strain alter the muscle-derived mesenchymal stem cell secretome to promote myogenesis. *Stem Cell Res. Ther.* 5, 1–12. doi: 10.1186/scrt463

Liu, J., Das, M., Yang, J., Ithychanda, S. S., Yakubenko, V. P., Plow, E. F., et al. (2015). Structural mechanism of integrin inactivation by filamin. *Nat. Struct. Mol. Biol.* 22, 1–9. doi: 10.1038/nsmb.2999

Luo, T. Z., Mohan, K., Iglesias, P. A., and Robinson, D. N. (2013). Molecular mechanisms of cellular mechanosensing. *Nat. Mater.* 12, 1063–1070. doi: 10.1038/nmat3772

Maartens, A. P., and Brown, N. H. (2015). The many faces of cell adhesion during *Drosophila* muscle development. *Dev. Biol.* 401, 62–74. doi: 10.1016/j.ydbio.2014.12.038

Maeda, E., Fleischmann, C., Mein, C. A., Shelton, J. C., Bader, D. L., and Lee, D. A. (2010). Functional analysis of tenocytes gene expression in tendon fascicles subjected to cyclic tensile strain. *Connect. Tissue Res.* 51, 434–444. doi: 10.3109/03008201003597056

Maeda, T., Sakabe, T., Sunaga, A., Sakai, K., Rivera, A. L., Keene, D. R., et al. (2011). Conversion of mechanical force into TGF-B-mediated biochemical signals. *Curr. Biol.* 21, 933–941. doi: 10.1016/j.cub.2011.04.007

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., et al. (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285, 895–898. doi: 10.1126/science.285.5429.895

Mammoto, T., and Ingber, D. E. (2010). Mechanical control of tissue and organ development. *Development* 137, 1407–1420. doi: 10.1242/dev.024166

Martin, D., Zusman, S., Li, X., Williams, E. L., Khare, N., DaRocha, S., et al. (1999). wing blister, a new *Drosophila* laminin chain required for cell adhesion

and migration during embryonic and imaginal development. *J. Cell Biol.* 145, 191–201. doi: 10.1083/jcb.145.1.191

Munger, J. S., and Sheppard, D. (2011). Cross talk among TGF-β signaling pathways, integrins, and the extracellular matrix. *Cold Spring Harb. Perspect. Biol.* 3, a005017–a005017. doi: 10.1101/cshperspect.a005017

Murchison, N. D., Price, B. A, Conner, D. A, Keene, D. R., Olson, E. N., Tabin, C. J., et al. (2007). Regulation of tendon differentiation by scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. *Development* 134, 2697–2708. doi: 10.1242/dev.001933

Nieves, B., Jones, C. W., Ward, R., Ohta, Y., Reverte, C. G., and Laflamme, S. E. (2010). The NPIY motif in the integrin b 1 tail dictates the requirement for talin-1 in outside-in signaling. *J. Cell Sci.* 123, 1216–1226. doi: 10.1242/jcs.056549

Noegel, A. A., Popowicz, G. M., Mu, R., Schleicher, M., Huber, R., and Holak, T. A. (2004). Molecular structure of the rod domain of dictyostelium filamin. *J. Mol. Biol.* 342, 1637–1646. doi: 10.1016/j.jmb.2004.08.017

North, T. E., Goessling, W., Peeters, M., Li, P., Ceol, C., Lord, A. M., et al. (2009). Hematopoietic stem cell development is dependent on blood flow. *Cell* 137, 736–748. doi: 10.1016/j.cell.2009.04.023

Olguín, P., Glavic, A., and Mlodzik, M. (2011). Intertissue mechanical stress affects frizzled-mediated planar cell polarity in the *Drosophila* notum epidermis. *Curr. Biol.* 21, 236–242. doi: 10.1016/j.cub.2011.01.001

Ordan, E., Brankatschk, M., Dickson, B., Schnorrer, F., and Volk, T. (2015). Slit cleavage is essential for producing an active, stable, non-diffusible short-range signal that guides muscle migration. *Development* 142, 1431–1436. doi: 10.1242/dev.119131

Ordan, E., and Volk, T. (2015). A non-signaling role of Robo2 in tendons is essential for Slit processing and muscle patterning. *Development* 142, 3512–3518. doi: 10.1242/dev.128157

Ordan, E., and Volk, T. (2016). Amontillado is required for *Drosophila* Slit processing and for tendon-mediated muscle patterning. *Biol. Open* 143, 1491–1501. doi: 10.1242/bio.020636

Otoole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., et al. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. *J.Cell Biol.* 124, 1047–1059. doi: 10.1083/jcb.124.6.1047

Pérez-Moreno, J. J., Bischoff, M., Martín-Bermudo, M. D., and Estrada, B. (2014). The conserved transmembrane proteoglycan Perdido/Kon-tiki is essential for myofibrillogenesis and sarcomeric structure in *Drosophila*. *J. Cell Sci.* 127, 3162–3173. doi: 10.1242/jcs.150425

Popov, C., Burggraf, M., Kreja, L., Ignatius, A., Schieker, M., and Docheva, D. (2015). Mechanical stimulation of human tendon stem/progenitor cells results in upregulation of matrix proteins, integrins and MMPs, and activation of p38 and ERK1/2 kinases. *BMC Mol. Biol.* 16:6. doi: 10.1186/s12867-015-0036-6

Popowicz, G. M., Schleicher, M., Noegel, A. A., and Holak, T. A. (2006). Filamins : promiscuous organizers of the cytoskeleton. *TRENDS Biochem. Sci.* 31, 411–419. doi: 10.1016/j.tibs.2006.05.006

Pryce, B. A, Watson, S. S., Murchison, N. D., Staverosky, J. A, Dünker, N., and Schweitzer, R. (2009). Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 136, 1351–1361. doi: 10.1242/dev.027342

Pudas, R., Kiema, T., Butler, P. J. G., Stewart, M., and Ylänne, J. (2005). For vertebrate filamin dimerization. *Structure* 13, 111–119. doi: 10.1016/j.str.2004.10.014

Schiffhauer, E. S., Luo, T., Mohan, K., Srivastava, V., Qian, X., Griffis, E. R., et al. (2016). Mechanoaccumulative elements of the mammalian actin cytoskeleton. *Curr. Biol.* 26, 1473–1479. doi: 10.1016/j.cub.2016.04.007

Schnorrer, F., Kalchhauser, I., and Dickson, B. J. (2007). The transmembrane protein Kon-tiki couples to dgrip to mediate myotube targeting in *Drosophila*. *Dev. Cell* 12, 751–766. doi: 10.1016/j.devcel.2007.02.017

Schwartz, A. G., Lipner, J. H., Pasteris, J. D., Genin, G. M., and Thomopoulos, S. (2013). Muscle loading is necessary for the formation of a functional tendon enthesis. *Bone* 55, 44–51. doi: 10.1016/j.bone.2013.03.010

Schweitzer, R., Chyung, J. H., Murtaugh, L. C., Brent, A. E., Rosen, V., Olson, E. N., et al. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 128, 3855–3866.

Schweitzer, R., Zelzer, E., and Volk, T. (2010). Connecting muscles to tendons: tendons and musculoskeletal development in flies and vertebrates. *Development* 137, 2807–2817. doi: 10.1242/dev.047498

Shwartz, Y., Blitz, E., and Zelzer, E. (2013). One load to rule them all: mechanical control of the musculoskeletal system in development and aging. *Differentiation* 86, 104–111. doi: 10.1016/j.diff.2013.07.003

Subramanian, A., Bunch, T., Wayburn, B., and Volk, T. (2007). Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in. *Development* 1278, 1269–1278. doi: 10.1242/dev.000406

Subramanian, A., Prokop, A., Yamamoto, M., Sugimura, K., Uemura, T., Betschinger, J., et al. (2003). Shortstop recruits EB1/APC1 and promotes microtubule assembly at the muscle-tendon junction. *Curr. Biol.* 13, 1086–1095. doi: 10.1016/S0960-9822(03)00416-0

Subramanian, A., and Schilling, T. F. (2015). Tendon development and musculoskeletal assembly: emerging roles for the extracellular matrix. *Development* 142, 4191–4204. doi: 10.1242/dev.114777

Takagi, J., Konstantin, S., Springer, T. A., and Walz, T. (2003). Structure of integrin a5b1 in complex with fibronectin. *EMBO J.* 22, 4607–4615. doi: 10.1093/emboj/cdg445

Tseng, Y., An, K. M., Esue, O., and Wirtz, D. (2004). The bimodal role of filamin in controlling the architecture and mechanics of F-actin networks. *J. Biol. Chem.* 279, 1819–1826. doi: 10.1074/jbc.M306090200

Ueda, K., Ohta, Y., and Hosoya, H. (2003). The carboxy-terminal pleckstrin homology domain of ROCK interacts with filamin-A. *Biochem. Biophys. Res. Comun.* 301, 886–890. doi: 10.1016/S0006-291X(03)00048-2

Vega-Macaya, F., Manieu, C., Valdivia, M., Mlodzik, M., and Olguín, P. (2016). Establishment of the muscle-tendon junction during thorax morphogenesis in *Drosophila* requires the rho-kinase. *Genetics* 204, 1139–1149. doi: 10.1534/genetics.116.189548

Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T. A., Plow, E. F., et al. (2002). A structural mechanism of integrin aIIb3 "inside-out" activation as regulated by its cytoplasmic face. *Cell* 110, 587–597. doi: 10.1016/S0092-8674(02)00906-6

Volk, T., and VijayRaghavan, K. (1994). A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo. *Development* 120, 59–70.

Wang, N., Naruse, K., Stamenović, D., Fredberg, J. J., Mijailovich, S. M., Tolić-Nørrelykke, I. M., et al. (2001). Mechanical behavior in living cells consistent with the tensegrity model. *Proc. Natl. Acad. Sci. U.S.A.* 98, 7765–7770. doi: 10.1073/pnas.141199598

Wayburn, B., and Volk, T. (2009). LRT, a tendon-specific leucine-rich repeat protein, promotes muscle-tendon targeting through its interaction with Robo. *Development* 136, 3607–3615. doi: 10.1242/dev.040329

Weitkunat, M., Kaya-Çopur, A., Grill, S. W., and Schnorrer, F. (2014). Tension and force-resistant attachment are essential for myofibrillogenesis in *Drosophila* flight muscle. *Curr. Biol.* 24, 705–716. doi: 10.1016/j.cub.2014.02.032

Wipff, P. J., Rifkin, D. B., Meister, J. J., and Hinz, B. (2007). Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J. Cell Biol.* 179, 1311–1323. doi: 10.1083/jcb.200704042

Xu, B., Song, G., Ju, Y., Li, X., Song, Y., and Watanabe, S. (2012). RhoA/ROCK, cytoskeletal dynamics, and focal adhesion kinase are required for mechanical stretch-induced tenogenic differentiation of human mesenchymal stem cells. *J. Cell. Physiol.* 227, 2722–2729. doi: 10.1002/jcp.23016

Yarnitzky, T., Min, L., and Volk, T. (1997). The *Drosophila* neuregulin homolog Vein mediates inductive interactions between myotubes and their epidermal attachment cells. *Genes Dev.* 11, 2691–2700. doi: 10.1101/gad.11.20.2691

Zhang, M., Kalinec, G. M., Urrutia, R., Billadeau, D. D., and Kalinec, F. (2003). ROCK-dependent and ROCK-independent control of cochlear outer hair cell electromotility. *J. Biol. Chem.* 278, 35644–35650. doi: 10.1074/jbc.M301668200

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frontiers in Cell and Developmental Biology

OPINION
published: 05 April 2017
doi: 10.3389/fcell.2017.00032

Putting Cells into Context

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Keywords: cell signaling, mechanobiology, extracellular matrix, artificial matrices, material sciences

CELLS LIVE IN A COMPLEX WORLD

It may sound blatantly obvious, but we have to remind ourselves occasionally that *in vivo* cells experience an environment with a level of complexity far beyond experimental reach. The developing organism is a highly complex system, where each cell receives a multitude of cues of diverse nature at any given time point. Only the comprehensive integration of all these multivalent interactions determines the actual signaling state and hence the behavior of a cell.

The analysis of biological questions is mainly inspired by a reductionist approach adopted from the "exact sciences," where it has been proven immensely successful. That is, we are used to break down our experimental setup to a manageable number of variables. This of course is inherently contradictory to the complexity of biological systems. While simplification may be the only viable option for the experimenter to dissect biological function down to detail, it has also influenced our perspective toward the experimental systems applied. For example, studies of intracellular signaling pathways are typically performed with cultured cells. Culturing cells in an *in vitro* setting became a standard model system in biomedical research and with it in cell and developmental biology. These simplified systems allow for the dissection of molecular interactions and pathways and are aimed to deepen and mechanistically understand cellular behavior. While cell cultures have generated a wealth of information into cellular function, the data obtained *in vitro* frequently are in conflict with *in vivo* observations. One reason for this discrepancy is that these analyses focus on the cell as a closed functional system, thus conceptually unhinging it from its environment.

In a living organism, cells are embedded in extracellular matrix (ECM) with diverse but also organ-specific properties. Cells attach to the ECM mainly via focal adhesions, which on the inside are linked to the cytoskeleton (**Figure 1A**). The ECM has long been seen as a mere scaffold providing support and shape; however, in the past decades it has become clear that the ECM has also an instructive character (see Adams and Watt, 1993; Tsang et al., 2010). Like a color palette ECMs come in many shades with different molecular composition, resulting in manifold chemical and physical characteristics (Rozario and DeSimone, 2010). We focus here on a rather simple but often overlooked property that provides tissues with their rigidity or elasticity. It is these mechanical properties that emerged as a decisive factor mediating information flow (see Mammoto et al., 2013). There is a multitude of interactions between cells and their ECM and it is now well accepted that cells perceive the substrate's mechanical cues and integrate them into their intracellular signal transduction pathways, gene expression and cell fate decisions. In this sense cells can be both writers and readers of ECM and its cues, implying crosstalk between cells via the ECM. Besides determining mechanical tissue properties, cells build a matrix with spatial decoration of specific growth factors to thereby modulate their local availability. Emerging data even suggest that intracellular signaling pathways integrate external biomechanical cues directly by altering the phosphorylation state of cytosolic signaling proteins (Kopf et al., 2012, 2014; Ashe, 2016).

OPEN ACCESS

Edited by:
José Lozano,
University of Málaga, Spain

Reviewed by:
Stefano Biressi,
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Vania Braga,
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *16 January 2017*
Accepted: *21 March 2017*
Published: *05 April 2017*

Citation:
Stricker S, Knaus P and Simon H-G (2017) Putting Cells into Context. Front. Cell Dev. Biol. 5:32. doi: 10.3389/fcell.2017.00032

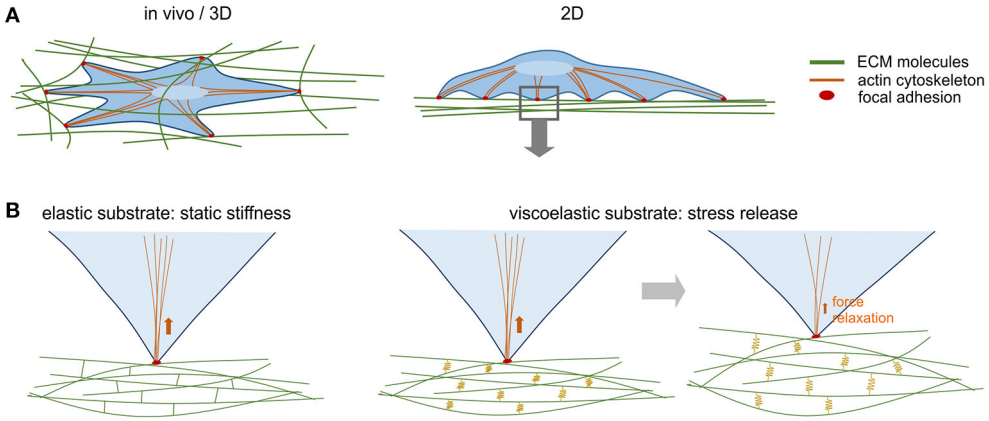

FIGURE 1 | Mechanical interactions between cells and ECM. (A) *In vivo*, cells interact with their tissue-specific ECM mainly via focal adhesions that are linked to the actin cytoskeleton inside the cell. Via focal adhesions cells exert mechanical force to the ECM and are able to deform it and thereby measure its stiffness. In 2D *in vitro* culture, cells contact their substrate via focal adhesions on the basal side. **(B)** Standard synthetic elastic matrices where fibrillary proteins are covalently cross linked have a static stiffness profile, i.e., they deform as the cells apply force, but do not dissipate the force. The ECM in living tissues is viscoelastic and undergoes stress relaxation. Synthetic viscoelastic substrates, in which ionic crosslinking allows a certain degree of flexibility (here symbolized by springs) dissipate energy through the substrate so that the cell can gradually re-shape its ECM environment, leading to a decrease in the substrates elastic modulus over time.

BIOMECHANICAL PROPERTIES OF THE ECM AND ITS INFLUENCE ON CELLS

For a long time it has been known that cells can, based on intrinsic sensing mechanisms, differentially respond to, for example, growth factor signaling (e.g., Nakagawa et al., 1989). The importance of the mechanical aspect, however, has only gained wider attention in recent years. In 2006, a landmark study from the Discher group showed that *in vivo* tissues exhibit an elastic modulus in the range of below 1 kPa (brain) up to over 100 kPa (ossified bone). Moreover, they provided the first evidence that this property has an instructive character on the behavior of progenitor cells, specifically mesenchymal stem cells (MSCs). The cells sensed their microenvironment by attaching and applying force to the substrate. Plating MSCs on polyacrylamide substrates of varying stiffness revealed a differentiation potential correlating with the stiffness of the *in vivo* tissue; in other words, MSCs seeded on extremely soft substrates differentiated along the neural fate, while cells seeded on hard substrates differentiated along the osteogenic fate, and intermediate stiffness substrates supported differentiation along a myogenic fate (Engler et al., 2006).

Experiments using dynamic modulation of substrate stiffness further revealed that cells initially cultured on soft (0.5 kPa) or stiff (40 kPa) polyacrylamide hydrogels and then transferred to gels of the opposite stiffness had the capacity to revert their gene expression profile from neurogenic to osteogenic, and *vice versa* (Lee et al., 2014). However, while the cells displayed a remarkable potential switching lineage specification, MSCs transferred from stiff to soft substrates maintained elevated osteogenesis markers; thus, they kept a memory of their previous culture conditions indicating a certain degree of irreversible, likely epigenetically fixed, lineage commitment.

Just recently it has been discovered that partial matrix stress relaxation is another fundamental signal in cell-ECM interactions (**Figure 1B**). Stress relaxation means that the force cells exert on the ECM dissipates, and over time ECM resistance decreases. Chaudhuri et al. engineered alginate polysaccharide hydrogels that are, independent from their initial elastic modulus, also tunable in their viscoelasticity. Thus, they mimic the remodeling of the matrix microenvironment over time. MSCs embedded in 17 kPa-stiff hydrogels with a rapid rate of stress relaxation demonstrated enhanced spreading, proliferation and osteogenic differentiation (Chaudhuri et al., 2016). In addition to force dissipation it is likely that remodeling of the ECM by Matrix Metalloproteinase (MMP) activity contributes to this behavior *in vivo*.

How do cells perceive these stimuli and translate them into transcriptional activity? It was shown that mechanotransduction of ECM stiffness toward MSC differentiation critically depends on YAP (yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) signaling. In this context, YAP/TAZ were not activated by the "canonical" Hippo/LATS cascade, but by cytoskeletal tension and Rho-GTPase activity (Dupont et al., 2011). Interestingly, deregulation of YAP/TAZ signaling has been linked to disease conditions characterized by ECM stiffness changes such as fibrosis (Liu et al., 2015), and in this context apparently a circuit with TGFβ and WNT signaling pathways exists (Piersma et al., 2015). It is noteworthy that the interplay of ECM stiffness and mechanosensing itself impinges on the expression of profibrotic genes, driving a feed forward vicious cycle (Parker et al., 2014).

Another example are muscle resident stem cells, the so-called satellite cells (SCs). SCs reside in a specific niche underneath the myofiber's rigid basal lamina where they are kept in a quiescent state that is dependent on different factors including the collagen,

glycoprotein and proteoglycan-rich ECM (e.g., Brohl et al., 2012; Bentzinger et al., 2013). In an injury situation SCs are activated and form new muscle, but importantly they also self-renew. After isolation and culture, the expansion of SCs *in vitro* and their expression of myogenic transcription factors was shown to be influenced by the elasticity of the culturing substrate (Gilbert et al., 2010), a feature recently utilized to create artificial niches maintaining satellite cell quiescence *ex vivo* (Quarta et al., 2016).

The importance of the biochemical and biophysical properties of extracellular matrices on myogenesis has been coherently demonstrated in a vertebrate *in vivo/in vitro* regeneration model. During amphibian limb and cardiac regeneration, the collagen/laminin-rich matrix typical for differentiated tissues is temporarily replaced by a transitional matrix of reduced stiffness composed of hyaluronic acid, tenascin-C and fibronectin that is surprisingly similar to the type found in developing structures (Calve et al., 2010; Mercer et al., 2013). Employing these regeneration-permissive ECMs in *in vitro* cultures, Calve et al. demonstrated an instructive role of distinct ECM components promoting cell fragmentation, proliferation, migration and differentiation of *ex vivo* skeletal muscle cells (Calve et al., 2010). In addition, using a polydimethylsiloxane (PDMS)-ECM culture system that allowed for the modulation of both, stiffness and matrix composition they could further demonstrate that ECM type and substrate stiffness over a range of 2–100 kPa combine to control migration as well as differentiation state of skeletal muscle cells (Calve and Simon, 2012).

Mechanical and biophysical properties of the ECM are provided by coordinated synthesis and secretion of matrix components with protein or sugar backbones and biologically active epitopes that result in a network of different biomolecules. Dynamic post-translational modifications including MMP cleavage further shape a characteristic local signaling environment. These native 3D structures also serve as versatile surfaces for the binding of growth factors—either in their active form or inactive preform—which, often as a consequence of mechanical stress, will be released in a spatially controlled manner. Mimicking these complex *in vivo* conditions using a 3D bioreactor with a collagen scaffold as a simplified *in vitro* culture system, the Knaus and Petersen groups provided evidence that biomechanical signaling is directly integrated into the BMP/Smad pathway (Kopf et al., 2012). Coapplication of mechanical stress and BMP stimulation resulted in increased and prolonged phosphorylation of Smads, the direct target of the transmembrane BMP receptor kinases. As a consequence, distinct target genes, including known mechanotransducers, were upregulated in a synergistic manner.

CONSEQUENCES FOR *IN VITRO* STUDIES

Clearly, studying the complex *in vivo* interactions of cell-growth factor, cell-cell, and cell-matrix interactions and their downstream intracellular signal transduction and gene expression pathways, we will also in the future have to rely on simplified *in vitro* culture systems. As by default cells apparently integrate mechanical and biochemical inputs, the cellular behavior experimentally determined is in consequence dependent on the *in vitro* culture conditions and not necessarily reflect cellular behavior seen *in vivo*.

The standard tissue culture method is still the plastic dish, with an elastic modulus of approximately 10^6 kPa way out of the physiological range. When a more natural environment is desired, plastic dishes are at best coated with a thin layer of mostly collagenous matrices such as gelatine or Matrigel™. This, however, rather serves as a functionalization of the surface toward better cell adherence rather than altering the mechanical properties of the substrate. In light of the growing body of evidence from the emerging field of mechanobiology we have to change course.

Time has come to move on to more comprehensive *in vitro* culture systems that better simulate the complex *in vivo* conditions. Recent approaches employing engineered biopolymers as mimetics of the natural environment provide new opportunities to develop more physiological cell culture procedures. The material sciences have made available a range of different tested hydrogels; of particular interest are those made from biologically inert polymers including polyacrylamide, PDMS, alginate, and polyethylene glycol (PEG). All of these synthetic polymers allow, to various degrees, for the tuning of stiffness over a range of 2–40 kPa (similar to that observed in natural tissues), presentation of native matrix-derived peptide epitopes, and/or binding and release of growth factors. Ideally, these cell culture models would be in a 3D architecture resembling the *in vivo* context as closely as possible. However, building a perfect mimetic of the *in vivo* environment is virtually impossible in a standard cell culture experiment when analyzing, for example, intracellular signaling using current routine reporter assays. It therefore appears as a minimal requirement for a more comprehensive experimental design to at least consider the biomechanical properties of the tissue of origin, i.e., the mechanical modulus. A realistic rational approach could be 2D culturing techniques with softer synthetic matrix substrates (as compared to the hard plastic dish) that mimic the *in vivo* viscoelastic conditions, which we think would greatly improve the reliability of *ex vivo*/*in vitro* experimentation and improve comparability to *in vivo* data. It is important to note that the fabrication of such biomimetic matrices in the laboratory is still challenging and coupled to an operating expense that clearly hinders their standard application. However, custom products are beginning to enter the market and it is foreseeable that a panel of matrix solutions will become available in the near future tailored to many if not most individual experimental needs.

Importantly, embracing more physiological cell culture conditions might generate a fundamentally new understanding of how extracellular cues, both insoluble and soluble, are integrated and stored to guide cellular behavior. These immediate biological goals would further help to achieve current and future therapeutic challenges in humans (see Sommerfeld and Elisseeff, 2016).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

Research in the Stricker lab on this topic is funded by the Berlin-Brandenburg School for Regenerative Therapies (BSRT). In the Knaus lab this research topic is funded by the DFG (FOR2165) and the Berlin-Brandenburg School for Regenerative Therapies (BSRT). This manuscript was inspired during a sabbatical stay of HGS at the Knaus lab, who was supported through a Kennedy Sabbatical Award of Northwestern University's Department of Pediatrics and a gift of the Family of Michael P. Polsky.

REFERENCES

Adams, J. C., and Watt, F. M. (1993). Regulation of development and differentiation by the extracellular matrix. *Development* 117, 1183–1198.

Ashe, H. L. (2016). Modulation of BMP signalling by integrins. *Biochem. Soc. Trans.* 44, 1465–1473. doi: 10.1042/BST20160111

Bentzinger, C. F., Wang, Y. X., von Maltzahn, J., Soleimani, V. D., Yin, H., and Rudnicki, M. A. (2013). Fibronectin regulates Wnt7a signaling and satellite cell expansion. *Cell Stem Cell* 12, 75–87. doi: 10.1016/j.stem.2012.09.015

Brohl, D., Vasyutina, E., Czajkowski, M. T., Griger, J., Rassek, C., Rahn, H. P., et al. (2012). Colonization of the satellite cell niche by skeletal muscle progenitor cells depends on Notch signals. *Dev. Cell* 23, 469–481. doi: 10.1016/j.devcel.2012.07.014

Calve, S., Odelberg, S. J., and Simon, H. G. (2010). A transitional extracellular matrix instructs cell behavior during muscle regeneration. *Dev. Biol.* 344, 259–271. doi: 10.1016/j.ydbio.2010.05.007

Calve, S., and Simon, H. G. (2012). Biochemical and mechanical environment cooperatively regulate skeletal muscle regeneration. *FASEB J.* 26, 2538–2545. doi: 10.1096/fj.11-200162

Chaudhuri, O., Gu, L., Klumpers, D., Darnell, M., Bencherif, S. A., Weaver, J. C., et al. (2016). Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat. Mater.* 15, 326–334. doi: 10.1038/nmat4489

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., et al. (2011). Role of YAP/TAZ in mechanotransduction. *Nature* 474, 179–183. doi: 10.1038/nature10137

Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell* 126, 677–689. doi: 10.1016/j.cell.2006.06.044

Gilbert, P. M., Havenstrite, K. L., Magnusson, K. E., Sacco, A., Leonardi, N. A., Kraft, P., et al. (2010). Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. *Science* 329, 1078–1081. doi: 10.1126/science.1191035

Kopf, J., Paarmann, P., Hiepen, C., Horbelt, D., and Knaus, P. (2014). BMP growth factor signaling in a biomechanical context. *Biofactors* 40, 171–187. doi: 10.1002/biof.1137

Kopf, J., Petersen, A., Duda, G. N., and Knaus, P. (2012). BMP2 and mechanical loading cooperatively regulate immediate early signalling events in the BMP pathway. *BMC Biol.* 10:37. doi: 10.1186/1741-7007-10-37

Lee, J., Abdeen, A. A., and Kilian, K. A. (2014). Rewiring mesenchymal stem cell lineage specification by switching the biophysical microenvironment. *Sci. Rep.* 4:5188. doi: 10.1038/srep05188

Liu, F., Lagares, D., Choi, K. M., Stopfer, L., Marinkovic, A., Vrbanac, V., et al. (2015). Mechanosignaling through YAP and TAZ drives fibroblast activation and fibrosis. *Am. J. Physiol. Lung. Cell Mol. Physiol.* 308, L344–L357. doi: 10.1152/ajplung.00300.2014

Mammoto, T., Mammoto, A., and Ingber, D. E. (2013). Mechanobiology and developmental control. *Annu. Rev. Cell Dev. Biol.* 29, 27–61. doi: 10.1146/annurev-cellbio-101512-122340

Mercer, S. E., Odelberg, S. J., and Simon, H. G. (2013). A dynamic spatiotemporal extracellular matrix facilitates epicardial-mediated vertebrate heart regeneration. *Dev. Biol.* 382, 457–469. doi: 10.1016/j.ydbio.2013.08.002

Nakagawa, S., Pawelek, P., and Grinnell, F. (1989). Extracellular matrix organization modulates fibroblast growth and growth factor responsiveness. *Exp. Cell Res.* 182, 572–582. doi: 10.1016/ 0014-4827(89)90260-7

Parker, M. W., Rossi, D., Peterson, M., Smith, K., Sikstrom, K., White, E. S., et al. (2014). Fibrotic extracellular matrix activates a profibrotic positive feedback loop. *J. Clin. Invest.* 124, 1622–1635. doi: 10.1172/JCI71386

Piersma, B., Bank, R. A., and Boersema, M. (2015). Signaling in fibrosis: TGF-beta, WNT, and YAP/TAZ converge. *Front. Med. (Lausanne)* 2:59. doi: 10.3389/fmed.2015.00059

Quarta, M., Brett, J. O., DiMarco, R., De Morree, A., Boutet, S. C., Chacon, R., et al. (2016). An artificial niche preserves the quiescence of muscle stem cells and enhances their therapeutic efficacy. *Nat. Biotechnol.* 34, 752–759. doi: 10.1038/nbt.3576

Rozario, T., and DeSimone, D. W. (2010). The extracellular matrix in development and morphogenesis: a dynamic view. *Dev. Biol.* 341, 126–140. doi: 10.1016/j.ydbio.2009.10.026

Sommerfeld, S. D., and Elisseeff, J. H. (2016). Time to relax: mechanical stress release guides stem cell responses. *Cell Stem Cell* 18, 166–167. doi: 10.1016/j.stem.2016.01.020

Tsang, K. Y., Cheung, M. C., Chan, D., and Cheah, K. S. (2010). The developmental roles of the extracellular matrix: beyond structure to regulation. *Cell Tiss. Res.* 339, 93–110. doi: 10.1007/s00441-009-0893-8

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