INNOVATIVE MODELS IN BONE BIOLOGY: WHAT CAN BE LEARNED FROM RARE BONE DISEASES?

EDITED BY: Elisabeth Marelise W. Eekhoff, Teun J. De Vries, Wim Van Hul and Ralph Sakkers

PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88974-987-4
DOI 10.3389/978-2-88974-987-4

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

INNOVATIVE MODELS IN BONE BIOLOGY: WHAT CAN BE LEARNED FROM RARE BONE DISEASES?

Topic Editors:
Elisabeth Marelise W. Eekhoff, VU Medical Center, Netherlands
Teun J. De Vries, Academic Centre for Dentistry Amsterdam, VU Amsterdam, Netherlands
Wim Van Hul, University of Antwerp, Belgium
Ralph Sakkers, University Medical Center Utrecht, Netherlands

Citation: Eekhoff, E. M. W., De Vries, T. J., Van Hul, W., Sakkers, R., eds. (2022). Innovative Models in Bone Biology: What can be Learned From Rare Bone Diseases? Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-987-4

Table of Contents

04 ***Editorial: Innovative Models in Bone Biology: What can be Learned From Rare Bone Diseases?***
Teun J. de Vries, Wim Van Hul and E. Marelise Eekhoff

07 ***LRP5, Bone Density, and Mechanical Stress: A Case Report and Literature Review***
Nicholas G. Norwitz, Adrian Soto Mota, Madhusmita Misra and Kathryn E. Ackerman

14 ***Chordin-Like 1 Improves Osteogenesis of Bone Marrow Mesenchymal Stem Cells Through Enhancing BMP4-SMAD Pathway***
Tao Liu, Bo Li, Xin-Feng Zheng, Sheng-Dan Jiang, Ze-Zhu Zhou, Wen-Ning Xu, Huo-Liang Zheng, Chuan-Dong Wang, Xiao-Ling Zhang and Lei-Sheng Jiang

28 ***Fibrodysplasia Ossificans Progressiva (FOP): A Segmental Progeroid Syndrome***
Robert J. Pignolo, Haitao Wang and Frederick S. Kaplan

36 ***High Fidelity of Mouse Models Mimicking Human Genetic Skeletal Disorders***
Robert Brommage and Claes Ohlsson

46 ***WNT Signaling and Bone: Lessons From Skeletal Dysplasias and Disorders***
Yentl Huybrechts, Geert Mortier, Eveline Boudin and Wim Van Hul

64 ***Human Fibroblasts as a Model for the Study of Bone Disorders***
Lauria Claeys, Nathalie Bravenboer, Elisabeth M. W. Eekhoff and Dimitra Micha

71 ***The Osteocyte as the New Discovery of Therapeutic Options in Rare Bone Diseases***
Janak L. Pathak, Nathalie Bravenboer and Jenneke Klein-Nulend

85 ***Activin-A Induces Fewer, but Larger Osteoclasts From Monocytes in Both Healthy Controls and Fibrodysplasia Ossificans Progressiva Patients***
Ton Schoenmaker, Esmée Botman, Merve Sariyildiz, Dimitra Micha, Coen Netelenbos, Nathalie Bravenboer, Angele Kelder, E. Marelise W. Eekhoff and Teun J. De Vries

97 ***Zebrafish: A Resourceful Vertebrate Model to Investigate Skeletal Disorders***
Francesca Tonelli, Jan Willem Bek, Roberta Besio, Adelbert De Clercq, Laura Leoni, Phil Salmon, Paul J. Coucke, Andy Willaert and Antonella Forlino

125 ***Massively Parallel Sequencing for Rare Genetic Disorders: Potential and Pitfalls***
Aideen M. McInerney-Leo and Emma L. Duncan

frontiers in Endocrinology

EDITORIAL
published: 31 March 2022
doi: 10.3389/fendo.2022.892799

Editorial: Innovative Models in Bone Biology: What can be Learned From Rare Bone Diseases?

Teun J. de Vries[1], *Wim Van Hul*[2] *and E. Marelise Eekhoff*[3]*

[1] Department of Periodontology, Academic Centre for Dentistry Amsterdam, University of Amsterdam and Vrije Universiteit, Amsterdam, Netherlands, [2] Center of Medical Genetics, Antwerp University Hospital, University of Antwerp, Antwerp, Belgium, [3] Department of Internal Medicine, Section Endocrinology, Amsterdam University Medical Center (Amsterdam UMC), Amsterdam Bone Center, Vrije Universiteit Amsterdam, Amsterdam, Netherlands

Keywords: cell models, rare bone diseases, animal models, fibrodysplasia ossificans progressiva (FOP), fibroblast, osteocytes, osteoclasts, genetics

Editorial on the Research Topic

Innovative Models in Bone Biology: What can be Learned from Rare Bone Diseases?

OPEN ACCESS

Edited and reviewed by:
Jonathan H. Tobias,
University of Bristol, United Kingdom

***Correspondence:**
E. Marelise Eekhoff
emw.eekhoff@amsterdamumc.nl

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *09 March 2022*
Accepted: *10 March 2022*
Published: *31 March 2022*

Citation:
de Vries TJ, Van Hul W and Eekhoff EM (2022) Editorial: Innovative Models in Bone Biology: What can be Learned From Rare Bone Diseases? Front. Endocrinol. 13:892799. doi: 10.3389/fendo.2022.892799

INTRODUCTION

Since the elucidation of the human genome in 2000, all human genes are known. Subsequently, medical science has bloomed in identifying disease-specific causative mutations. For rare bone diseases, pivotal discoveries of causal genes were for instance the *SOST* gene encoding sclerostin for Van Buchem disease and sclerosteosis (1) or *ACVR1* for fibrodysplasia ossificans progressiva (2). For broadening mechanistic insight, such discoveries require animal- and cell-based models, for instance mouse models with knocked-out or induced expression of the mutated gene (3) or induced pluripotent stem cells (4). Such disease-tailored models were at the forefront of mechanistical discoveries and can lead to therapeutical intervention in the near future (5). The current topic with its 10 contributions, hopes to contribute to the new and still remaining challenges in the field of rare bone diseases by identifying current models or by refining suitable and innovative models.

NEW ANIMAL MODELS IN RARE BONE DISEASE RESEARCH

Knock-out mice have been available since the early 1990s, soon followed-up by inducible knock-out mice. These developments have turned out to be valuable for elucidating mechanisms in common bone diseases such as periodontitis (6). Brommage and Ohlson have summarized the state-of-the art of mouse models in bone research and their utility for the human equivalent. An impressive 96% (249 out of 260) of genes that were studied in mice, mimicked a known human variant with skeletal

anomalies. In the past decade, zebrafish models have come to the forefront as new models for studying rare bone diseases. Tonelli et al. introduce us to the bone biology of zebrafish and demonstrate that this model is relatively easy for manipulating genes, for instance using CRISPR-Cas9 technology, that can be relevant for rare bone diseases.

CELL-BASED MODELS FROM PATIENTS WITH RARE BONE DISEASES

To gain mechanistical insight, knowledge of the causative cell type in rare bone diseases should be the starting point for *in vitro* studies. Appropriate cell models to study rare bone diseases could be challenging, but the most appropriate model seems bone cells that are isolated from biopsies from patients. Thus, one could consider *ex vivo* material of bone chips with viable osteocytes still present (Pathak et al.). Osteocytes produce a variety of proteins and signaling molecules such as sclerostin, cathepsin K, Wnts, DKK1, DMP1, IGF1, and RANKL/OPG to regulate osteoblast and osteoclast activity. Various genetic abnormality-associated rare bone diseases are related to disrupted osteocyte functions is the case in Van Buchem's disease and sclerosteosis, which are related to non-functional sclerostin. Pathak et al. suggest that future research in rare bone diseases could also aim at restoring function of osteocytes. Fibrodysplasia ossificans progressiva is a rare bone disease where bone biopsy-related bone cells cannot be obtained since this could lead to worsening of the disease. Useful alternatives to study osteogenesic aspects, are skin (7) or periodontal ligament fibroblasts, scraped and isolated from extracted teeth (8). Claeys et al. describe the state-of-the art of fibroblast models in bone research. The osteoclast has been entirely neglected in FOP research, a disease with more bone. Schoenmaker et al. have used monocytes isolated from peripheral blood from controls and FOP patients to study the effect of FOP ligand and bone morphogenetic protein (BMP) Activin-A on osteoclast formation. Although no disease specific effect was observed, interestingly, this ligand caused fewer but larger osteoclasts. Therefore, studies aimed at elucidating rare bone disease mechanisms, may also contribute to more fundamental knowledge on the formation of multinucleated cells. Bone marrow derived mesenchymal stem cells (BMSCs) is yet another example of an appropriate cell model for osteogenesis that could be used in rare bone diseases. By manipulating its expression in BMSCs, Liu et al. show an important role for Chordin-like1 in increasing BMP4 driven osteogenesis. In a series of complementary experiments, the relationship between Chordin-like1 and BMP4 was established, culminating in experiments with bone defects and positive effects of Chordin-like1 on bone healing. Mild phenotypes of rare bone diseases may manifest later in life. Norwitz et al. describe a case of a novel LRP5 mutation in a professional runner, who turned out to be osteoporotic at the age of 18. Here, genetics overrules the bone dogma that impact loading improves bone quality. Huybrechts et al. update the current knowledge of Wnt signaling and rare bone disease. The overview of the skeletal and extra-skeletal phenotypes of the different monogenic skeletal disorders were linked to deviations in the WNT signaling pathway.

NEW PERSPECTIVES

Our era has gradually unveiled mysteries of many rare bone diseases by identifying genes, ligands, and pathways that are causative. Nevertheless, despite this tremendous progress, one could also step back and take the liberty to place an old disease into a new framework. Pignolo et al. have done this for FOP, by comparing clinical symptoms that coincide between progeria, or expedited aging, and FOP. Progeroid features that may primarily be associated with mutations in *ACVR1* include osteoarthritis, hearing loss, alopecia, subcutaneous lipodystrophy, myelination defects, heightened inflammation, menstrual abnormalities, and perhaps nephrolithiasis.

For finding the genetical cause of rare bone diseases, technological innovations in the field of sequencing, such as massively parallel sequencing (MPS), have broad potential applications. MacInernery-Leo and Duncan describe the historical development of finding causative mutations and demonstrate that MPS has high potential for future findings of new genetic insight in rare bone diseases. This technique speeds up discovery of causative genes from years to weeks.

CONCLUSION

The 10 contributions to this topic on innovative models for rare bone diseases have demonstrated the progress of rare bone disease models in research. For future research, a lot can be expected from CRISPR-Cas9 restored or induced gene function, in combination with induced pluripotent stem cells, since this could build reliable and clean read-out models, where only the mutation is induced or restored. Technological advances in speed of sequencing will faster and more accurately than ever identify novel mutations. Together with our increased biological understanding of the various rare bone diseases, it can be anticipated that clinicians will have more comprehensive guidelines for intervention for the benefit of the patient. In this way, it can be foreseen that quality of life will increase of patients with rare bone diseases.

AUTHOR CONTRIBUTIONS

TV initiated writing, WH and EE contributed to editing the draft text. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Claire E. Carlin, Giacomina Brunetti and Katherine A. Staines, editors of Frontiers in Endocrinology, for managing three manuscripts that had a conflict of interest with the editors of this topic.

REFERENCES

1. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, et al. Increased Bone Density in Sclerosteosis is Due to the Deficiency of a Novel Secreted Protein (SOST). *Hum Mol Genet* (2001) 10(5):537–43. doi: 10.1093/hmg/10.5.537
2. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, et al. A Recurrent Mutation in the BMP Type I Receptor ACVR1 Causes Inherited and Sporadic Fibrodysplasia Ossificans Progressiva. *Nat Genet* (2006) 5):525–7. doi: 10.1038/ng1783
3. Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, et al. ACVR1R206H Receptor Mutation Causes Fibrodysplasia Ossificans Progressiva by Imparting Responsiveness to Activin A. *Sci Transl Med* (2015) 7(303):303ra137. doi: 10.1126/scitranslmed.aac4358
4. Hino K, Ikeya M, Horigome K, Matsumoto Y, Ebise H, Nishio M, et al. Neofunction of ACVR1 in Fibrodysplasia Ossificans Progressiva. *Proc Natl Acad Sci USA* (2015) 112(50):15438–43. doi: 10.1073/pnas.1510540112
5. de Ruiter RD, Smilde BJ, Pals G, Bravenboer N, Knaus P, Schoenmaker T, et al. Fibrodysplasia Ossificans Progressiva: What Have We Achieved and Where Are We Now? Follow-up to the 2015 Lorentz Workshop. *Front Endocrinol.* (2017) 12:732728. doi: 10.3389/fendo.2021.732728
6. de Vries TJ, Andreotta S, Loos BG, Nicu EA. Genes Critical for Developing Periodontitis: Lessons From Mouse Models. *Front Immunol* (2017) 8:1395. doi: 10.3389/fimmu.2017.01395
7. Micha D, Voermans E, Eekhoff MEW, van Essen HW, Zandieh-Doulabi B, Netelenbos C, et al. Inhibition of Tgfβ Signaling Decreases Osteogenic Differentiation of Fibrodysplasia Ossificans Progressiva Fibroblasts in a Novel In Vitro Model of the Disease. *Bone* (2016) 84:169–80. doi: 10.1016/j.bone.2016.01.004
8. de Vries TJ, Schoenmaker T, Micha D, Hogervorst J, Bouskla S, Forouzanfar T, et al. Periodontal Ligament Fibroblasts as a Cell Model to Study Osteogenesis and Osteoclastogenesis in Fibrodysplasia Ossificans Progressiva. *Bone* (2018) 109:168–77. doi: 10.1016/j.bone.2017.07.007

Conflict of Interest: 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

CASE REPORT
published: 26 March 2019
doi: 10.3389/fendo.2019.00184

LRP5, Bone Density, and Mechanical Stress: A Case Report and Literature Review

Nicholas G. Norwitz[1,2]*, *Adrian Soto Mota*[1], *Madhusmita Misra*[2,3,4] and *Kathryn E. Ackerman*[2,4,5]

[1] Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom, [2] Harvard Medical School, Boston, MA, United States, [3] Division of Pediatric Endocrinology, Massachusetts General Hospital, Boston, MA, United States, [4] Neuroendocrine Unit, Massachusetts General Hospital, Boston, MA, United States, [5] Divisions of Sports Medicine and Endocrinology, Boston Children's Hospital, Boston, MA, United States

The Wnt-β-catenin pathway receptor, low-density lipoprotein receptor-related protein 5 (LRP5), is a known regulator of bone mineral density. It has been hypothesized that specific human polymorphisms in *LRP5* impact bone density, in part, by altering the anabolic response of bone to mechanical loading. Although experiments in animal models support this hypothesis, there is limited evidence that *LRP5* polymorphisms can alter the anabolic response of bone to mechanical loading in humans. Herein, we report a young male who harbors a rare *LRP5* missense mutation (A745V) and who provides potential proof of principle for this mechanotransduction hypothesis for low bone density. The subject had no history of fractures until age 18, a year into a career in competitive distance running. As he continued to run over the following 2 years, his mileage threshold to fracture steadily and rapidly decreased until he was diagnosed with severe osteoporosis (lumbar spine BMD Z-score of −3.2). By contextualizing this case within the existing *LRP5* and mechanical stress literature, we speculate that this represents the first documented case of an individual in whom a genetic mutation altered the anabolic response of bone to mechanical stress in a manner sufficient to contribute to osteoporosis.

Keywords: bone mineral density, LRP5, mechanical stress, osteoporosis, Wnt-β-catenin signaling

OPEN ACCESS

Edited by:
Teun J. De Vries, VU University Amsterdam, Netherlands

Reviewed by:
Natasha Appelman-Dijkstra, Leiden University Medical Center, Netherlands
Michaël R. Laurent, University Hospitals Leuven, Belgium

***Correspondence:**
Nicholas G. Norwitz
nicholas.norwitz@dpag.ox.ac.uk

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *04 February 2019*
Accepted: *06 March 2019*
Published: *26 March 2019*

Citation:
Norwitz NG, Mota AS, Misra M and Ackerman KE (2019) LRP5, Bone Density, and Mechanical Stress: A Case Report and Literature Review. Front. Endocrinol. 10:184. doi: 10.3389/fendo.2019.00184

BACKGROUND

Low-density lipoprotein receptor-related protein 5 (LRP5) is a 1,615 amino acid transmembrane receptor for the conserved Wnt-β-catenin signaling pathway, a pathway known to regulate bone metabolism in humans. In canonical Wnt-β-catenin signaling, a Wnt ligand binds to a binding site created by the 1st and 3rd β-propeller domains of LRP5 and to its co-receptor, Frizzled. This enables LRP5 to sequester a cytoplasmic destruction complex and, thereby, prevent the degradation of the protein β-catenin. Subsequently, β-catenin translocates into the nucleus, where it interacts with TCF/LEF family transcription factors and alters gene expression to promote bone formation (1) (**Figure 1A**). Genome-wide association studies (GWAS) have repeatedly classified *LRP5* as a key mediator of bone mineral density (BMD) (2–4), including the largest GWAS to date, which identified *LRP5* as a BMD and fracture risk locus at a significance level of $p < 1.0 \times 10^{-21}$ (5).

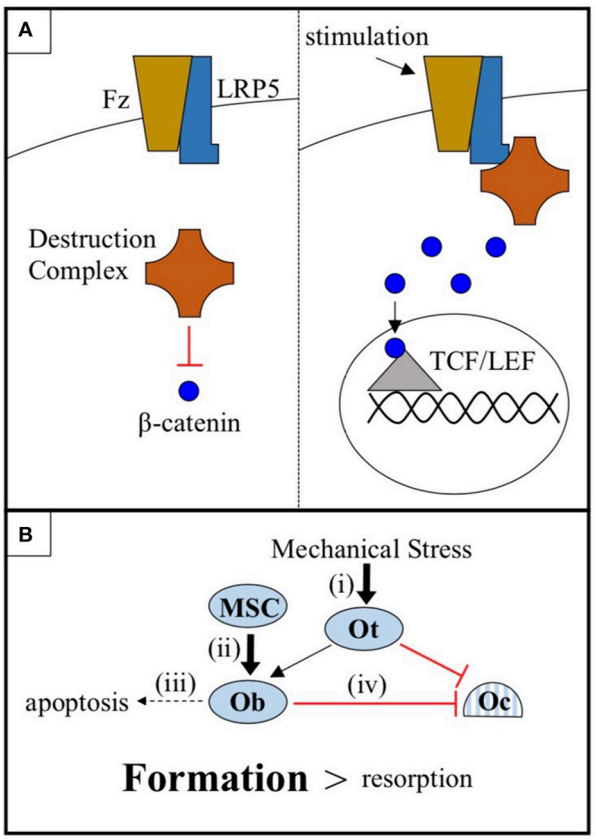


FIGURE 1 | (A) The cytoplasmic destruction complex constitutively targets β-catenin for degradation. Mechanical or chemical stimulation of the Wnt-β-catenin pathway receptor pair LRP5-Frizzled (Fz), in cells of the osteoblast linage, causes LRP5 to sequester the destruction complex, allowing β-catenin to accumulate and translocate to the nucleus where it interacts with TCF/LEF family transcription factors and promotes osteogenic gene expression. **(B)** Osteocytes (Ot) sense mechanical stress and respond by increasing Wnt-β-catenin signaling and coordinating the anabolic activities of osteoblasts (Ob) and the catabolic activities of osteoclasts (Oc). Wnt-β-catenin signaling, in addition to (i) sensitizing osteocytes to mechanical stress, (ii) promotes the differentiation of mesenchymal stem cells (MSC) into osteoblasts, (iii) prevents osteoblast apoptosis, and (iv) increases osteoprotegerin expression by osteoblasts, thus inhibiting osteoclast-mediated bone resorption. The net effect is a shift in favor of bone formation over bone resorption.

LRP5 mutations are known to cause disorders of both low and high BMD. Recessive loss-of-function mutations in *LRP5* cause osteoporosis-pseudoglioma syndrome (OPPG), a condition characterized by severe osteoporosis and occasional ocular abnormalities (1, 6), whereas gain-of-function mutations in *LRP5* are associated with abnormally high BMD (7). Furthermore, *LRP5* demonstrates haploinsufficiency (6, 8–11). In fact, dominant loss-of-function mutations in *LRP5* are among the most common causes of familial exudative vitreoretinopathy (FEVR), a congenital eye defect that often presents with a comorbid low BMD phenotype (10, 11). Of note, *LRP5* haploinsufficiency appears to affect BMD in men more severely than in women (12–15). In addition to GWAS and clinical associations, *LPR5* heterozygous ($LPR5^{+/-}$) mouse models reliably exhibit low BMD (16–18). Consistent with data from human studies, the loss-of-function phenotype is more severe in male mice than in female mice, with male mice exhibiting lower relative BMDs, shortened femurs during their youth, and a reduced osteogenic response to mechanical stress (17).

There are a number of mechanisms by which LRP5-mediated Wnt-β-catenin signaling in cells of the osteoblast lineage may promote bone growth. These include (i) sensitizing osteocytes to mechanical stress, (ii) promoting the differentiation of mesenchymal stem cells (MSC) into osteoblasts, (iii) preventing osteoblast apoptosis, and (iv) increasing osteoblast expression of osteoprotegerin to decrease osteoclastogenesis (19–24) (**Figure 1B**). While each of these mechanisms likely plays a part in mediating the regulatory effects of the LRP5 protein on BMD, the mechanical stress model (i) is the focus of this report. There is an abundance of mouse data to support this model. First, *LRP5* gain-of-function mutations in mice do not appear to increase basal rates of bone formation in the absence of mechanical stimulation, but more than double bone formation in response to mechanical stress (25, 26). Second, *LRP5* gain-of-function enhances the expression of bone formation genes in response to mechanical stress (24). Third, conditional knockout of *LRP5* in murine osteocytes, cells which are believed to serve as the mechanosensors of bone, diminishes the osteogenic response to mechanical stress, whereas activation of Wnt-β-catenin signaling in osteocytes is sufficient to increase the osteogenic response (27–29). Thus, data from mice support a model in which the LRP5 receptor influences BMD, at least in part, by regulating mechanotransduction.

Three clinical observations of patients bearing *LRP5* mutations also support the mechanical stress model. First, *LRP5* mutations do not appear to affect calcium homeostasis, anabolic or catabolic hormones, collagen synthesis, or basal levels of bone turnover, even in patients with severe osteoporosis (9, 13). Second, *LRP5* gain-of-function mutations can increase BMD without affecting bone shape or causing bony lesions, which are observed in genetic conditions that simply increase basal osteoblast activity or decrease basal osteoclast activity (30). Third, *LRP5* gain-of-function mutations cause the greatest enhancement of BMD in load bearing bones (30).

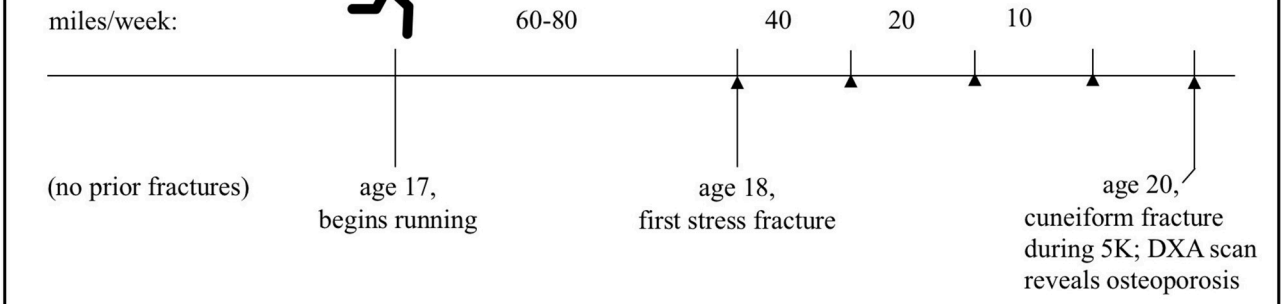
Two population-based studies add yet another level of support to the mechanical stress model. In a subset of 868 men from the Framingham Offspring Study Cohort, a polymorphism in exon 10 of *LRP5* appeared to negatively affect the interaction between physical activity and BMD. Specifically, men homozygous for the common allele exhibited a positive correlation between physical activity and BMD; heterozygous men exhibited no correlation; and men homozygous for the less common allele exhibited a negative correlation between physical activity and BMD (31). Similar data were reported from the Odense Androgen Study. In this study of 783 men aged 20–30, the *LRP5* polymorphisms A1330V and V667M were associated with low BMD in physically active men, but not in sedentary men (32). Although these two independent studies each suggest that polymorphisms in *LRP5* can alter the anabolic response of bone to mechanical stress in men, they were limited by the fact that they assessed physical activity using questionnaires.

We report a 23-year-old male ex-distance runner who presented with primary osteoporosis and a rare *LRP5* variant, A745V in exon 10, at age 20. His mutation, medical history, and athletic history complement and build upon the mouse models, clinical observations, and epidemiological data introduced above. In brief, this case represents potential proof of principle for the mechanical stress model and suggests the possibility that *LRP5* mutations contribute to low BMD, in part, by blunting the anabolic response of bone to mechanical stress.

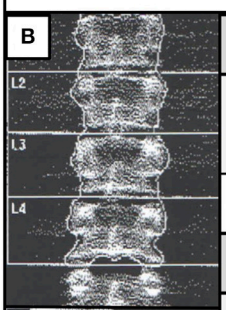
CASE REPORT

The Caucasian male subject was the product of an uncomplicated pregnancy, although he did exhibit shortened femurs *in utero*, similar to *LRP5* loss-of-function male mice (17). He demonstrated no signs of any chronic health condition during his highly active youth or adolescence, during which he engaged in a variety of sports, including basketball, soccer, rugby, and martial arts. He began competitive distance running at age 17. For over 1 year, he consistently ran between 60 and 80 miles per week without sustaining any bone injuries. At age 18, he sustained a stress fracture in his right lateral tibial plateau. Subsequent to this initial stress fracture, he began to experience stress fractures at progressively lower mileage thresholds. After fracture resolution, physical therapy, and a gradual return to running, he sustained further tibial, femoral, and sacral alar stress fractures when running 40, 20, and even 10 miles per week, consistent with the notion that his bones were weakening as he continued to run (**Figure 2A**).

A



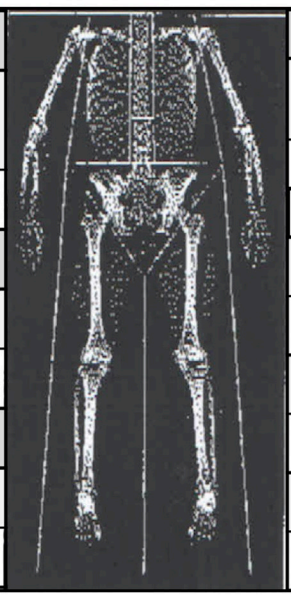
B



BMD of Lumbar Spine		
g/cm^2	T-score	Z-score
0.742	-3.20	**-3.20**

BMD of Total Hip		
g/cm^2	T-score	Z-score
0.735	-2.00	**-2.00**

BMD of Femoral Neck		
g/cm^2	T-score	Z-score
0.628	-2.20	**-2.20**



BMD of Total Body (Minus Head)		
BMD (g/cm^2)	T-score	Z-score
1.046	-1.60	**-1.50**

C Endocrine Assessment

Testosterone ng/dL	**TSH** µIU/mL	**24-hr Cortisol** µg
591 (250-1100)	0.99 (0.27-4.20)	34 (3.5-45)
PTH pg/mL	**Alk Phos** U/L	**NTX/creatinine** ratio
15 (10-65)	83 (45-115)	49 (21-66)
Calcium mg/dL	**25-OH-Vit D** ng/mL	**BMI** kg/m^2
9.7 (8.5-10.5)	48 (30-100)	21.1 (>17.5)

FIGURE 2 | (A) Diagram of the subject's running and stress fracture history. The subject had no history of fractures during his childhood and began distance running at age 17. He successfully ran 60–80 miles per week for over 1 year before experiencing his first fracture in his right lateral tibial plateau. Over the subsequent years, as he continued to run, his mileage threshold to fracture decreased precipitously (stress fractures are represented by arrowheads). At age 20, he fractured his right cuneiform during a 5-kilometer road race. A follow-up of the unusual foot fracture revealed osteoporosis. **(B)** DXA scan of the subject's lumbar spine, total hip, femoral neck, and total body (minus head) at time of diagnosis. These data are consistent with the notion that the subject's load-bearing bones failed to adapt to the mechanical stress of running. **(C)** Endocrine assessment at time of diagnosis. Reference ranges are given in parentheses and BMI >17.5 kg/m^2 is used because this threshold is a surrogate marker for Relative Energy Deficiency in Sport (RED-S) in men (33, 34).

At age 20, he sustained a complete fracture of his right cuneiform during a 5-kilometer run. A dual-energy X-ray absorptiometry (DXA) scan was performed given this history of recurrent fractures and this revealed a lumbar spine BMD Z-score of −3.2, total hip BMD Z-score of −2.0, femoral neck BMD Z-score of −2.2, and total body (minus head) BMD Z-score of −1.5 (**Figure 2B**). At the time of diagnosis, the subject had a normal BMI (21.1 kg/m^2), normal resting metabolic rate (1,613 kcal/day, measured by respirometry vs. 1,604 kcal/day, calculated using the Harris-Benedict equation), normal testosterone, TSH, 24-h urine free cortisol, PTH, alkaline phosphatase, urinary N-terminal telopeptide/creatinine, calcium, and 25-OH-Vitamin D (**Figure 2C**). All other electrolytes, hormones, and kidney and liver function tests were unremarkable, and the subject, now 23, has exhibited no meaningful signs of endocrine dysfunction in the years since initial evaluation.

A genetic screen revealed an undocumented paternally-inherited polymorphism (A745V) in the *LRP5* gene. His father, a 54-year-old with a BMI of 37.2 kg/m^2, did not exhibit low BMD at the lumbar spine, total hip, or femoral neck (T-scores of 0.0, 0.9, and 0.1, respectively); however, the father did exhibit a radial BMD T-score of −2.6 (age-adjusted Z-score of −2.0). The subject completed a 13-month course of teriparatide, which increased his lumbar spine BMD Z-score from −3.2 to −2.7, and he is currently on denosumab.

The subject's only other health condition is ulcerative colitis, which was diagnosed at age 22, 4 years after his first fracture. The colitis is mild and localized to the cecum and sigmoid colon. As the subject never exhibited evidence of malabsorption/malnutrition or systemic inflammation (his high sensitivity CRP was consistently measured to be low both before and after his colitis diagnosis), was never on chronic glucocorticoids, and had absolutely no symptoms of this condition at the time that he was having the fractures, it is unlikely that his ulcerative colitis contributed to his low BMD. This opinion was unanimously shared by three independent gastroenterological consults.

DISCUSSION

The properties of the A745V variant suggest that it likely contributed to the subject's osteoporosis. A745V is extremely rare, with a minor allele frequency of 0.0008 in the Genome Aggregation Database (254/282476 alleles; 0 homozygotes), and is perfectly conserved among mammals, birds, snakes, fish, and even the Drosophila homolog of *LRP5*. It is located within the Wnt-ligand-binding 3rd β-propeller domain, adjacent to two other residues (N740, from the Framingham Study, and W734) mutations in which are also associated with low BMD in humans (6, 31) (**Figure 3**). Other alanine to valine missense mutations in *LRP5* have been reported to contribute to low BMD. The A745V variant was predicted to be consequential in *in silico* models and was reported to contribute to a case of FEVR, which is often associated with low BMD (3, 32, 35) (**Figure 4**).

Despite the evidence supporting the consequence of the A745V variant mentioned in the previous paragraph, the proposition that this inherited genetic mutation was a major contributor to the subject's osteoporosis raises two important questions: (1) Why is there a discrepancy between the subject's BMD and that of his father? (2) If the subject's low BMD

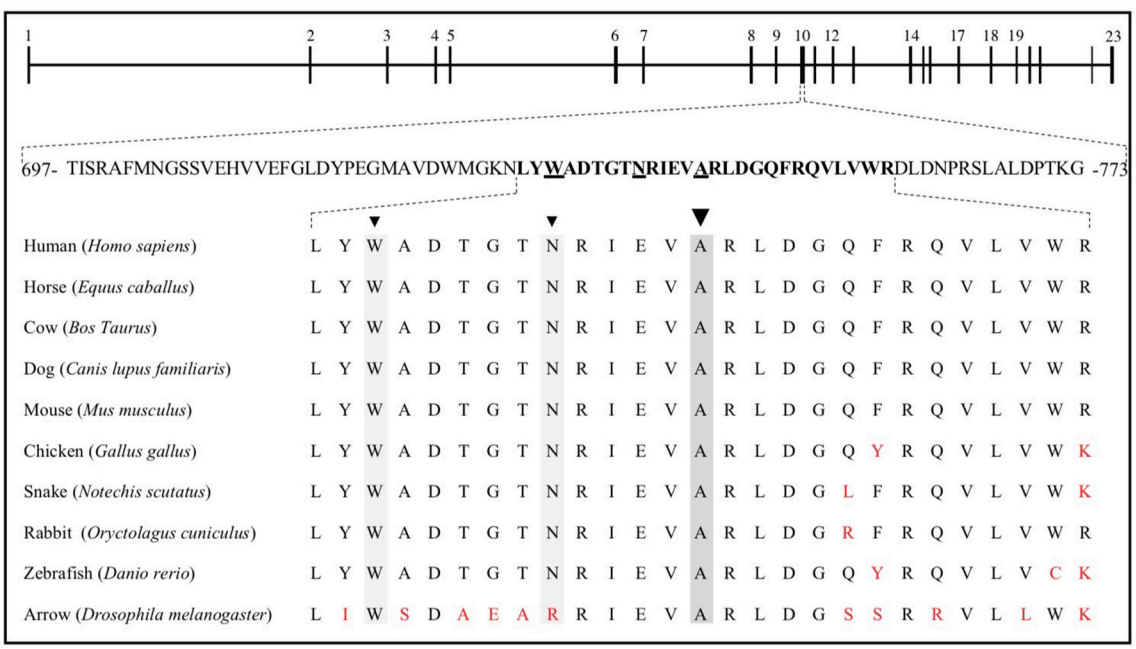


FIGURE 3 | The *LRP5* gene is composed of 23 exons, coding for 1,615 amino acids. Exon 10 includes residues 697 to 773, 27 of which are sequence aligned with the corresponding horse, cow, dog, mouse, chicken, snake, rabbit, and zebrafish *LRP5* sequences, as well as with that of the homologous protein in Drosophila, arrow. W734 (6), N740 (31), and A745 are underlined and identified by arrowheads. Red letters represent nonconserved residues.

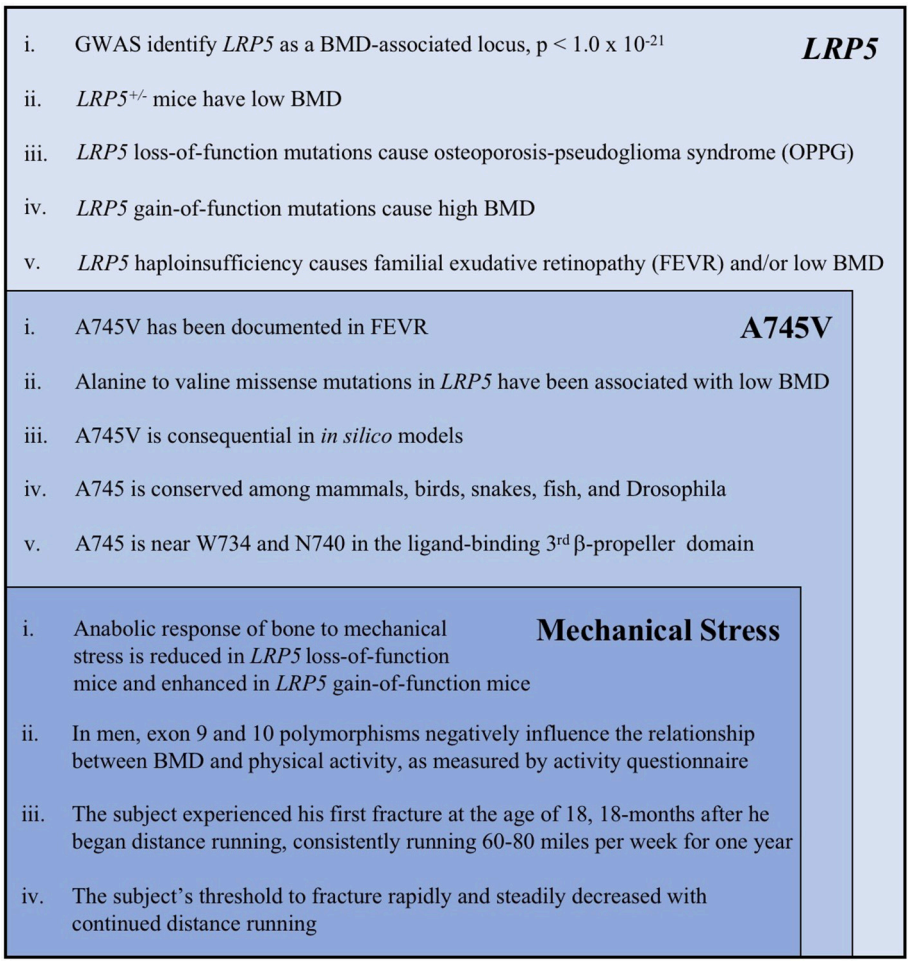


FIGURE 4 | A summary of the key evidence supporting the role of *LRP5*, the A745V variant, and mechanical stress in the etiology of the subject's osteoporosis. Each category of evidence builds upon, and is inset within, the previous category. The following references correspond to each line of evidence: ***LRP5*** (i). Trajanoska et al. (5) (ii). Sawakami et al. (17), Clement-Lacroix et al. (16), Yadav et al. (18) (iii). Gong et al. (6), Joiner et al. (1) (iv). Johnson et al. (30), Johnson (7) (v). Toomes et al. (11), Qin et al. (10). **A745V** (i). Pefkianaki et al. (35) (ii). Brixen et al. (32), Estrada et al. (3) (iii). Pefkianaki et al. (35) (iv). NCBI sequence analyzer and alignment tools were used to assess conservation (v). Gong et al. (6), Joiner et al. (1). **Mechanical Stress** (i). Sawakami et al. (17), Zhao et al. (29), Robinson et al. (24), Johnson (7), Niziolek et al. (25) and others (see text) (ii). Brixen et al. (32), Kiel et al. (31) (iii, iv). Information from the subject's medical history.

is attributable to a congenital genetic defect, why did it only manifest with fractures over 1 year into his running career when he was a young adult? The discrepancy between the father's and son's BMDs may be explained, in part, by the variable expressivity observed repeatedly with *LRP5* mutations. In the first report of this A745V variant, the carrier father exhibited only subclinical symptoms (35); and probands heterozygous for inherited *LRP5* mutations often exhibit BMDs significantly lower than those of their carrier parents (8, 9). It is also possible that the father's higher BMI (37.2 kg/m^2) was somewhat protective for his BMD, or that it artifactually increased his BMD. Adipose tissue can inflate DXA measurements of BMD, particularly at the spine, hip, and femur, where overestimates can approach 30% (36). By contrast, radial DXA cannot be easily confounded by soft tissue, suggesting that the radius may be a more accurate BMD measurement site for heavier individuals (37). Therefore, the father's radial BMD T-score of −2.6 (age-adjusted Z-score of −2.0) may reflect the pathogenicity of the A745V allele. Finally, and most interestingly, we speculate that the subject's running interacted with his genetics to precipitate his osteoporosis.

The proposition that the A745V polymorphism altered the anabolic response of the subject's bones to mechanical stress not only provides a potential explanation for why his phenotype is more severe than that of his father, but can also explain the peculiar chronology of his fracture history (**Figure 2A**). If the subject's bones were not able to adapt appropriately to the mechanical stress imposed by distance running, one would expect that he would only begin to experience fractures after a sustained period of habitual distance running, as was indeed the case in our patient. In addition, one would predict that continued distance running would continue to weaken his bones

and increase his susceptibility to fracture, as it did. Interestingly, the subject's DXA scan revealed that his lumbar spine, total hip, and femoral neck BMD Z-scores, all of which represent load-bearing sites, were notably lower than his total body (minus head) BMD Z-score (**Figure 2B**). This observation mirrors the observation that *LRP5* gain-of-function kindred exhibit the greatest increases in BMD at load-bearing sites (30). Notably, the subject's phenotype was more severe at the lumbar spine than at the hip and femur. Counterintuitively, this is also what the mechanotransduction model predicts. Although the spine, hip, and femur are all load-bearing sites, *LRP5* polymorphisms have been reported to alter mechanotransduction in trabecular bone more so than in cortical bone, and the spine has the highest proportion of trabecular bone of these sites (26). The mechanical stress response model is further consistent with data from *LRP5* mouse models, which collectively show that *LRP5* gain-of-function increases bone formation specifically in response to mechanical stress and that *LRP5* loss-of-function reduces the response of bone to mechanical stress in a dose-dependent manner (17, 24, 25, 29). Furthermore, results of the Framingham Cohort and Odense Androgen Studies suggest that *LRP5* polymorphisms can affect the interaction between physical activity and BMD in men, such that men carrying particular polymorphisms do not appear experience the increases in BMD usually associated with weight-bearing activities. Notably, the physical activity data from these studies were limited to self-report questionnaires (31, 32) (**Figure 4**).

Our report has certain limitations, chief among these being that the subject had no DXA scans available for comparison before his first tibial stress fracture or during his running career. Therefore, we cannot rule out the possibility that the *LRP5* mutation substantially impacted his BMD before the start of his running career, or confirm that his BMD decreased with continued running (as suggested by his decreasing mileage threshold to fracture). We also could not assess the degree to which other factors, such as the subject's ulcerative colitis or nutritional status, may have independently, or by interacting with the A745V variant, contributed to the subject's low BMD. In fact, at the time of diagnosis, when the A745V variant was undocumented and its significance unrecognized, Relative Energy Deficiency in Sport (RED-S) was proposed as a diagnosis of exclusion (33, 34). While the subject's normal BMI (19–22 kg/m^2) and endocrine assessments made this a less likely diagnosis, it remains possible that insufficient nutritional intake during the high-mileage period of his running career contributed to some extent to his low BMD. Nevertheless, the fact that this young man has osteoporosis, harbors a rare mutation in a gene that is known to modify the response of bone to mechanical stress in animal models (perhaps in a sex-specific manner), and underwent a discrete period of intense mechanical loading during which he became increasingly prone to fracture, suggests that the subject may represent the first documented case of a genetic mutation that contributes to osteoporosis, in part, by altering the anabolic response of bone to mechanical stress. Future work in needed to enhance our understanding of the genetic contributions of *LRP5* to mechanotransduction in bone.

ETHICS STATEMENT

Written informed consent was obtained from the participant for the publication of this case report and any potentially-identifying information/images.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by K24 HD071843 (grant to MM).

REFERENCES

1. Joiner DM, Ke J, Zhong Z, Xu HE, Williams BO. LRP5 and LRP6 in development and disease. *Trends Endocrinol Metab.* (2013) 24:31–9. doi: 10.1016/j.tem.2012.10.003
2. Brent Richards J, Zheng HF, Spector TD. Genetics of osteoporosis from genome-wide association studies: advances and challenges. *Nat Rev Genet.* (2012) 13:576–88. doi: 10.1038/nrg3228
3. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet.* (2012) 44:491–501. doi: 10.1038/ng.2249
4. Rivadeneira F, Styrkársdottir U, Estrada K, Halldórsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat Genet.* (2009) 41:1199–206. doi: 10.1038/ng.446
5. Trajanoska K, Morris JA, Oei L, Zheng H, Evans DM, Kiel DP, et al. Assessment of the genetic and clinical determinants of fracture risk : genome wide association and mendelian randomisation study. *Br Med J.* (2018) 362:1–14. doi: 10.1136/bmj.k3225
6. Gong Y, Slee R, Fukai N, Rawadi G, Roman-Roman S, Reginato A, et al. LDL Receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell.* (2001) 107:513–23.
7. Johnson ML. The high bone mass family - the role of Wnt/Lrp5 signaling in the regulation of bone mass. *J Musc Neuronal Inter.* (2004) 4:135–8.
8. Hartikka H, Mäkitie O, Männikkö M, Doria AS, Daneman A, Cole WG, et al. Heterozygous mutations in the LDL receptor-related protein 5 (LRP5) gene are associated with primary osteoporosis in children. *J Bone Min Res.* (2005) 20:783–9. doi: 10.1359/JBMR.050101
9. Korvala J, Jüppner H, Mäkitie O, Sochett E, Schnabel D, Mora S, et al. Mutations in LRP5 cause primary osteoporosis without features of OI by reducing Wnt signaling activity. *BMC Med Genet.* (2012) 13:26. doi: 10.1186/1471-2350-13-26
10. Qin M, Hayashi H, Oshima K, Tahira T, Hayashi K, Kondo H. Complexity of the genotype-phenotype correlation in familial exudative vitreoretinopathy with mutations im the LRP5 and/or FZD4 genes. *Hum Mut.* (2005) 26:104–12. doi: 10.1002/humu.20191
11. Toomes C, Bottomley HM, Jackson RM, Towns KV, Scott S, Mackey DA, et al. Mutations in LRP5 or FZD4 underlie the common familial exudative vitreoretinopathy locus on chromosome 11q. *Am J Hum Genet.* (2004) 74:721–30. doi: 10.1086/383202

12. Armstrong VJ, Muzylak M, Sunters A, Zaman G, Saxon LK, Price JS, et al. Wnt/β-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor α. *J Biol Chem.* (2007) 282:20715–27. doi: 10.1074/jbc.M703224200
13. Balemans W, Wim VH. Minireview: the genetics of low-density lipoprotein receptor-related protein 5 in bone: a story of extremes. *Endocrinology*. (2007) 148:2622–9. doi: 10.1210/en.2006-1352
14. Dubrow SA, Hruby PM, Akhter MP. Gender specific LRP5 influences on trabecular bone structure and strength. *J Musc Neuronal Inter.* (2007) 7:166–73.
15. Ferrari SL, Deutsch S, Choudhury U, Chevalley T, Bonjour J-P, Dermitzakis ET, et al. Polymorphisms in the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with variation in vertebral bone mass, vertebral bone size, and stature in whites. *Am J Hum Genet*. (2004) 74:866–75. doi: 10.1086/420771
16. Clement-Lacroix P, Ai M, Morvan F, Roman-Roman S, Vayssiere B, Belleville C, et al. Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci USA*. (2005) 102:17406–11. doi: 10.1073/pnas.0505259102
17. Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, et al. The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. *J Biol Chem.* (2006) 28:23698–711. doi: 10.1074/jbc.M601000200
18. Yadav VK, Ryu JH, Suda N, Tanaka KF, Gingrich JA, Schütz G, et al. Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell*. (2008) 135:825–37. doi: 10.1016/j.cell.2008.09.059
19. Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblast by β-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. *J Biol Chem.* (2005) 280:41342–51. doi: 10.1074/jbc.M502168200
20. Glass DA, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, et al. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell*. (2005) 8:751–64. doi: 10.1016/j.devcel.2005.02.017
21. Kolpakova E, Olsen B. Wnt/β-catenin—a canonical tale of cell-fate choice in the vertebrate skeleton. *Dev Cell.* (2005) 8:626–7. doi: 10.1016/j.devcel.2005.04.008
22. Manolagas SC, Almeida M. Gone with the Wnts: β-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism. *Mol Endocrinol.* (2007) 21:2605–14. doi: 10.1210/me.2007-0259
23. Papachroni KK, Karatzas DN, Papavassiliou KA, Basdra EK, Papavassiliou AG. Mechanotransduction in osteoblast regulation and bone disease. *Trends Mol Med.* (2009) 15:208–16. doi: 10.1016/j.molmed.2009.03.001
24. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, et al. Wnt/β-catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem.* (2006) 281:31720–8. doi: 10.1074/jbc.M602308200
25. Niziolek PJ, Warman ML, Robling AG. Mechanotransduction in bone tissue: The A214V and G171V mutations in Lrp5 enhance load-induced osteogenesis in a surface-selective manner. *Bone*. (2012) 51:459–65. doi: 10.1016/j.bone.2012.05.023
26. Saxon LK, Jackson BF, Sugiyama T, Lanyon LE, Price JS. Analysis of multiple bone responses to graded strains above functional levels, and to disuse, in mice *in vivo* show that the human Lrp5 G171V High Bone Mass mutation increases the osteogenic response to loading but that lack of Lrp5 activity reduces it. *Bone*. (2011) 49:184–93. doi: 10.1016/j.bone.2011.03.683
27. Javaheri B, Stern AR, Lara N, Dallas M, Zhao H, Liu Y, et al. Deletion of a single β-catenin allele in osteocytes abolishes the bone anabolic response to loading. *J Bone Min Res.* (2014) 29:705–15. doi: 10.1002/jbmr.2064
28. Tu X, Delgado-Calle J, Condon KW, Maycas M, Zhang H, Carlesso N, et al. Osteocytes mediate the anabolic actions of canonical Wnt/β-catenin signaling in bone. *Proc Natl Acad Sci USA*. (2015) 112:E478–E486. doi: 10.1073/pnas.1409857112
29. Zhao L, Shim JW, Dodge TR, Robling AG, Yokota H. Inactivation of Lrp5 in osteocytes reduces Young's modulus and responsiveness to the mechanical loading. *Bone*. (2013) 54:35–43. doi: 10.1016/j.bone.2013.01.033
30. Johnson M, Picconi B, Recker R. The gene for high bone mass. *Endocrinologist*. (2002) 12:445–53. doi: 10.1097/01.ten.0000029417.07585.81
31. Kiel DP, Ferrari SL, Cupples LA, Karasik D, Manen D, Imamovic A, et al. Genetic variation at the low density lipoprotein receptor-related protein 5 (LRP5) locus modulates Wnt signaling and the relationship of physical activity with bone mineral density in men. *Bone*. (2007) 40:587–96. doi: 10.1016/j.bone.2006.09.029
32. Brixen K, Beckers S, Peeters A, Piters E, Balemans W, Nielsen TL, et al. Polymorphisms in the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with peak bone mass in non-sedentary men: results from the Odense Androgen Study. *Calcif Tissue Int.* (2007) 81:421–9. doi: 10.1007/s00223-007-9088-z
33. Elliott-Sale K, Tenforde A, Parziale A, Holtzman B, Ackerman K. Endocrine effects of relative energy deficiency in sport. *Int J Sport Nutr Exerc Metab.* (2018) 28:335–49. doi: 10.1123/ijsnem.2018-0127
34. Mountjoy M, Sundgot-Borgen JK, Burke LM, Ackerman KE, Blauwet C, Constantini N, et al. IOC consensus statement on relative energy deficiency in sport (RED-S): 2018 update. *Br J Sports Med.* (2018) 52:687–97. doi: 10.1136/bjsports-2018-099193
35. Pefkianaki M, Hasanreisoglu M, Suchy SF, Shields CL. Familial exudative vitreoretinopathy with a novel LRP5 mutation. *J Pediatr Ophthalmol Strabism.* (2016) 53:39–42. doi: 10.3928/01913913-20160719-02
36. Bolotin HH. DXA *in vivo* BMD methodology: an erroneous and misleading research and clinical gauge of bone mineral status, bone fragility, and bone remodelling. *Bone*. (2007) 41:138–54. doi: 10.1016/j.bone.2007.02.022
37. Trivitayaratana W, Trivitayaratana P. The accuracy of bone mineral density at the distal radius on non-forearm osteoporosis identification. *J Med Assoc.* (2001) 84:566–71.

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 Endocrinology

ORIGINAL RESEARCH
published: 12 June 2019
doi: 10.3389/fendo.2019.00360

Chordin-Like 1 Improves Osteogenesis of Bone Marrow Mesenchymal Stem Cells Through Enhancing BMP4-SMAD Pathway

Tao Liu[1†], Bo Li[1†], Xin-Feng Zheng[1], Sheng-Dan Jiang[1], Ze-Zhu Zhou[1], Wen-Ning Xu[1], Huo-Liang Zheng[1], Chuan-Dong Wang[2], Xiao-Ling Zhang[2] and Lei-Sheng Jiang[1*]*

[1] Spine Center, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Department of Orthopedic Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China

OPEN ACCESS

Edited by:
Teun J. De Vries,
Vrije Universiteit (VU)
Amsterdam, Netherlands

Reviewed by:
Gonzalo Sanchez-Duffhues,
Leiden University Medical
Center, Netherlands
Dimitra Micha,
University Medical Center
Amsterdam, Netherlands

***Correspondence:**
Xiao-Ling Zhang
xlzhang@shsmu.edu.cn
Lei-Sheng Jiang
jiangleisheng@xinhuamed.com.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: 17 January 2019
Accepted: 21 May 2019
Published: 12 June 2019

Citation:
Liu T, Li B, Zheng X-F, Jiang S-D, Zhou Z-Z, Xu W-N, Zheng H-L, Wang C-D, Zhang X-L and Jiang L-S (2019) Chordin-Like 1 Improves Osteogenesis of Bone Marrow Mesenchymal Stem Cells Through Enhancing BMP4-SMAD Pathway. Front. Endocrinol. 10:360. doi: 10.3389/fendo.2019.00360

Chordin-like 1 (CHRDL1) is a secreted glycoprotein with repeated cysteine-rich domains, which can bind to BMPs family ligands. Although it has been reported to play important roles in several systems, the exact roles of CHRDL1 on human bone mesenchymal stem cells (hBMSCs) osteogenesis remain to be explored. The present study aimed to investigate the roles of CHRDL1 on the osteogenic differentiation of hBMSCs and the underlying molecular mechanisms. We found that CHRDL1 was upregulated during hBMSCs osteogenesis, and rhBMP-4 administration could enhance CHRDL1 mRNA expression in a dose and time dependent manner. Knockdown of CHRDL1 did not affect hBMSCs proliferation, but inhibited the BMP-4-dependent osteogenic differentiation, showing decreased mRNA expression levels of osteogenic markers and reduced mineralization. On the contrary, overexpression of CHRDL1 enhanced BMP-4 induced osteogenic differentiation of hBMSCs. Moreover, *in vivo* experiments by transplanting CHRDL1 gene modified hBMSCs into nude mice defective femur models displayed higher new bone formation in CHRDL1 overexpression groups, but lower new bone formation in CHRDL1 knockdown groups, compared with control groups. In consistent with the bone formation rate, there were increased CHRDL1 protein expression in new bone formation regions of defective femur in CHRDL1 overexpression groups, while reduced CHRDL1 protein expression in CHRDL1 knockdown groups compared with control groups. These indicate that CHRDL1 can promote osteoblast differentiation *in vivo.* Furthermore, the mechanisms study showed that CHRDL1 improved BMP-4 induced phosphorylation of SMAD1/5/9 during osteogenic differentiation of hBMSCs. Besides, promotion of osteogenic differentiation and the activation of SMAD phosphorylation by CHRDL1 can be blocked by BMP receptor type I inhibitor LDN-193189. In conclusion, our results suggested that CHRDL1 can promote hBMSCs osteogenic differentiation through enhancing the activation of BMP-4-SMAD1/5/9 pathway.

Keywords: CHRDL1, hBMSCs, BMP-4, SMAD, RUNX2, osteogenic differentiation

INTRODUCTION

Bone size and shape are precisely modeled and remodeled throughout life to ensure the structure and integrity of the skeleton (1). Bone remodeling is maintained by the regulation of two essential cell types, namely, the bone resorption osteoclasts and matrix-forming osteoblasts (2). Osteoporosis develops when the rate of osteoclastic bone resorption exceeds that of osteoblastic bone formation, which leads to loss of BMD and deterioration of bone structure and strength (3). Although osteoclast suppression machineries have been the focus of many bone studies, osteogenesis of BMSCs and its underlying mechanisms are also essential issues of bone remodeling (4).

Bone formation is mediated by osteoblasts recruited from bone mesenchymal cells (5), which can also differentiate into cells of other lineages, including myoblasts, chondrocytes, and adipocytes. The fate determination of bone marrow mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is tightly controlled by several early regulators including: Wnt/β-catenin signaling, bone morphogenetic proteins (BMPs), hedgehog proteins, endocrine hormones, epigenetic regulators, and various growth factors. Among them, BMPs are known to exhibit high osteogenic activity (6).

BMPs usually function through BMPs-SMAD signaling pathway by binding to and signaling through types II and I BMP receptors, which are transmembrane serine/threonine kinases (7). Upon ligand binding, the types II(BMPR-II and ActR-IIA and ActR-IIB) and I(BMPR- I A or ALK-3,BMPRIB or ALK-6, and ActR- IA or ALK-2) receptors form a heterotetrameric complex (8), resulting in the phosphorylation of the type I receptor by the type II kinase domain. The phosphorylation of type I receptor facilitates the type I receptor to bind and phosphorylate SMAD1/5/9 proteins. Once phosphorylated, SMAD1/5/9 proteins form heterodimeric complexes with SMAD4 and translocate into the nucleus where they interact with other transcription factors, such as RUNX2, and stimulate the differentiation of BMSCs into osteoblasts (9). BMSCs committed to osteogenesis continue to develop the genetic profile and morphology of the osteoblast, expressing genes such as alkaline phosphatase, osteoprotegerin, type I collagen, and later osteocalcin (10). Osteogenic capability of BMPs, such as BMP-2 and BMP-7 have already been vastly studied and their recombinant proteins are currently being investigated in human clinical trials of craniofacial deformities, fracture healing, and spine fusion. However, several reports described the heterotopic ossification associated with their use which restricted their application (11, 12). It's imperative to further explore the osteogenic function and underlying regulation mechanism of other BMPs.

BMPs are functionally regulated by a class of intra and extracellular BMP-binding proteins, termed BMP antagonists, such as noggin, chordin, short gastrulation (Sog), twisted gastrulation (Tsg), and gremlin. BMP antagonists usually bind BMP family ligands and prevent their contact with receptors, thus inhibiting signaling. Chordin-like 1 (CHRDL1) is a secreted glycoprotein, which is structurally related to certain BMP antagonists and plays important roles in several systems, including angiogenesis (13), neural stem cell fate determination and neurogenesis (14), kidney protection from acute and chronic injuries (15), and suppression of tumor growth and metastasis(16, 17). Most of these functions are fulfilled by acting as BMP-4 antagonist. However, the role of CHRDL1 in human osteoblast differentiation induced by BMPs remains ambiguous. The intent of this study is to investigate the direct effect of CHRDL1 on human bone remodeling and the mechanisms involved.

MATERIALS AND METHODS

Isolation and Expansion of Human BMSC (hBMSCs)

This study was approved by the Institutional Ethics Committee of Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. Two male and two female patients averaged 45.7 years (range, 39–52 years) were recruited in this study. They all accepted traumatic femoral or tibia shaft fracture treatment by intramedullary nailing. Bone marrow samples from these patients were obtained with written consent, and patients presented with osteoporosis, other orthopedic or systemic diseases were excluded from the study.

Bone marrow blood aspirated during reaming from the femur or tibia of each donor was filtered through a 100 μm nylon mesh cell strainer. The filtrate was incubated in a 10 cm dish containing basal medium (BM) [low glucose Dulbecco's modified Eagle's medium (#SH30021.01; HyClone, USA) supplemented with 10% fetal bovine serum (#16000-044; Gibco, AUS), 100 U/mL penicillin G, and 100 mg/L streptomycin (#SV30010; HyClone, USA)] at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were discarded 72 h after cell-culture. Adherent cells were washed twice with phosphate buffered saline (PBS). Adherent hBMSCs cultured in complete medium were replaced every 2 days. When the cells achieved 90% confluence, the cultures were detached with 0.25% trypsin (#25200072; Gibco, AUS) and stored or reseeded for the following culture. Cells were used for subsequent experiments at passages 3 to 7.

Identification of hBMSC Lineage

Approximately 4×10^5 hBMSCs at passage 3 were incubated with 1 ug fluorescein isothiocyanate-conjugated mouse anti-human monoclonal antibodies at room temperature for 45 min. After washing with fluorescence-activated cell sorting (FACS) buffer (PBS with 10% bovine serum albumin and 1% sodium azide) at 376 g for 5 min, the stained cells were suspended in 250 μl of ice-cold FACS buffer and then analyzed with FACS (BD Biosciences, USA). For each sample, 1×10^4 events were counted. The percentage of positive signals was analyzed using technical flow cytometry. The antibodies, including CD-29 (#555443; BD Pharmingen), CD-34 (#555822; BD Pharmingen), CD-44 (#555478; BD Pharmingen), CD-45 (#566156; BD Pharmingen), CD-90 (#551401; BD Pharmingen), and CD-105 (#323208; Biolegend), were used in this study.

Multi-Lineage Differential Potential

For osteogenic differentiation assays, the culture-expanded cells were seeded at 1×10^5 cells/well in a six-well culture plate and cultured in a complete culture medium until confluence. The cells were then cultured in BM or osteogenic medium(BM supplemented with 1 nM dexamethasone (#D4902; Sigma–Aldrich), 50 mM ascorbic acid(#A4403; Sigma–Aldrich), and 20 mM β-glycerolphosphate (#G9891; Sigma–Aldrich). The culture medium was changed every 3 days. The cells were cultured for 21 days for the assessment of Alizarin red S (#A5533; Sigma–Aldrich)staining.

For chondrogenic differentiation assays, culture-expanded cells were resuspended in BM at a density of 1×10^7 cells/ml. A total of 20 ul cell suspension was carefully added to a 12-well plate. The cells were allowed to adhere at 37°C in 5% CO_2 for 2 h, followed by the addition of 1 mL of BM or chondrogenic medium [BM supplemented with 10 ng/mL recombinant human transforming growth factor (TGF) beta 1 (#7666-MB-005; R&D Systems) and 50 ng/mL recombinant human insulin-like growth factor 1 (#6630-GR-025; R&D Systems)]. The culture medium was changed every 3 days. Micromasses were fixed for paraffin sectioning and Alcian blue staining after 28 days.

For adipogenic differentiation assays, culture-expanded cells were seeded at 1×10^5 cells/well in a six-well plate and then cultured with BM. Upon reaching confluence, the medium was replaced with BM or adipogenic medium, wherein the BM was supplemented with 500 nM dexamethasone, 0.5 mM isobutylmethylxanthine (#I7018; Sigma–Aldrich), 50 mM indomethacin (#I7378; Sigma–Aldrich), and 10 mg/mL insulin (#I3536; Sigma–Aldrich). The medium was changed every 3 days. The cells were cultured for 14 days for oil red O (#O0625; Sigma–Aldrich) staining.

siRNA Transfection

siRNA transfection was conducted using Lipofectamine 3000 (#L3000015;Thermo Fisher) according to the manufacturer's instruction. The sequences of siRNA targeting CHRDL1 (GenePharma Co., Ltd., Shanghai, China) were as follows: siRNA1: Sense 5′-GCAGCUGUUCGGAGGGAAATT dTdT-3′ and antisense 5′-UUUCCCUCCGAACAGCUGCTT dTdT-3′; siRNA2: Sense 5′-GCAAGCAUCAGGAACCAUUTT dTdT-3′ and antisense 5′-AAUGGUUCCUGAUGCUUGCTT dTdT-3′; siRNA3: Sense 5′-GCCUGUGUAUGAGUCUGUATT dTdT-3′ and antisense 5′-UACAGACUCAUACACAGGCTT dTdT-3′.

The sequences of negative control siRNA (NC-siRNA) that does not target any human gene product: 5′-UUCUCCGAACGUGUCACGUTT dTdT-3′ and anti-sense 5′-ACGUGACACGUUCGGAGAATT dTdT-3′.

Lentiviral Transduction Overexpression Study

CHRDL1 gene was ligated into pLVX-IRES-puro to construct the CHRDL1 overexpression plasmid. The pLVX-IRES-puro and pLVX-IRES-puro-CHRDL1 were transfected into the HEK293T viral packaging cell line together with pSPAX2 and pMD 2.G. Exactly 48 h after transfection, the harvested cells were used for real-time PCR or Western blot analysis.

ALP Staining

On the 7th day of osteogenic induction, ALP staining was performed. Cells were washed thrice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The samples were then stained with 0.1% naphthol AS-Biphosphate (Sigma-Aldrich, St. Louis, MO, USA) and 2% fast violet B (Sigma-Aldrich). After 30 min incubation at 37°C, the cell layer was washed thrice with deionized water to remove the dissociative dye and was observed under a digital camera.

ALP Activity Assay

Cell layers were rinsed with PBS in triplicate and then lysated with lysis buffer containing 0.5% Triton, 50 mM of Tris–HCl, and 5 mM of $MgCl_2$ (Sigma). ALP activity was assayed at 37°C in a buffer containing 0.1 M 2-amino-2-methyl-1-propanol (Wako), 2 mM $MgCl_2$, and p-nitrophenyl phosphate (pNPP) for 30 min. The reaction was terminated by adding of 200 ml of 2 M NaOH per 200 μl of reaction mixture. Absorbance values were measured at 405 nm using pNPP (Sigma-Aldrich) as the substrate.

Alizarin Red Staining and Calcium Assay

On the 14th day of osteogenic induction, cells were fixed in 75% ice-cold ethanol for 1 h and rinsed with distilled water. Cells were then stained with Alizarin Red S solution (Sigma) for 15 min until orange-red in color. After staining, the cells were washed thrice with deionized water, and observed under a digital camera. All experiments were repeated independently in triplicate.

Cell Proliferation Assay

We performed CCK-8 assay according to the manufacturer's instructions. Serum-starved synchronized hBMSCs were seeded at 1×10^4 cells/well in 96-well plates and cultured for 0, 24, 48 or 72 h and 10 ul Cell Counting Kit-8 solution (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added into the well. After 4 h incubation with CCK-8, cell proliferation was measured by reading optical density value at a wavelength of 450 nm.

Real-Time PCR

Total RNA weighing about 1 ug was isolated using TRIzol reagent (Invitrogen) and reverse transcribed using the PrimeScript RT Master Mix cDNA Synthesis Kit (Takara, Japan) to obtain first strand cDNA. Real-time PCR was performed with a Roche LC 480 system using SYBR1 Premix (TaKaRa, Inc., Dalian, China) according to the instructions of the manufacturer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Data were analyzed using the comparison Ct ($2^{-\Delta\Delta Ct}$) method and expressed as the fold change relative to GAPDH. Each sample was presented in mean with the standard error of triplicate.

Primer sequences were as follows: GAPDH: forward, 5′-AGGTCGGTGTGAACGGATTTG-3′; reverse, 5′-GGGG TCGTTGATGG CAACA-3′; CHRDL1: forward, 5′- CC TGGAACCTTATGGGTTGGT-3′; reverse, 5′-AACATTTG GACATCTGACTCGG-3′; ALP: forward, 5′-ACCACCAC GAGAGTGAACCA-3′; reverse, 5′-CGTTGTCTGAGTAC

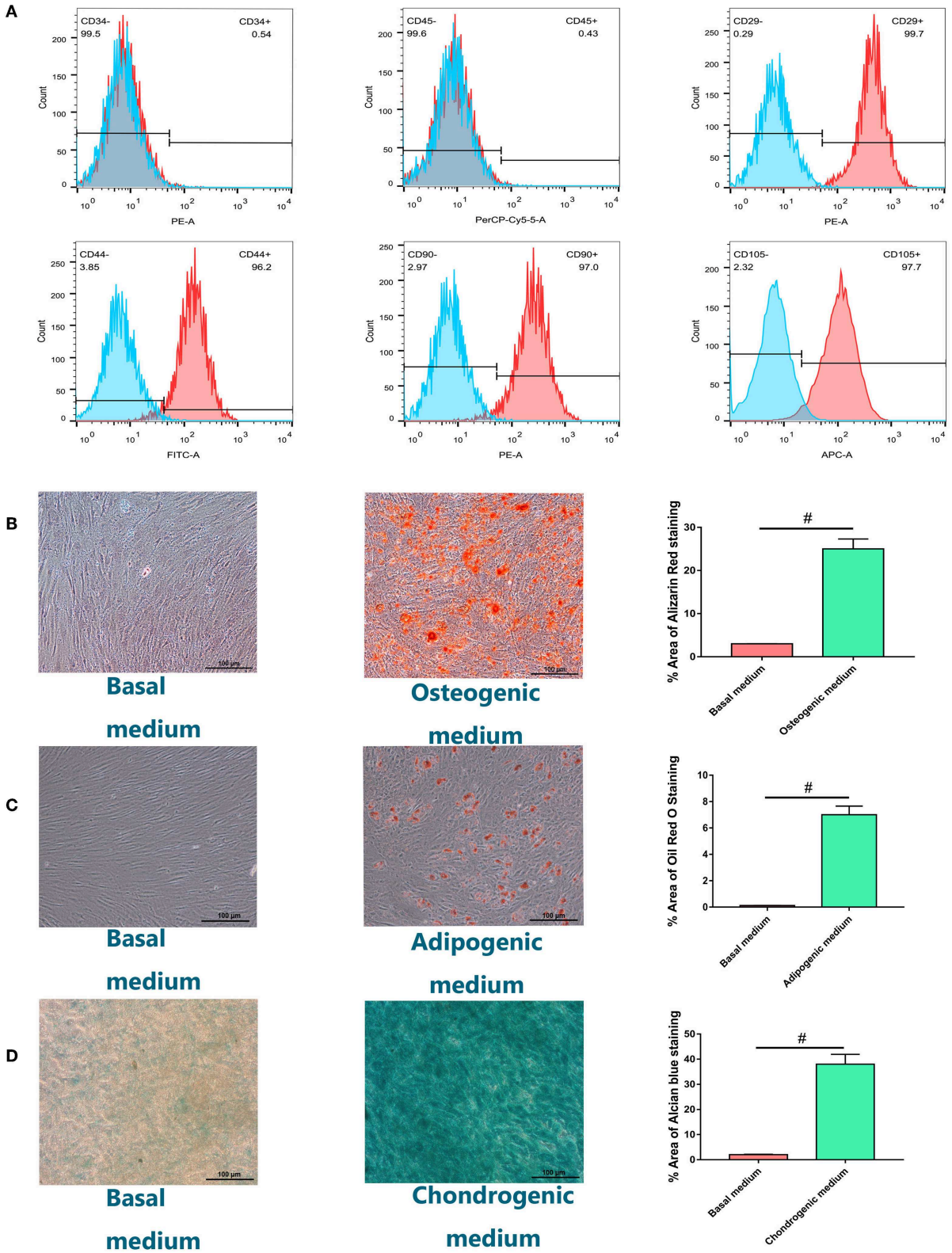


FIGURE 1 | Identification and characterization of human bone marrow-derived mesenchymal stem cells (hBMSCs). **(A)** Flow cytometry analysis of the expression of positive (CD29, CD44, CD90, CD105) and negative (CD34, CD45) cell surface markers of hBMSCs. **(B)** Alizarin Red S staining for hBMSCs culturing for 21 days in osteogenic medium or basal medium. **(C)** Adipogenic differentiation potential of hBMSCs *in vitro*. Oil Red O staining for hBMSCs culturing for 14 days in adipogenic

(Continued)

FIGURE 1 | medium or basal medium. **(D)** Chondrogenic differentiation potential of hBMSCs *in vitro*. Alcian blue staining for hBMSCs culturing for 28 days in chondrogenic medium or basal medium with the method of micromass (magnification: ×100). Quantification of positively stained area recognized by image J was also shown in graph. All experiments were repeated independently in triplicate. Data were presented as mean ± SD ($n = 3$); $^{\#}P < 0.01$.

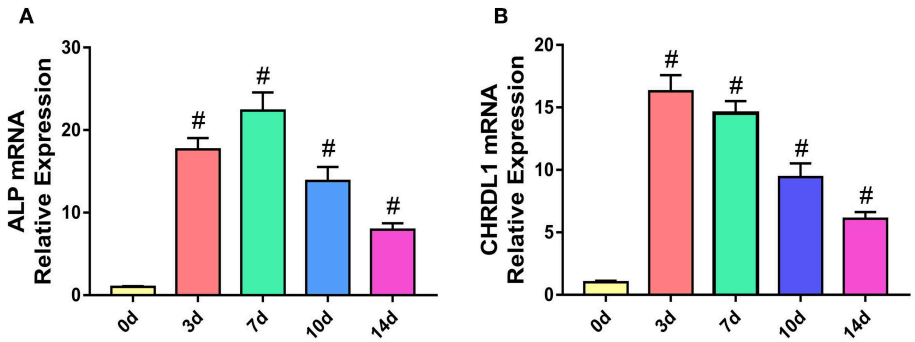


FIGURE 2 | The expression models of CHRDL1 and ALP during the osteogenic differentiation of hBMSCs. hBMSCs were cultured with osteogenic medium, and total RNA was collected at 0, 3, 7, 10, and 14 days, and the mRNA expression levels of ALP **(A)** and CHRDL1 **(B)** were evaluated by real-time quantitative PCR. GAPDH was used as internal control. All experiments were repeated independently in triplicate. Data were presented as mean ± SD ($n = 3$); $^{\#}P < 0.01$ vs. 0 day.

CAGTCCC; COL1A1: forward, 5′-GAGGGCCAAGACGA AGACATC-3′; reverse, 5′-CAGATCACGTCATCGCAC AAC-3′; osteopontin (OPN): forward, 5′-CTGTGTTGGT GGAGGATGTCTGC-3′; reverse, 5′-GTCGGCGTTTGG CTGAGAAGG−3′; OCN: forward, 5′-GACAAGTCC CA CACAGCAACT-3′; reverse, 5′-GGACATGAAGGCTTTGT CAGA-3′.

Western Blot Analysis

Cells were washed with ice-cold Dulbecco's PBS and total protein lysates were extracted with cell lysis buffer RIPA (Biocolors, R0095) containing 1% PMSF (Meilunbio, MA0001). For western blot analysis, 20 ug of proteins was resolved on 10% SDS–PAGE gels (Bio-Rad, Richmond, CA) and transferred to polyvinylidene difluoride membranes (Merch, ISEQ00010).The membranes were blocked with TBS containing 5% (w/v) non-fat dry milk and 0.1% Tween-20 for 1 h, and then incubated at 4°C overnight with the appropriate antibodies, including CHRDL1 (Abcam, ab103369), BMPRII (CST, 6979), RUNX2 (Santa Cruz, 10758), p-SMAD1/5/9 (CST, 13820), SMAD1/5/9 (Santa Cruz, 6031), and GAPDH (CST, 5174). Blots were developed with horseradish peroxidase-labeled secondary antibody and visualized using the enhanced chemiluminescence detection system (Millipore, Billerica, MA) according to the manufactures' instructions.

Surgical Procedure and Cell Transplantation

We used mice femoral shaft cortical bone defect model *in vivo* experiments, and all animal experiments were approved by the Laboratory Animal Institutions Committee. Animal care was provided in accordance with the Institutional Guidelines. Eight-week-old male BALB/C nude mice (Vital River Laboratory Animal Technology Co., Ltd. Beijing, China) were i.p. anesthetized with 1.5% pentobarbital sodium (40 mg/kg). A decimal bone defect 0.8 mm in diameter was performed on the femoral shafts. hBMSCs were suspended in the medium mixture and Matrigel (BD Bioscience), and 5–10^5 cells/femoral shaft was transplanted into the defective lesions. hBMSCs were infected with si-CHRDL1 or pLVX- CHRDL1 before transplantation. Control mice underwent the same surgical operation except for transplantation of hBMSCs infected with pLVX-vector or NC-siRNA.

Statistical Analysis

All statistical analysis was performed using SPSS (version 16.0; SPSS, Inc., Chicago, IL). All quantitative data were presented as the mean ± SD at least three separate experiments, each performed with triplicate samples and analyzed by Student's *t*-test or one-way ANOVA. All tests were two-sided with a *P*-value of 0.05 was used as the boundary of statistical significance.

RESULTS

Identification and Characterization of hBMSCs

Isolated cells all expressed MSC markers CD29, CD44, CD90, and CD105 and did not express leukocyte and hematopoietic markers CD45 and CD34, respectively (**Figure 1A**). Most cells formed mineralized calcium deposits after 21 days of osteogenic differentiation, which were confirmed by Alizarin Red staining (**Figure 1B**). The adipogenic differentiation capacity of hBMSCs was confirmed by oil red O staining. Lipid droplets were detected 14 days after adipogenesis induction and were not observed in host medium (**Figure 1C**). After 28 days of micromass culture and induction, the cartilage differentiation

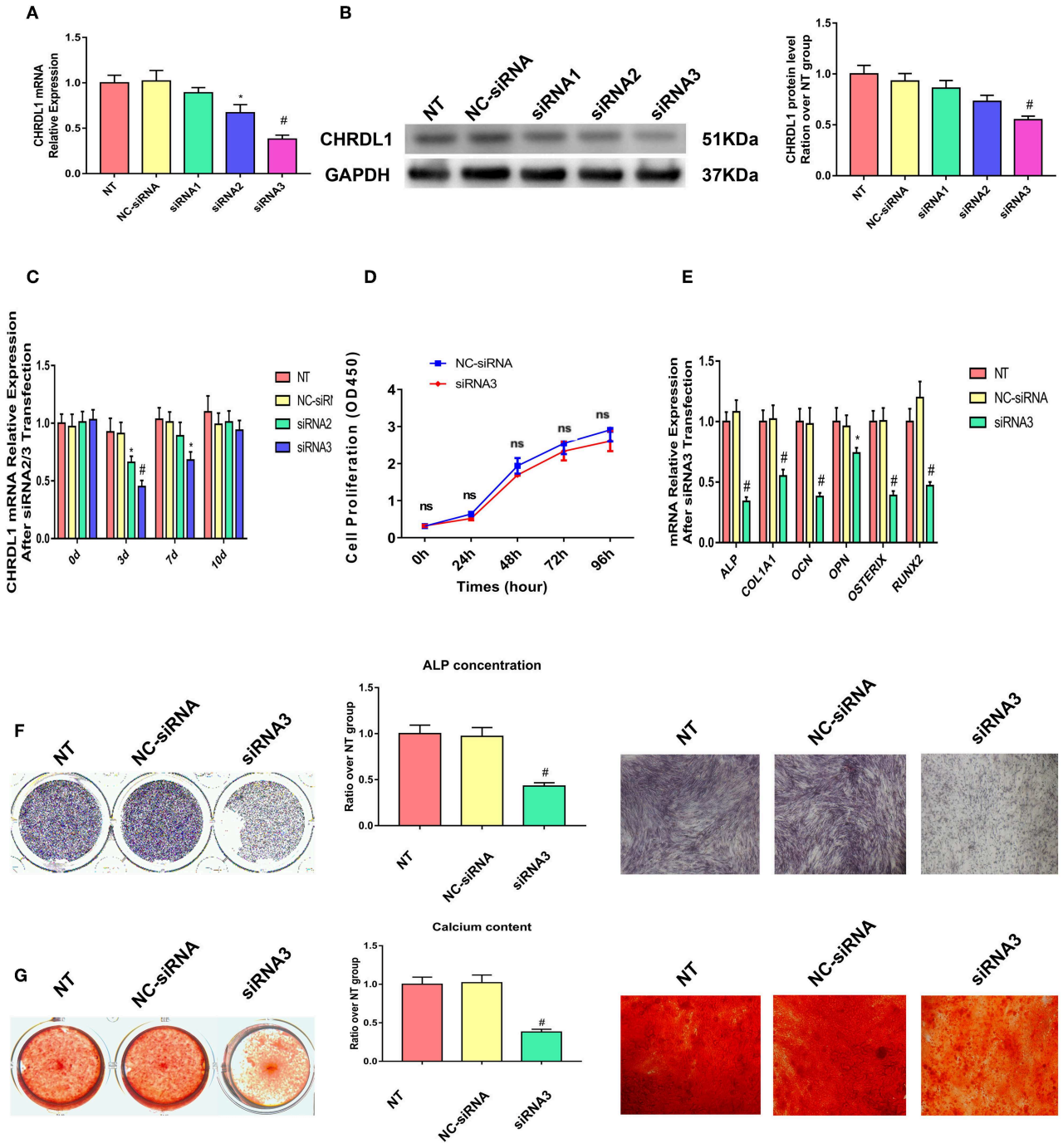


FIGURE 3 | Knockdown of CHRDL1 suppressed hBMSCs osteogenesis. Three siRNAs were generated to suppress CHRDL1 expression. **(A,B)** CHRDL1 mRNA and protein expression levels were detected 72 h after siRNAs transfection. Densitometric analysis of immunoblot band intensities for CHRDL1 normalized by GAPDH were also detected. **(C)** CHRDL1 expression was detected at 0, 3, 7, and 10 days after siRNA2 or siRNA3 transfection. **(D)** *In vitro* growth of NC-siRNA and siRNA3 transfected hBMSCs were measured by CCK8 assay at 24, 48, 72, 96 h after transfection. **(E)** The mRNA expression levels of OCN, COL1A1, OPN, ALP, OSX, and RUNX2 were all detected using real-time quantitative PCR at 48 h after osteogenic induction and 72 h after siRNA3 transfection. **(F,G)** ALP staining, ALP quantitative analysis and Alizarin Red staining were performed to detect osteogenesis of hBMSCs after siRNA3 transfection. All experiments were repeated independently in triplicate. Data were presented as mean ± SD ($n = 3$) (*$P < 0.05$ and $^{\#}P < 0.01$).

of hBMSC was verified by positive staining with Alcian blue staining. Compared with chondrogenic induction in host medium, chondrogenic induction in micromass culture resulted in high Alcian blue staining (**Figure 1D**). Quantification of positively stained area recognized by image J was also shown in graph.

The Expression of CHRDL1 Increased During Osteogenesis of hBMSCs

To understand the role of CHRDL1 during the process of osteogenesis, we determined the mRNA expression profile of CHRDL1 and early osteogenic marker ALP in hBMSCs cultured under osteogenic differentiation medium by using real-time PCR.

During the process of osteogenic differentiation in hBMSCs, CHRDL1 mRNA expression, followed a similar distribution to that of ALP. CHRDL1 mRNA expression levels were detectable on day 0. During the first 7 days in culture, CHRDL1 expression levels peaked on day 3. During these days, the cells exhibited elevated ALP expression but the peak level appeared on day 7, which slightly lagged behind that of CHRDL1. CHRDL1 and ALP mRNA levels gradually declined on day 10 and then further declined to approximately half of the peak levels on day 14 (**Figures 2A,B**). These data showed that CHRDL1 gene was expressed in osteoblastic cells and its expression levels were regulated time dependently along with the osteogenic differentiation process.

Suppression of CHRDL1 Decreased Osteogenesis of hBMSCs

Three siRNAs were generated to suppress CHRDL1 expression to explore the possible function of CHRDL1 during osteogenic differentiation. Exactly 72 h after transfection, CHRDL1 siRNA2 and siRNA3 significantly reduced in corresponding CHRDL1 mRNA expression and protein levels (**Figures 3A,B**) in the culture supernatant compared with transfection of control siRNA. Transfection of CHRDL1 siRNA1 did not change CHRDL1 expression at mRNA and protein levels significantly. We conducted a time-course study with siRNA2 and siRNA3. Reduced CHRDL1 mRNA expression was observed in siRNA2- or siRNA3-transfected cells from day 0 to day 10 after transfection compared with non-transfected (NT) and control siRNA-transfected groups with the lower value that occurred 3 days after transfection by siRNA3 (**Figure 3C**). Given that siRNA3 achieved the best suppression effect on CHRDL1 expression, we conducted the following experiments with CHRDL1 siRNA3 only.

To make sure if CHRDL1 knockdown could affect hBMSCs proliferation, we conducted CCK8 assay according to the

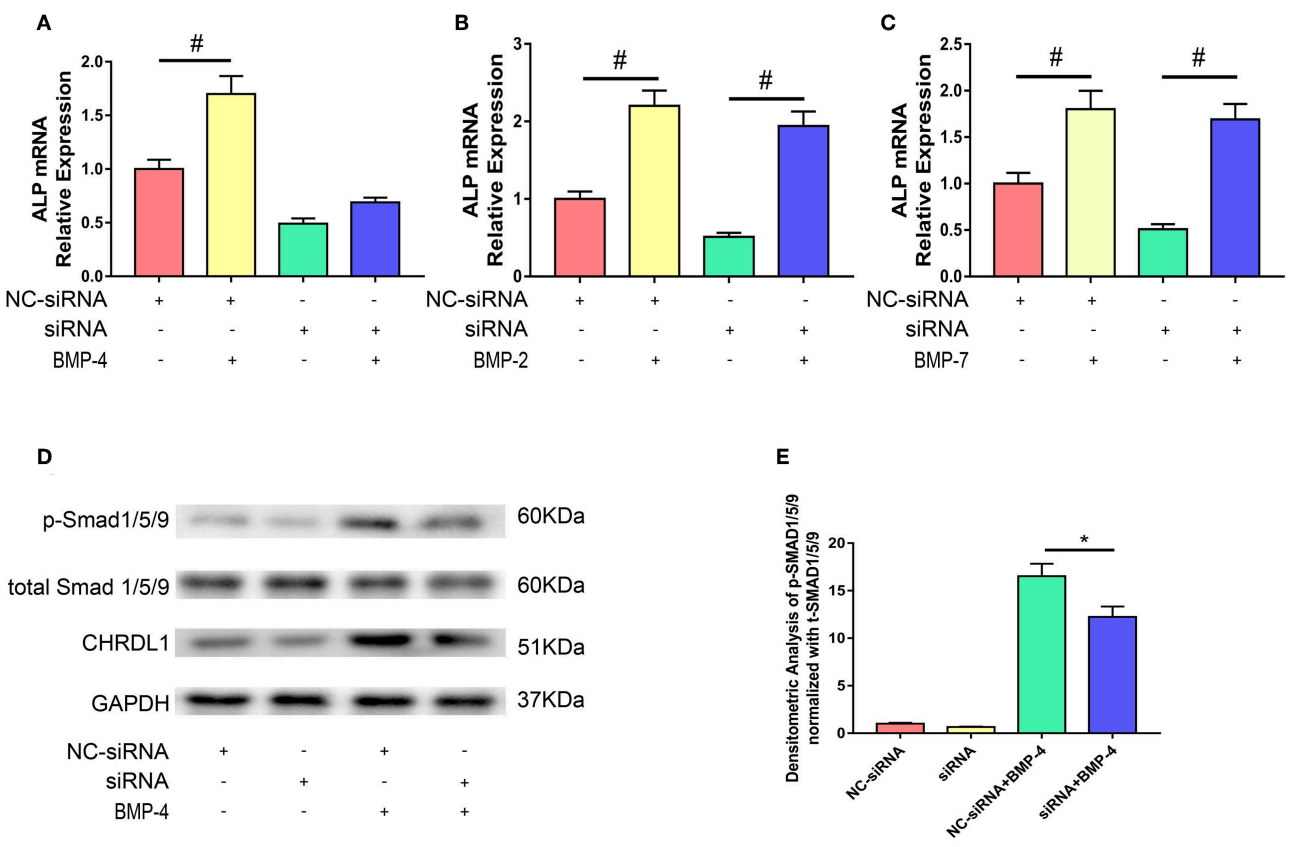


FIGURE 4 | Knockdown of CHRDL1 inhibited BMP4-induced osteoblast differentiation of hBMSCs. hBMSCs were either transfected with si-CHRDL1 or NC-siRNA, 24 h later, both groups were induced by BMP-2, BMP-4, or BMP-7. 3 days after transfection, mRNAs were harvested for real-time quantitative PCR **(A-C)**. Western blotting was used to detect levels of CHRDL1, p-SMAD1/5/9, and total SMAD1/5/9 in hBMSCs transfected with NC-siRNA and si-CHRDL1 either with or without treatment with rhBMP-4 **(D)**. Relative levels of p-SMAD/t-SMAD were plotted graphically in panel **(E)**. The experiment was repeated three times. Data were reported as mean ± SE ($n = 3$) ($^{*}P < 0.05$ and $^{\#}P < 0.01$).

instructions. Fluorescence multi-well plate reader (Infinite M200 PRO, TECAN, Switzerland) with the optical density value set at a wavelength of 450 nm was used to detect siRNA3 and NC-siRNA transfected hBMSCs proliferation, no difference was detected at 24,48,72, 96 h after transfection between two groups (**Figure 3D**).

Exactly 24 h after transfection with CHRDL1 siRNA3, hBMSCs were induced by osteogenic medium without exogenous BMPs addition, and 72 h after transfection, the cells were harvested for quantitative PCR, mRNA expression levels of osteogenesis genes, such as ALP, OCN, OPN, COL1A1, OSTERIX, and RUNX2 were also significantly reduced compared with the control group (**Figure 3E**). ALP staining and ALP quantitative analysis showed that after 7 days of osteogenic induction, the transfection of CHRDL1 siRNA3 reduced the ALP activity of cells significantly (**Figure 3F**). Alizarin Red staining showed that the group transfected with CHRDL1 siRNA3 exhibited less calcium deposition than control groups. Similar results were obtained in photomicrographs (**Figure 3G**). A rescue experiment was performed by adding rhCHRDL1 protein (0.1 ug/ml) (Abcam, ab164881) to osteogenic medium 24 h after siRNA3 transfection. mRNA expression levels of osteogenesis related genes decreased by CHRDL1 suppression were largely rescued by rhCHRDL1 protein administration 72 h after transfection. ALP staining showed similar results after 7 days of osteogenic induction. (**Supplementary Figures 1A**,**B**).

CHRDL1 Potentiate Osteogenesis Function of BMP-4

We next used the siRNA system to assess the possible role of CHRDL1 in the BMP-SMAD signaling pathway. hBMSCs were either transfected with si-CHRDL1 or NC-siRNA, 24 h later, both groups were induced by 0.1 ug/ml recombinant human BMP-2, BMP-4, and BMP-7 addition to test the dependence of BMPs function on CHRDL1. Three days after transfection, cells were harvested for quantitative PCR, rhBMP-4 administration can slightly rescue the decreased ALP mRNA level caused by CHRDL1 suppression (**Figure 4A**), whereas rhBMP-2 and rhBMP-7 substantially increased ALP mRNA levels (**Figures 4B,C**). Thus, we speculated that the induction of osteogenesis by BMP-4 depends on the presence of CHRDL1. Western blot was conducted to detect the level of SMAD-1/5/9 phosphorylation in response to rhBMP-4. Compared with cells transfected with NC-siRNA, hBMSCs that suppressed CHRDL1 by siRNA3, showed decreased p-SMAD-1/5/9 (**Figure 4D**) in a statistically significant fashion (**Figure 4E**). These data indicated that the function of BMP-4 was enhanced by and depended on the presence of CHRDL1 through BMP-SMAD signaling pathway during hBMSCs osteogenesis.

Induced CHRDL1 mRNA Expression by BMP-4 Treatment

Given that the function of CHRDL1 on hBMSC osteogenesis was fulfilled via BMP-4, we next assessed the change of CHRDL1 mRNA expression during hBMSCs osteogenesis differentiation induced by 72 h rhBMP-4 treatment. The expression of CHRDL1 mRNA was induced by rhBMP-4 in a dose-dependent manner. In the range of 0–1 ug/ml rhBMP-4, increased concentrations of BMP-4 induced high expression of CHRDL1, and 1 ug/ml rhBMP-4 induced the peak expression. mRNA expression of CHRDL1 declined as the concentration of BMP-4 increased from 1 to 50 mg/ml (**Figure 5A**). CHRDL1 mRNA was also induced by BMP-4 in a time-dependent manner. The level of CHRDL1 mRNA induced by 0.5 ug/ml rhBMP-4 increased gradually with time and increased significantly at 48 and 72 h after the addition of rhBMP-4, and after 96 h the upward trend became gentle (**Figure 5B**).

Since CHRDL1 could be induced by BMP-4, we wonder if CHRDL1 expression could be blocked by BMP type I kinase inhibitor LDN-193189. We detected hBMSCs ALP and CHRDL1 mRNA expression levels after 72 h induction by rhBMP-4 with LDN-193189 administration. LDN-193189 (100 nM) significantly decreased ALP and CHRDL1 mRNA expression which was upregulated by rhBMP4 (**Supplementary Figure 2**).

CHRDL1 Overexpression Enhances BMP-4 Induced Osteogenesis

We next examined whether CHRDL1 overexpression promoted osteogenic differentiation of hBMSCs. Lentivirus that expressed

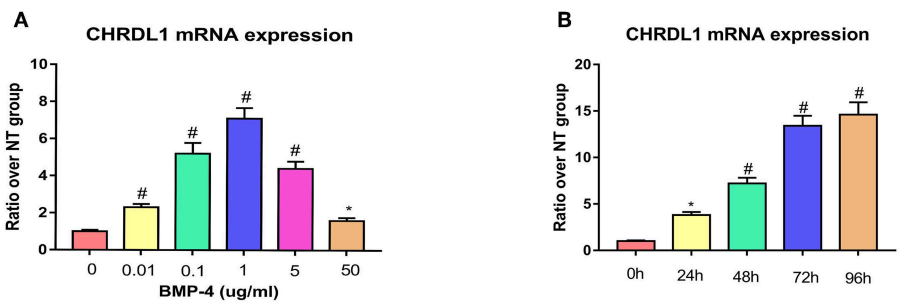


FIGURE 5 | BMP-4 induced CHRDL1 expression in a time- and dose-dependent manner. **(A)** CHRDL1 mRNA expression of hBMSCs was tested using real-time quantitative PCR after treatment with indicated doses of rhBMP-4 for 72 h. $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. the group without BMP-4 treatment. **(B)** CHRDL1 mRNA expression of hBMSCs was tested with real-time quantitative PCR after treatment with 0.5 ug/ml rhBMP-4 for indicated time. All experiments were repeated independently in triplicate. Data were presented as mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. the CHRDL1 expression level at 0 h.

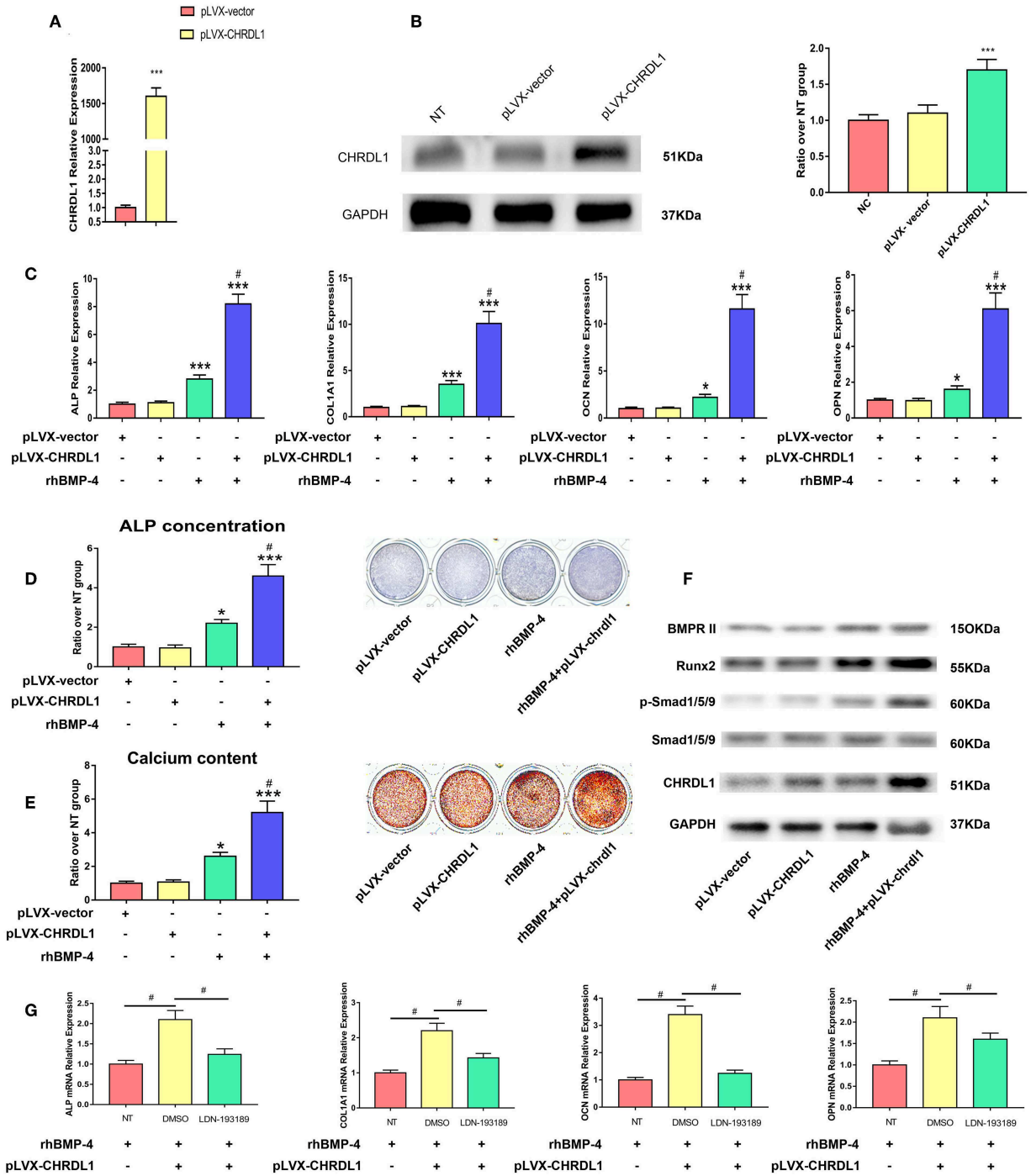


FIGURE 6 | CHRDL1 overexpression enhanced BMP-4-induced osteoblast differentiation *in vitro*. **(A,B)** CHRDL1 mRNA expression and protein levels were detected at 72 h after transfection of pLVX-CHRDL1. $^{***}P < 0.01$ compared with pLVX-vector. **(C)** The mRNA levels of COL1A1, ALP, OCN, and OPN were detected at 72 h after pLVX-CHRDL1 transfection and 48 h after osteogenic induction. $^{*}P < 0.05$; $^{***}P < 0.01$ vs. pLVX-vector transfected sample; and $^{\#}P < 0.01$ vs. rhBMP-4 administrated sample. **(D,E)** ALP and Alizarin Red staining after transfection with pLVX-CHRDL1 and rhBMP-4 addition separately or in combination when cultured in osteogenic induction medium for 14 days. Data are presented as mean ±SD ($n = 3$). $^{*}P < 0.05$; $^{***}P < 0.01$ vs. pLVX-vector transfected sample; and $^{\#}P < 0.001$ vs. rhBMP-4 administrated sample. **(F)** Western blot analysis of BMPR II, p-Smad1/5/9, total Smad1/5/9, Runx2, CHRDL1, and GAPDH at 48 h after transfection with pLVX-CHRDL1 and rhBMP-4 addition separately or in combination. GAPDH was used as loading control. **(G)** mRNA levels of ALP, COL1A1, OCN, and OPN were detected 48 h after treating with LDN-193189 or its vehicle (DMSO). Data were presented as mean ± SD ($n = 3$); ($^{*}P < 0.05$ and $^{\#}P < 0.01$). All experiments were repeated independently in triplicate.

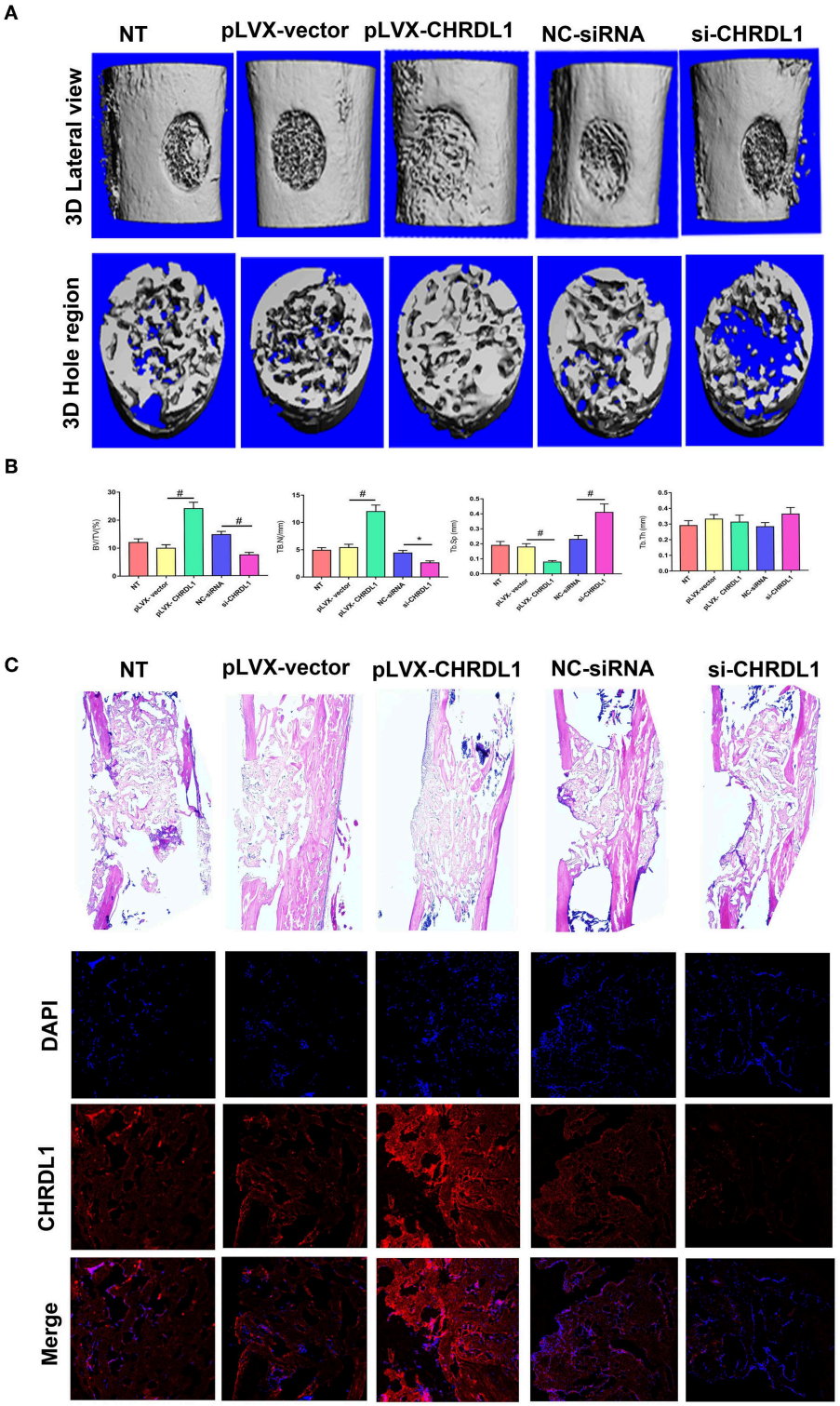


FIGURE 7 | Knockdown and overexpression of CHRDL1 affected bone repair in a mouse model of femoral bone defect. **(A)** Lateral views of 3D reconstruction of defective femur (top panel) and mineralized bone formed in hole region (lower panel) by micro-CT. Representative images of each group. **(B)** 3D structural parameters of trabecular BV/TV, Tb.N, Tb.Sp, and Tb.Th of mineralized bone formed in hole region by micro-CT; **(C)** H & E staining also shows new bone accumulation in hole regions of si-CHRDL1 and pLVX-CHRDL1 treated mice. CHRDL1 expression detected by immunofluorescent assay around newly formed bone in each group were also shown. (Original magnification: 100×). All experiments were repeated independently in triplicate. (*P <0.05 and $^{\#}P$ <0.01).

human CHRDL1 (pLVX- CHRDL1) under the control of the pLVX-vector were generated. pLVX-CHRDL1 was transduced into cultures of hBMSCs, and CHRDL1 mRNA and protein expression levels were analyzed by PCR and Western blot analysis 72 h after transfection. All these expressions increased substantially in a dose-dependent manner compared with cells transduced with control virus (pLVX-vector) (**Figures 6A,B**).

We then tested hBMSCs osteogenic differentiation affected by pLVX- CHRDL1 alone, BMP-4 administration, or by both. 24 h after transfection, hBMSCs were cultured in osteogenic differentiation medium, 48 h later, osteogenic marker expression was tested by real-time PCR. In cells transfected with pLVX-CHRDL1 alone, no significant effect on osteogenic marker expression was detected. However, 0.5 ug/ml rhBMP-4 application cells had increased osteogenesis and showed significantly higher ALP and COL1A1 mRNA levels and slightly higher late-osteogenic markers, such as OPN and OCN. Combined administration with rhBMP-4 and pLVX-CHRDL1 significantly enhanced ALP and COL1A1 mRNA levels over and above that of rhBMP-4 alone and also significantly increased mRNA expression of OPN and OCN (**Figure 6C**).

24 h after pLVX- CHRDL1 transfection, hBMSCs were induced for 7 or 21 days for ALP staining and Alizarin Red staining. Cells transfected with pLVX-CHRDL1 did not change the intensity of ALP and Alizarin Red staining compared with the control of the pLVX-vector, whereas rhBMP-4 administration significantly increased the staining, and they could increase further by combination of BMP-4 administration and pLVX-CHRDL1 transduction (**Figures 6D,E**). All these results confirmed our hypothesis that CHRDL1 acts to enhance BMP-4-mediated osteogenesis of hBMSCs.

To verify the relationship of CHRDL1 and BMP-4 during the osteogenic differentiation induced by them, we investigated the effect of CHRDL1 on downstream of the BMP-4-SMAD signaling pathway events during osteoblastic differentiation 48 h after pLVX-CHRDL1 transduction. BMP-4 singly application increased p-SMAD-1/5/9 level, and little such effect was observed in hBMSCs transfected with pLVX- CHRDL1. However, BMP-4 administration combined with pLVX- CHRDL1 transduction increased the phosphorylation of SMAD-1/5/9 in a statistically significant manner compared with BMP-4 alone (**Figure 6F**), indicating that CHRDL1 potentiates BMP-4 activity by increasing the activation level of SMAD-1/5/9.

In vitro gain of function experiment was also conducted with rhCHRDL1 addition instead of pLVX- CHRDL1 transfection. 0.1ug/ml rhCHRDL1 was added to the osteogenic medium 72 h for hBMSCs osteogenesis. Compared with control group, osteogenic related genes mRNA expression, ALP staining, as well as western blotting all showed results similar to those of pLVX- CHRDL1 transfection (**Supplementary Figure 3**). These results further confirmed osteogenic function of secreted glycoprotein CHRDL1.

To further confirm the functional connection between CHRDL1 and BMP-4, we examined the effect of LDN-193189 on pLVX- CHRDL1 transfection and rhBMP-4 administrated hBMSCs osteogenesis. 24 h after pLVX- CHRDL1 or pLVX-vector transfection, LDN193189 (100 nM) in its vehicle DMSO was applied during rhBMP-4 induced osteogenesis. 72 h after transfection, osteoblastic genes, such as COL1A1, ALP, OCN, and OPN mRNA expressions, were detected. Compared with pLVX-vector group, pLVX-CHRDL1 group showed higher mRNA levels, whereas descended mRNA levels were detected when treating with LDN-193189 (**Figure 6G**). All these data suggested that CHRDL1 enhanced BMP-4 function by increasing SMAD-1/5/9 phosphorylation level.

In vivo Study

To verify the role of gene CHRDL1 in osteogenesis *in vivo*, we performed two different experiments that used either a gain or loss of CHRDL1 function strategy. For gain of function experiment, hBMSCs transfected with pLVX-CHRDL1 or pLVX- vector were transplanted into the femoral defective lesions of immunocompromised mice. Two weeks after implantation, micro-CT showed that pLVX- CHRDL1 transduced cells generated more newly formed bone than cells that were transfected with pLVX-vector (**Figure 7A**). Percentage of bone volume (BV) to total tissue volume (TV) of callus was calculated to be significantly higher in pLVX- CHRDL1 group compared with both sham and pLVX- vector groups 3 weeks post-transplantation. Mineralized bone defects in pLVX-CHRDL1 group showed increased trabecular number (Tb.N), decreased trabecular separation (Tb.Sp), and unaltered trabecular thickness (Tb.Th) (**Figure 7B**). Newly formed bone detected by immunofluorescent assay showed that CHRDL1 expression in the pLVX- CHRDL1 group was higher than in the pLVX-vector group. consistently, H&E staining also showed increased new bone formation in the hole regions of defective femur of mice in PLVX- CHRDL1 group compared with the control groups (**Figure 7C**). These findings confirmed our previous *in vitro* experiments that showed CHRDL1 can enhance hBMSCs osteogenesis.

For loss of function experiment, hBMSCs transfected with si-CHRDL1 had dramatically reduced bone formation compared with cells transfected with NC-siRNA (**Figure 7A**) 2 weeks after implantation. si-CHRDL1 group showed significantly reduced percentage of BV to total TV, decreased Tb.N, increased Tb.Sp, and similar Tb.Th compared with two control groups 3 weeks post-transplantation (**Figure 7B**). CHRDL1 expression in the fibrous tissue around newly formed bone in the si-CHRDL1 group was lower and fewer new bone formation was also detected in H&E staining of si-CHRDL1 group (**Figure 7C**). Reduction in bone formation appeared to be due to a diminished ability of the si-CHRDL1 transfected hBMSCs to undergo differentiation, consistent with the reduction in the expression of osteogenesis markers, ALP, COL1A1, OCN, and OPN mRNA *in vitro* experiments.

We also conducted *in vivo* gain of function experiment by injecting rhCHDRL1 (0.1 ug/ml) mixed with Matrigel to the femoral defective lesions. Similar to the results of pLVX-CHRDL1 transfected hBMSCs transplantation, rhCHDRL1 local injection also significantly generated more new bone compared

with the control group, assessed by micro-CT and HE staining 2 weeks after operation (**Supplementary Figure 4**).

To rule out the possibility of the secreted protein, CHRDL1, promote bone formation via affecting other cells such as: resident osteoblasts, osteoclasts and fibroblasts, immunohistochemical staining for Osteoprotegerin (OPG), tartrate-resistant acidic phosphatase (TRAP) and Gomori methenamine silver staining were conducted. No significant difference of stained area quantification in each experiment was detected between si-CHRDL1 and NC-siRNA group (**Supplementary Figure 5**), and these results may indicate these cell groups may not get involved in CHRDL1 potentiated osteogenesis.

DISCUSSION

Recent studies have advanced our understanding of the cellular events and signals that are involved in bone metabolism, and at the center of these advancements is the demonstration of the role of BMP signaling in skeletal biology (7). The BMP signaling pathway is highly conserved and vital to the development of various systems. Since the first BMP was discovered by Marshall Urist in the 1960s (18), more than 22 members of the BMP family have been identified to be critical molecules for osteoblastic activation and bone formation. These molecules are members of the TGF family, among them, BMP-4 is generally known for its critical roles in embryonic, hematopoietic, and mesenchymal developments, and has been identified as a regulator of cartilage and bone formation (19).

Although BMP-4 has been reported to work through other mechanisms (20), it usually functions through BMPs-SMAD signaling pathway. Many downstream molecules have been reported concerning bone formation in this signaling pathway, however, the complex regulation mechanism in several levels has not been fully understood. In addition to intracellular regulation, such as inhibitory SMADs, miRNAs, and methylation, extracellular regulation by "BMP antagonists" has been regarded as a pivotal morphogenetic mechanism of the BMPs-SMAD signaling pathway. BMP antagonists are a set of structurally distinct secreted proteins with repeated cysteine-rich (CR) domains, which bind to the BMP family ligands and prevent their contact with receptors, inhibiting BMP signaling. Secreted proteins with CR domains, such as chordin, Sog, Tsg, noggin, and gremlin-2, inhibit BMPs-SMAD signaling pathway via binding to BMP receptors (21). These observations indicate that BMP-antagonist expression is detrimental to bone formation. However, not all proteins containing CR domains are BMP inhibitors, some exert both stimulatory and antagonistic effects in different contexts. BMPER antagonizes or enhances BMP signaling, depending on the assay (22). Xiao also revealed that BMPER stimulates bone formation by coupling angiogenesis (23). Noggin plays varying roles during osteogenesis, inhibiting osteogenesis by preventing BMPs from binding to their receptors on the cell surface in some animal models, such as mice (24), and enhances osteogenesis by inducing BMP-2 and OCN in hBMSCs (25). Kielin/chordin-like protein is another BMP enhancer with CR domains, increasing the affinity of the ligand to the receptor and enhancing the stability of the ligand-receptor complex (26), further attenuating the pathology of renal fibrotic disease.

These studies extend our understanding of the role of "BMP antagonists," revealing that proteins containing CR domain might also potentiate BMP-SMAD signaling and further benefit bone formation. CHRDL1 is a secreted glycoprotein containing three characteristic CR repeats structurally related to that of BMP antagonists and is reported to interact with several members of the BMP family, for example, BMP-4,−7, and−5 and TGF- β. CHRDL1 functions as a BMP antagonist in several systems, however, the exact role of CHRDL1 concerning osteogenesis is unclear. Hugo Fernandes et al. conducted an *in vitro* study, and showed that CHRDL1 upregulated hBMSC proliferation but unaffected osteogenic differentiation (27). However, hBMSC osteogenesis was induced only by BMP-2 in his study, whereas CHRDL1 was reported to interact with BMP-4 rather than BMP-2. Thus, their conclusion may be incomplete. Our results suggested that si-CHRDL1 did not affect hBMSCs proliferation but suppressed osteogenesis. And a stimulatory effect of CHRDL1 on osteogenesis of human BMSCs with the presence of BMP-4 was also detected. In addition, although hBMSCs with reduced CHRDL1 by knockdown showed increased phosphorylation of p-SMAD-1/5/9 in response to rhBMP-4 addition, phosphorylation level was significantly lower when compared with cells transfected with NC-siRNA, while CHRDL1 overexpression plus rhBMP-4 significantly upregulated SMAD1/5/9 phosphorylation, and RUNX2 protein level. Both loss and gain of function experiments consistently suggested that the induction of osteogenesis by BMP-4 was enhanced by and depended on the presence of CHRDL1. Furthermore, we noticed that BMP-4 induced CHRDL1 mRNA expression can be blocked by the addition of LDN193189, a specific BMP type I kinase inhibitor. The positive effect of combined administration of CHRDL1 and BMP-4 was also alleviated by LDN193189, these results further confirmed that osteogenesis of CHRDL1 was controlled by the BMP-4-SMAD pathway.

In vitro, single CHRDL1 overexpression or rhCHRDL1 addition did not significantly increase ALP activity and calcification as well as the expression of several osteoblastic genes, whereas combined addition of CHRDL1 and rhBMP-4 significantly promoted hBMSC osteogenesis. Whereas, *in vivo*, CHRDL1 could singly promote osteogenesis in femur bone defect models. The difference between *in vivo* and *in vitro* experiments may be explained by the presence of endogenous BMP-4 in bone defect models, which obviated the need for extra rhBMP-4 addition, indicating that CHRDL1 promotes osteogenesis depending on the presence of BMP-4.

In summary, the mechanism of CHRDL1 on bone formation may lie in a positive feedback loop and can be explained as follows. During hBMSCs osteogenesis, BMP-4 induced CHRDL1 expression, and BMP-4 activity was potentiated by CHRDL1. CHRDL1 further sensitized BMSCs to BMP-4, maintained the enhanced BMP-mediated signaling. As a CR domain containing "BMP antagonists," CHRDL1 does not inactivate BMP-4 but rather acts as a novel inducer of hBMSCs osteogenesis through

BMP4-SMAD signaling pathway although the possible roles of other BMPs in CHRDL1-regulated osteogenesis cannot be completely ruled out. Our study confirmed osteogenesis function of rhCHRDL1 *in vivo*, which lays a foundation for its clinical application, although its systemic or local concentrations need to be further studied to understand the diagnostic and therapeutic potential of CHRDL1.

In conclusion, our study indicated that osteogenesis induction by BMP-4 was enhanced by and depended on the presence of CHRDL1. The ability of CHRDL1 to enhance BMP-4 activity might be an important mechanism to elucidate the mechanisms of hBMSCs osteogenic differentiation and bone remodeling, and CHRDL1 might be a potential treatment target for metabolic and developmental bone diseases.

AUTHOR CONTRIBUTIONS

TL designed and conducted the study, contributed to the data analysis, and writing the manuscript. BL collected and analyzed data. S-DJ and X-FZ supervised the statistical analysis and contributed to writing the manuscript. Z-ZZ, W-NX, H-LZ, and C-DW wrote and revised the paper. L-SJ and X-LZ supervised this study and contributed to study design, data analysis, and writing the manuscript.

FUNDING

This work was financially supported by the National Natural Science Foundation of China (81772374).

ACKNOWLEDGMENTS

We would like to thank Cheng-Long Wang, Sheng-Zhou Shan, Fei Xiao, and Xiao-Ying Zhao for helpful discussions and feedback.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00360/full#supplementary-material

Supplementary Figure 1 | rhCHRDL1 rescued hBMSCs osteogenesis suppressed by si-CHRDL1. **(A)** mRNA expression levels of osteogenesis related genes and were detected at 72 h after si-CHRDL1 transfection and 48 h after rhCHRDL1 protein (0.1 ug/ml) administration. **(B)** ALP staining was detected after 7 days of osteogenic induction. Data were presented as mean ± SD ($n = 3$); $^{*}P < 0.05$; $^{***}P < 0.001$ vs. NC-siRNA transfected sample; and $^{\#}P < 0.001$ vs. siRNA3 transfected sample. All experiments were repeated independently in triplicate.

Supplementary Figure 2 | BMP type I kinase inhibitor LDN-193189 blocked ALP and CHRDL1 mRNA expression upregulated by rhBMP4. ALP **(A)** and CHRDL1 **(B)** mRNA expression levels were detected after 72 h rhBMP-4 induction in combination with LDN-193189 administration. Data were presented as mean ± SD ($n = 3$); ($^{\#}P < 0.01$). All experiments were repeated independently in triplicate.

Supplementary Figure 3 | rhCHRDL1 enhanced BMP-4-induced osteoblast differentiation *in vitro*. Osteogenesis related gene mRNA expression levels **(A)** and ALP staining **(B)** after addition of rhCHRDL1 and rhBMP-4 addition separately or in combination when cultured in osteogenic induction medium for 72 h. Data were presented as mean ± SD ($n = 3$); $^{*}P < 0.05$; $^{***}P < 0.01$ vs. NC group sample; and $^{\#}P < 0.01$ vs. rhBMP-4 administrated sample. **(C)** Western blot analysis of BMPR II, p-Smad1/5/9, total Smad1/5/9, Runx2 and GAPDH at 72 h after rhCHRDL1 and rhBMP-4 addition separately or in combination. GAPDH was used as loading control. All experiments were repeated independently in triplicate.

Supplementary Figure 4 | rhCHRDL1 addition promoted bone repair in a mouse model of femoral bone defect. **(A)** Representative images of lateral views of 3D reconstruction of defective femur and mineralized bone formed in hole region by micro-CT. **(B)** H & E staining also shows new bone accumulation in hole regions of control group and rhCHRDL1 treated mice. (Original magnification: 100×). All experiments were repeated independently in triplicate.

Supplementary Figure 5 | Knockdown of CHRDL1 did not affect osteoblasts, osteoclasts and fibroblasts in femoral bone defect model. Quantification of positively stained area of of OPG staining **(A)**, TRAP staining **(B)**, and Gomori methenamine silver staining **(C)** recognized by image J was also shown in graph. All experiments were repeated independently in triplicate.

REFERENCES

1. Xie H, Cui Z, Wang L, Xia Z, Hu Y, Xian L, et al. PDGF-BB secreted by preosteoclasts induces angiogenesis during coupling with osteogenesis. *Nat Med.* (2014) 20:1270–8. doi: 10.1038/nm.3668
2. Ikebuchi Y, Aoki S, Honma M, Hayashi M, Sugamori Y, Khan M, et al. Coupling of bone resorption and formation by RANKL reverse signalling. *Nature.* (2018) 561:195–200. doi: 10.1038/s41586-018-0482-7
3. Weitzmann M, Ofotokun I. Physiological and pathophysiological bone turnover - role of the immune system. *Nat Rev Endocrinol.* (2016) 12:518–32. doi: 10.1038/nrendo.2016.91
4. Pawaputanon Na Mahasarakham C, Ezura Y, Kawasaki M, Smriti A, Moriya S, Yamada T, et al. BMP-2 enhances Lgr4 gene expression in osteoblastic cells. *J Cell Physiol.* (2016) 231:887–95. doi: 10.1002/jcp.25180
5. Lee S, Long F. Notch signaling suppresses glucose metabolism in mesenchymal progenitors to restrict osteoblast differentiation. *J Clin Invest.* (2018) 128:5573–586. doi: 10.1172/JCI96221
6. Wu M, Chen G, Li YP. TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Res.* (2016) 4:16009. doi: 10.1038/boneres.2016.9
7. Lowery J, Rosen V. The BMP pathway and its inhibitors in the skeleton. *Physiol Rev.* (2018) 98:2431–52. doi: 10.1152/physrev.00028.2017
8. Yadin D, Knaus P, Mueller TD. Structural insights into BMP receptors:specificity, activation and inhibition. *Cytokine Growth Factor Rev.* (2016) 27:13–34. doi: 10.1016/j.cytogfr.2015.11.005
9. Liang C, Peng S, Li J, Lu J, Guan D, Jiang F, et al. Inhibition of osteoblastic Smurf1 promotes bone formation in mouse models of distinctive age-related osteoporosis. *Nat Commun.* (2018) 9:3428. doi: 10.1038/s41467-018-05974-z
10. Jensen ED, Gopalakrishnan R, Westendorf JJ. Regulation of gene expression in osteoblasts. *Biofactors.* (2010) 36:25–32. doi: 10.1002/biof.72
11. Krishnan L, Priddy LB, Esancy C, Klosterhoff BS, Stevens HY, Tran L, et al. Delivery vehicle effects on bone regeneration and heterotopic ossification induced by high dose BMP-2. *Acta biomater.* (2017) 49:101–12. doi: 10.1016/j.actbio.2016.12.012
12. Papanagiotou M, Dailiana ZH, Karachalios T, Varitimidis S, Hantes M, Dimakopoulos G, et al. Heterotopic ossification after the use of recombinant human bone morphogenetic protein-7. *World J Ortho.* (2017) 8:36–41. doi: 10.5312/wjo.v8.i1.36

13. Kane R, Godson C, O'Brien C. Chordin-like 1, a bone morphogenetic protein-4 antagonist, is upregulated by hypoxia in human retinal pericytes and plays a role in regulating angiogenesis. *Mol Vis.* (2008) 14:1138–48.
14. Gao W, Zhang S, Zhang H, Wan B, Yin Z. Chordin-like protein 1 promotes neuronal differentiation by inhibiting bone morphogenetic protein-4 in neural stem cells. *Mol Med Rep*. (2013) 7:1143–8. doi: 10.3892/mmr.2013.1310
15. Larman B, Karolak M, Adams D, Oxburgh L. Chordin-like 1 and twisted gastrulation 1 regulate BMP signaling following kidney injury. *J Am Soc Nephrol*. (2009) 20:1020–31. doi: 10.1681/ASN.2008070768
16. Pei Y, Zhang Y, Lei Y, Wu D, Ma T, Liu X. Hypermethylation of the CHRDL1 promoter induces proliferation and metastasis by activating Akt and Erk in gastric cancer. *Oncotarget.* (2017) 8:23155–66. doi: 10.18632/oncotarget.15513
17. Cyr-Depauw C, Northey J, Tabariès S, Annis M, Dong Z, Cory S, et al. Chordin-like 1 suppresses bone morphogenetic protein 4-induced breast cancer cell migration and invasion. *Mol Cell Biol.* (2016) 36:1509–25. doi: 10.1128/MCB.00600-15
18. Urist MR. Bone:formation by autoinduction. *Science*. (1965) 150:893–99. doi: 10.1126/science.150.3698.893
19. Zhuang W, Ge X, Yang S, Huang M, Chen P, Zhang X, et al. Upregulation of lncRNA MEG3 promotes osteogenic differentiation of mesenchymal stem cells from multiple myeloma patients by targeting BMP4 transcription. *Stem Cells.* (2015) 33:1985–97. doi: 10.1002/stem.1989
20. Peng H, Wright V, Usas A, Gearhart B, Shen H, Cummins J, et al. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest.* (2002) 110:751–9. doi: 10.1172/JCI200215153
21. Chang C. Agonists and antagonists of TGF-beta family ligands. *Cold Spring Harb Perspect Biol*. (2016) 8:21923. doi: 10.1101/cshperspect.a021923
22. Serpe M, Umulis D, Ralston A, Chen J, Olson D, Avanesov A, et al. The BMP-binding protein Crossveinless 2 is a short-range, concentration-dependent, biphasic modulator of BMP signaling in Drosophila. *Dev Cell.* (2008) 14:940–53. doi: 10.1016/j.devcel.2008.03.023
23. Xiao F, Wang C, Gao Y, Zhang X, Chen X. BMPER enhances bone formation by promoting the osteogenesis-angiogenesis coupling process in mesenchymal stem cells. *Cell Physiol Biochem.* (2018) 45:1927–39. doi: 10.1159/000487969
24. Cui Z, Sun J, Baljon J, Fan J, Kim S, Wu B, et al. Simultaneous delivery of hydrophobic small molecules and siRNA using Sterosomes to direct mesenchymal stem cell differentiation for bone repair. *Acta biomaterialia*. (2017) 58:214–24. doi: 10.1016/j.actbio.2017.05.057
25. Chen C, Uludag H, Wang Z, Jiang H. Noggin suppression decreases BMP-2-induced osteogenesis of human bone marrow-derived mesenchymal stem cells *in vitro*. *J Cell Biochem.* (2012) 113:3672–80. doi: 10.1002/jcb.24240
26. Lin J, Patel S, Cheng X, Cho E, Levitan I, Ullenbruch M, et al. Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. *Nat Med.* (2005) 11:387–93. doi: 10.1038/nm1217
27. Fernandes H, Dechering K, van Someren E, Steeghs I, Apotheker M, Mentink A, et al. Effect of chordin-like 1 on MC3T3-E1 and human mesenchymal stem cells. cells tissues organs (print) (2010) 191:443–52. doi: 10.1159/000281825

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 Endocrinology

REVIEW
published: 10 January 2020
doi: 10.3389/fendo.2019.00908

Fibrodysplasia Ossificans Progressiva (FOP): A Segmental Progeroid Syndrome

Robert J. Pignolo[1,2,3]*, *Haitao Wang*[1,2,3] and *Frederick S. Kaplan*[4,5,6]

[1] Department of Medicine, Mayo Clinic Alix School of Medicine, Rochester, MN, United States, [2] Department of Physiology-Biomedical Engineering, Mayo Clinic Alix School of Medicine, Rochester, MN, United States, [3] Kogod Center on Aging, Mayo Clinic Alix School of Medicine, Rochester, MN, United States, [4] Department of Orthopaedic Surgery, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States, [5] Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States, [6] Center for Research in FOP and Related Disorders, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, United States

Segmental progeroid syndromes are commonly represented by genetic conditions which recapitulate aspects of physiological aging by similar, disparate, or unknown mechanisms. Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disease caused by mutations in the gene for ACVR1/ALK2 encoding Activin A receptor type I/Activin-like kinase 2, a bone morphogenetic protein (BMP) type I receptor, and results in the formation of extra-skeletal ossification and a constellation of others features, many of which resemble accelerated aging. The median estimated lifespan of individuals with FOP is approximately 56 years of age. Characteristics of precocious aging in FOP include both those that are related to dysregulated BMP signaling as well as those secondary to early immobilization. Progeroid features that may primarily be associated with mutations in ACVR1 include osteoarthritis, hearing loss, alopecia, subcutaneous lipodystrophy, myelination defects, heightened inflammation, menstrual abnormalities, and perhaps nephrolithiasis. Progeroid features that may secondarily be related to immobilization from progressive heterotopic ossification include decreased vital capacity, osteoporosis, fractures, sarcopenia, and predisposition to respiratory infections. Some manifestations of precocious aging may be attributed to both primary and secondary effects of FOP. At the level of lesion formation in FOP, soft tissue injury resulting in hypoxia, cell damage, and inflammation may lead to the accumulation of senescent cells as in aged tissue. Production of Activin A, platelet-derived growth factor, metalloproteinases, interleukin 6, and other inflammatory cytokines as part of the senescence-associated secretory phenotype could conceivably mediate the initial signaling cascade that results in the intense fibroproliferative response as well as the tissue-resident stem cell reprogramming leading up to ectopic endochondral bone formation. Consideration of FOP as a segmental progeroid syndrome offers a unique perspective into potential mechanisms of normal aging and may also provide insight for identification of new targets for therapeutic interventions in FOP.

Keywords: progeroid syndrome, fibrodysplasia ossificans progressiva, activin A, ACVR1, cell senescence

OPEN ACCESS

Edited by:
Teun J. De Vries,
VU University
Amsterdam, Netherlands

Reviewed by:
Jan Josef Stepan,
Charles University, Czechia
Michaël R. Laurent,
University Hospitals Leuven, Belgium
Jenneke Klein-Nulend,
VU University
Amsterdam, Netherlands

***Correspondence:**
Robert J. Pignolo
pignolo.robert@mayo.edu

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *03 September 2019*
Accepted: *12 December 2019*
Published: *10 January 2020*

Citation:
Pignolo RJ, Wang H and Kaplan FS (2020) Fibrodysplasia Ossificans Progressiva (FOP): A Segmental Progeroid Syndrome. Front. Endocrinol. 10:908. doi: 10.3389/fendo.2019.00908

INTRODUCTION

Aging may be a unique biological process, since evolutionarily there appears to be an absence of genes specifically selected to cause it (1, 2). Rather, age-related changes may be the unprogrammed results of optimization for early reproductive success. Thus, senescence at the organismal level represents a phenomenon with low mechanistic conservation among disparate metazoans and so mechanisms of human aging do not necessarily have metazoan counterparts in every situation. For example, replicative senescence or the loss of proliferative capacity in replication-competent somatic tissues is not a potential mechanism for aging in organisms where soma compartments are post-mitotic, such as *C. elegans* (3).

A complementary approach to studying a model system for aging in a lower organism is to directly study human aging. Although this would closely capture aspects of aging that are relevant to humans, it does not obviate consideration for the highly polygenic nature of age-related pathologies (4), the confounding effects of outbreeding, or environmental effects based on where and how individuals live. An approach to providing a scientifically tractable system, at least with respect to the former, is to study genetic diseases whose phenotypes mimic at least some (i.e., "segmental") features of the usual human aging process (4, 5). Such segmental progeroid (i.e., premature or accelerated aging-like) syndromes are usually monogenic and may thus be simple enough to provide insights into the causes of their pathology. Studied within the context of theories for physiological aging, observations made in segmental progeroid syndromes may also explain certain aspects of normal aging. Despite being only partial phenocopies of normal aging (i.e., some tissues show aging features and other not), these segmental progeroid syndromes provide experimental tractability, with varying fidelity, that is the rationale for their use as paradigm for natural deteriorative changes that occur over time. Single-gene mutations that impact multiple aspects of the physiological aging phenotype may exert their action through developmental alterations that have consequences for post-maturational aging, and importantly, for regulation of the rates of post-maturational aging after normal development.

Here we propose that consideration of fibrodysplasia ossificans progressiva (FOP) as a segmental progeroid syndrome offers a unique perspective into potential mechanisms of normal aging and may also provide insight for identification of new targets for therapeutic interventions in FOP.

SEGMENTAL PROGEROID SYNDROMES AS A MODEL TO INVESTIGATE HUMAN AGING

Representative segmental progeroid syndromes are shown in **Table 1**. Several are monogenic or at least affect the same or similar pathways when more than one gene causes the same phenotype within the same syndrome. The putative mechanism(s) by which aging phenotypes are manifested are similar in several syndromes, including decreased genome maintenance and accelerated cellular senescence. All of the syndromes reduce mean lifespan or life expectancy.

In the case of FOP, possible mechanisms for generation of an accelerated aging phenotype include injury-induced senescence and overactive activin A signaling. In comparison to other segmental progeroid syndromes, FOP represents an opportunity to study two different mechanisms by which aging phenotypes may be produced. Injury-induced senescence, especially in soft tissue such as muscle, has recently been described (8, 9) and muscle injury is a known cause of episodic inflammatory exacerbations or flare-ups in FOP (10, 11). Increased signaling through the bone morphogenetic protein (BMP) pathway, especially by activin A, has been implicated in osteoarthritis, sarcopenia, neurodegeneration, and other features associated with aging and FOP (discussed below under section Segmental Progeroid Features of FOP). Furthermore, activin A is a component of the senescence-associated secretory phenotype (SASP) (12, 13) and in FOP the mutated ACVR1/ALK2 encoding Activin A receptor type I/Activin-like kinase 2 (ACVR1/ALK2), a BMP type I receptor, is exquisitely sensitive to increased levels (14). Thus, injury-induced senescence leading to increased production of activin A may precipitate flare-ups in FOP and increased BMP signaling through activating mutations in ACVR1 may contribute to accelerated age-related changes in certain tissues. To test this hypothesis it will be necessary to examine the senescent cell burden in FOP lesion formation using markers of senescence in both patient samples and mouse models of FOP as well as analysis of the SASP in mouse models of FOP.

FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP)

FOP is a strongly debilitating genetic disorder with hallmark features of congenital first toe malformations, progressive heterotopic ossification (HO) that produces normal bone at extra-skeletal locations, and accelerated features of aging (10, 11). The worldwide prevalence is 1/1,300,000–1/2,000,000 (15, 16). There is no ethnic, racial, gender, or geographic predilection to FOP. Early in life, episodic bouts of inflammatory soft tissue protuberances (i.e., exacerbations or flare-ups) develop which are often caused by injury, intramuscular injections, viral infections, muscular overuse, or fatigue (17, 18). These exacerbations convert connective tissues, including skeletal muscle, into HO. Tendons, ligaments, fascia, and aponeuroses are also affected, and together with transformed muscle, result in joint ankyloses and immobility. Atypical forms of FOP have been reported (19). Approximately 97% of patients with FOP harbor an activating mutation (617G > A; R206H) in ACVR1/ALK2 (6). Individuals with FOP variants also have heterozygous ACVR1 missense mutations in conserved amino acids. FOP is diagnosed clinically, with confirmation by genetic testing if available. The majority of FOP cases are sporadic (i.e., non-inherited mutations), but a small number of cases demonstrate germline transmission with inheritance in an autosomal dominant fashion (6). Although progressive HO is a hallmark feature, changes in early adulthood reminiscent of premature aging are evident.

TABLE 1 | Representative segmental progeroid syndromes including FOP (4–7).

Syndrome	Inheritance	Approximate mean life-span (years)	Causative mutation	Possible mechanistic relevance to natural aging
Down	*De novo* trisomy	60	Many genes involved in phenotype[a]	Decreased genome maintenance
Werner	Autosomal recessive	47	WRN	Decreased genome maintenance; altered DNA damage responses; accelerated cell senescence
Dyskeratosis congenita[b]	X-linked; autosomal dominant	Variable[c]	DKC1; TERC	Accelerated cell senescence
Cockayne	Autosomal recessive	20	CS-A (ERCC8); CS-8 (ERCC6)	Decreased genome maintenance
Hutchinson-Gilford	Dominant negative	12	LMNA	Altered DNA damage responses; accelerated cell senescence
Ataxia telangiectasia	Autosomal recessive	20	ATM	Decreased genome maintenance; Accelerated neurodegeneration; Reduced immune diversity
Berardinelli-Seip[d]	Autosomal recessive	40	AGPAT2; BSCL2	Altered insulin signaling; decreased membrane integrity; increased glycation damage
Fibrodysplasia ossifcans progressiva	Sporadic; autosomal dominant	56[e]	ACVR1 (ALK2)	Injury-induced senescence; overactive activin A-BMP pathway signaling

[a] *For examples, GATA1, JAK2, DSCR1, DYRK1A.*
[b] *Information shown for the two most common forms.*
[c] *Life expectancy ranges from infancy to 60s.*
[d] *Congenital generalized lipodystrophy type 1 and 2.*
[e] *Estimated median life expectation.*

Currently, there are no curative interventions, and the mainstay of treatment is focused on symptomatic relief using brief courses of high-dose corticosteroids for flare-ups, which may help to reduce the intense pain and edema associated with the early stages of ectopic bony lesion formation (10, 20). Steps to mitigate the likelihood of falls, decline in pulmonary function, and acquisition of viral infections are important prophylactic measures. The median life expectancy is about 56 years of age (7). Most patients require partial or complete assistance for ambulation by age 30, and common proximal causes of death include thoracic insufficiency syndrome and pneumonia (7). Factors contributing to the accelerated aging phenotype of FOP may be primarily related to ACVR1/ALK2 mutation, secondarily related to immobilization and disuse due to HO-associated joint ankyloses, or a combination of the two. Endpoints of current clinical trials focus on reducing heterotopic bone formation (20), but it is unclear if those therapies targeting mutant ACVR1/ALK2 signaling will also delay, prevent, or ameliorate the progeroid features of FOP. Furthermore, it is unclear if targeting the activin-A ligand, vs. the receptor or post-receptor pathways, will be sufficient to mitigate all aspects of the condition. Also, it is unknown if or how targeting activin-A and its signaling networks will impact its role in hypothalamic-pituitary-gonadal feedback.

SEGMENTAL PROGEROID FEATURES OF FOP

Progeroid features in FOP that may primarily be associated with mutations in ACVR1 include alopecia, subcutaneous lipodystrophy, hearing loss, myelination defects, osteoarthritis, heightened inflammation, menstrual abnormalities, and perhaps nephrolithiasis (**Table 2**).

TABLE 2 | Progeroid features in FOP.

System or tissue	Aging features in FOP	Characteristic(s) in FOP
Skin	Alopecia; Subcutaneous lipodystrophy (21–24)	Alopecia seen in both sexes (19, 25–27); lipodystrophy may be associated with jaw ankylosis or recurrent flare-ups
Central nervous	Hearing loss; Myelination defects	Conductive and sensorineural hearing loss (19, 28–30); re-myelination deficits (31–33)
Respiratory	Decreased vital capacity; Pulmonary hypertension	Restrictive pulmonary function (7, 34)
Bone	Osteoporosis; Fractures	Osteoporosis (secondary) (35)
Muscle	Sarcopenia (36–38)	Sarcopenia of disuse is prominent
Joint	Osteoarthritis (19, 39, 40)	Often symmetrical
Immune	Inflammation; Predisposition to respiratory infections (7)	Acute inflammatory episodes (flare-ups) (10); chronic inflammatory state (41–45)
Reproductive	Menstrual abnormalities	Amenorrhea (19, 46, 47)
Renal	Nephrolithiasis (48)	Three times more likely compared to general population (49)

Alopecia is frequently observed in individuals with FOP and clinically is seen in both males and females. Evidence suggests that BMP signaling is involved in the control of the hair cycle (25). Increased BMP signaling through expression of BMP4, or its inhibition by the antagonist Noggin, causes progressive alopecia (26). In androgen-dependent alopecia, elevated BMP signaling in early (refractory) telogen likely mediates the retention of quiescent bulge stem cells (27). The case for elevated BMP signaling in lipodystrophy is less direct. Increased Fra-1 causes severe lipodystrophy (21) and both BMP-2 and TGF-β stimulate AP-1 activities, including the DNA binding activity of Fra-1 (22). Alternative explanations for subcutaneous lipodystrophy include decreased caloric intake after jaw ankylosis and the effects of recurrent inflammatory flare-ups. With respect to the latter, activation of the NF-κβ pathway during periods of acute or chronic inflammation may contribute to loss of subcutaneous fat. For example, activation of the NF-κβ pathway due to ubiquitination defects has been associated with lipodystrophy (23, 24).

Conductive and sensorineural hearing loss are common in FOP (28) and with prebycusis. Conductive hearing loss occurs when sound waves are not relayed efficiently to the inner ear, while sensorineural hearing loss is related to sensory organ (cochlea and associated structures) dysfunction or damage to the vestibulocochlear nerve (cranial nerve VIII). In humans, *NOGGIN* (*NOG*) gene mutations are associated with a few autosomal dominant conditions like proximal symphalangism and multiple synostoses which are characterized by skeletal defects and fusion of adjacent bones. Synostosis of one or more ossicles in the ear promotes conductive hearing loss. Proper formation of the skeleton requires balanced levels of BMPs and Noggin and the conductive hearing loss in $Nog^{+/-}$mice results from an ectopic bridge of bone between the stapes and the tympanum, interfering with the normal mobility of the ossicle (29). BMP signaling is also required for inner ear development, including patterning of sensory regions in the cochlea that process sound (30). It is likely that hearing loss in FOP is due to increased BMP signaling very early in life affecting both/either the cochlear sensory regions and/or motion of ossicles. Later in life, synostosis of the ossicles due to HO may be the predominant cause of progressive hearing loss.

Demyelinated lesions and focal inflammatory changes of the CNS are seen in both mouse models of FOP and in CNS white matter lesions in FOP patients (31). BMP signaling is a potent inhibitor of oligodendroglial differentiation and remyelination (32), and gain-of-function mutations in ACVR1/ALK2 predictably enhance this potent inhibition. Dysregulated BMP signaling causes CNS demyelination, and CNS demyelination is one of the underlying mechanisms for the observed atypical neurologic phenotypes in FOP patients. With normal aging, decreased CNS remyelination becomes more prominent over time (33, 50). In addition, aging is associated with decreased hippocampal neurogenesis and concomitant hippocampus-dependent cognitive functions (51). There is an inverse relationship between CNS levels of BMP4 expression and noggin with age, with the former increasing substantially in the mouse dentate gyrus and the latter decreasing. This results in a profound elevation of phosphorylated-SMAD1/5/8, a key effector of BMP signaling. As with aging in mice, a large increase in BMP4 expression is seen in the dentate gyrus of older humans without known cognitive dysfunction (51). Increased BMP signaling is related to impairments in neurogenesis and to age-related cognitive changes (51) and aspects of these processes may be phenocopied in FOP.

Accelerated osteoarthritis is commonly found in FOP. Terminal differentiation of chondrocytes may be delayed or prevented by abrogation of BMP signaling in articular cartilage, and mitigation of this blockage or increased BMP signaling may then contribute to endochondral ossification and breakdown of cartilage matrix (39). In cartilage, TGFß and BMP are necessary for normal joint development and maintenance and their dysregulation has been associated with the pathogenesis of osteoarthritis. Interestingly, osteoarthritic patients have significantly higher serum levels of BMP-2 and BMP-4 compared to non-diseased humans and appear to characterize patients who have degenerative joint disease severe enough to require total joint replacement (40).

Heightened inflammation in FOP can be acute (as in episodic flare-ups) as well as chronic (as in an elevated pro-inflammatory state). The inflammatory nature of flare-ups in FOP is clinically obvious and well-described (41). In FOP patients without clinically evident HO, increased serum levels of cytokines, including IL3-, IL-7, IL-8, and IL-10, suggest a persistent pro-inflammatory state (42). So-called "inflammaging" refers to the chronic, sterile, low-grade inflammation which develops as part of normal aging, and is thought to contribute to the pathogenesis of multiple age-related diseases (43). In FOP, both acute and chronic inflammation may be related to the role of activin A in the initiation and persistence of the inflammatory response (44, 45).

Early menstrual abnormalities in FOP, including amenorrhea, are clinically recognized but have not been objectively studied or described. Roles for activin A in the ovulation cycle as well as in endometrial repair after menses have been reported and are perhaps causally related (46, 47).

Progeroid features in FOP that may secondarily be related to immobilization from progressive HO include decreased vital capacity, osteoporosis, fractures, sarcopenia, and predisposition to respiratory infections (**Table 2**). These manifestations represent an opportunity to study the contribution of disuse to the normal aging phenotype typified by the decreased physical activity, sedentary predilection, and increased likelihood of prolonged bed rest in older adults. If physiological aging is the result of primary aging processes interacting with or superimposed upon the pathophysiological consequences of inactivity (36), then specific characteristics of precocious aging in FOP due to disuse would be amenable to study in isolation. As an illustration, unloading of the normotopic skeleton due to bridging heterotopic bone results in osteoporosis. Another example is the increase in chest wall rigidity and decreases in elastic recoil and force-generating capacity of respiratory muscles that contribute to diminished vital capacity and predisposition to respiratory infection.

Some manifestations of precocious aging may be attributed to both primary and secondary effects of FOP. Sarcopenia in FOP likely represents the effects of both disuse atrophy due to joint ankyloses as well as increased activin A signaling causing both increased muscle catabolism and inhibition of myoblast differentiation (37, 38). Nephrolithiasis in FOP could be related to inadequate fluid intake due to functional difficulties in voiding, immobilization itself, and the effects of activin A on kidney function (48, 49).

INJURY, REPROGRAMMING *IN VIVO*, AND CELLULAR SENESCENCE

Injury, in general, is associated with accumulation of senescent cells [see (8) and **Figure 1**]. Growing evidence suggests that injury-induced reprogramming in skeletal muscle is facilitated by the accumulation of senescent cells at or near the site of damaged tissue (8). Bothe acute and chronic injury enables transcription-factor-mediated reprogramming in damaged muscle (8). The reprogramming effect of senescence appears to be due to the release of interleukin 6 (IL-6) and perhaps other components of the senescence-associated secretory phenotype (SASP) (8, 52). Senescence and the SASP facilitate the reprogramming of neighboring non-senescent cells but also recruit macrophages for the removal of necrotic tissue (8).

Paracrine release of IL-6 and other factors secreted by senescent cells promote reprogramming by Oct4, Sox2, Klf4, and c-Myc (OSKM) in non-senescent cells (53) into pluripotent cells (also called induced pluripotent stem cells or iPSCs). A direct relationship has been demonstrated between senescence and OSKM-driven reprogramming. In cells lacking p16INK4a/ARF (i.e., cells that do not undergo senescence), their ability to reprogram is severely compromised (9, 53). Furthermore, pharmacological inhibition of NF*k*B, a major driver of cytokine production and the SASP, reduces *in vivo* reprogramming (9). Aging, which is associated with higher levels of cellular senescence, also favors OSKM-driven reprogramming. Similarly, in physiological conditions of wound healing, senescence triggered by injury could promote cell dedifferentiation to mediate repair of damaged tissue (9, 53).

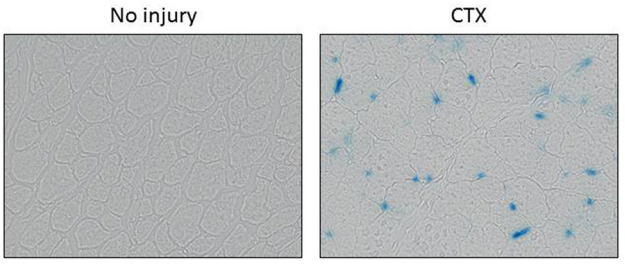


FIGURE 1 | Muscle injury-induced senescence. Senescence-associated β-galactosidase (SAβ-gal) staining of the tibialis anterior muscle of a wild-type mouse is shown without injury **(left)** and 5 days after injury via cardiotoxin (CTX) injection. SAβ-gal-stained cells appear blue. Images are courtesy of Haitao Wang, Ph.D., Mayo Clinic, Rochester, MN, USA).

ROLES OF CELLULAR SENESCENCE IN FOP LESION FORMATION

In FOP, injuries due to soft tissue trauma, viral infection, muscular stretching, and even fatigue due to overuse can precipitate a flare-up. Tissue damage causes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in response to microbial and endogenous injury in the setting of a hypoxic microenvironment (54, 55). As the result of tissue injury, senescent cells accumulate and potentially contribute to early events that enhance BMP signaling and facilitate the reprogramming of tissue-resident stem cells (**Figure 2**).

Senescence is a cellular response to damage characterized by an irreversible cell cycle arrest and then by the SASP (56, 57). The SASP produces at least two factors that can directly promote increased BMP signaling and stem cells reprogramming—activin A and IL-6, respectively (**Figure 2**). It is well-established that activin A stimulates BMP signaling in FOP cells, owing to the causative mutations in the ACVR1 gene. In addition to the permissive effects of IL-6 in reprogramming, FOP cells show an increased efficiency of iPSC generation (58). In normal cells, the generation of iPSCs is facilitated by transduction of mutant ACVR1 or SMAD1 or by the early addition of BMP4 during the reprogramming. ID genes, downstream targets of BMP-SMAD signaling, are important for iPSC generation and their signaling through this pathway can inhibit cell senescence due to p16/INK4A, which otherwise serves to prevent reprogramming (58). Thus, ID1 and other ID genes may serve to both enhance expansion of the FOP early lesion as well as stimulate production of osteochondral progenitor cells. Enhanced BMP signaling promotes a tremendous fibroproliferative response, perhaps further accelerated by secretion of platelet-derived growth factor (PDGF) and matrix metalloproteinases (MMPs) by the SASP (**Figure 2**). Osteochondral progenitor cells derived from reprogrammed stem cells ultimately contribute to the endochondral bone formation which is the hallmark of mature FOP lesions. Other events that may contribute to formation of heterotopic bone cannot be excluded (59). However, in mouse models it will be possible to demonstrate if senescence-mediated tissue reprogramming in FOP lesions shifts lineage determination from a myogenic to a chondrogenic fate after injury.

Senescent cells may play multiple roles in the formation of HO in FOP and drugs which target senescent cells and/or the SASP may be candidates for therapeutic interventions. Compounds which selectively clear senescent cells (so-called senolytics) were first described on the basis of targeting pro-survival networks in senescent cells (60). Compounds that reduce the SASP (i.e., senomodulators), including inhibitors of the JAK/STAT pathway that plays an important role in regulating cytokine production, reduce systemic and adipose tissue inflammation in old mice (61). Rapamycin, a senomodulator, may be of particular benefit in FOP, since it also reduces activin-A mediated mTOR signaling (62). Many senotherapeutic agents have been reported, are effective in delaying or alleviating multiple age-related conditions

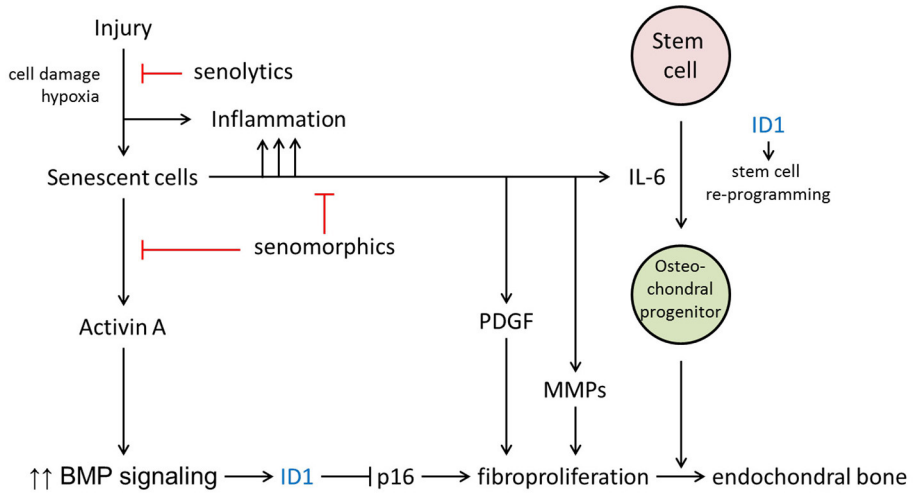


FIGURE 2 | Potential roles for cellular senescence in FOP lesion formation. The major hypothesized contributions of senescence are through the production of activin A, IL-6, and other components of the SASP. ⊤, inhibitory pathways; ⊤, inhibitory action of senotherapeutic drugs.

in pre-clinical models, and are now being evaluated in clinical trials (63, 64). Their potential use in FOP offers a novel therapeutic approach to injury-induced flare-ups in FOP which should be further explored. We propose that senescent cell clearance and/or reduction in the SASP will ameliorate HO formation in mouse models of FOP and can be translated for use in patients with FOP.

CONCLUSIONS

Monogenic segmental progeroid syndromes are important models for studying aspects of physiological aging. Features of precocious aging in FOP include both those that are related to dysregulated BMP signaling as well as those secondary to early immobilization and disuse. At the level of lesion formation in FOP, soft tissue injury resulting in hypoxia, cell damage, and inflammation may result in the accumulation of senescent cells as in aged tissue. Production of Activin A, interleukin 6, and other inflammatory cytokines as part of the SASP could mediate the initial signaling cascade that results in intense fibrosis as well as tissue-resident stem cell reprogramming prior to ectopic endochondral bone formation. This proposal requires experimental validation, but is amendable to testing in animal models. Consideration of FOP as a segmental progeroid syndrome may offer a unique perspective into potential mechanisms of normal aging, may increase understanding of BMP signaling as related to bone homeostasis and repair, and may also provide insight for identification of new targets for therapeutic interventions in FOP such as use of senotherapeutic drugs now in phase 1 and phase 2 clinical trials for aging-related conditions.

AUTHOR CONTRIBUTIONS

RP conceived the work, with substantial contributions from HW and FK. RP drafted the initial manuscript with HW and FK revising it critically. RP, HW, and FK gave final approval to the work and agreed to be accountable for all aspects.

ACKNOWLEDGMENTS

This work was enabled by support by the Radiant Hope Foundation (to RP, HW), the Robert and Arlene Kogod Professorship in Geriatric Medicine (to RP), and the Isaac and Rose Nassau Professorship of Orthopedic Molecular Medicine (to FK).

REFERENCES

1. Kirkwood TB. Human senescence. *Bioessays*. (1996) 18:1009–16. doi: 10.1002/bies.950181211
2. Partridge L, Gems D. The evolution of longevity. *Curr Biol*. (2002) 12:R544–6. doi: 10.1016/S0960-9822(02)01048-5
3. Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, et al. Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. *Nature*. (2002) 419:808–14. doi: 10.1038/nature01135
4. Martin GM. Genetics and aging; the Werner syndrome as a segmental progeroid syndrome. *Adv Exp Med Biol*. (1985) 190:161–70. doi: 10.1007/978-1-4684-7853-2_5
5. Martin GM. Genetic syndromes in man with potential relevance to the pathobiology of aging. *Birth Defects Orig Artic Ser*. (1978) 14:5–39.
6. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nat Genet*. (2006) 38:525–7. doi: 10.1038/ng1783
7. Kaplan FS, Zasloff MA, Kitterman JA, Shore EM, Hong CC, Rocke DM. Early mortality and cardiorespiratory failure in patients with

fibrodysplasia ossificans progressiva. *J Bone Joint Surg Am*. (2010) 92:686–91. doi: 10.2106/JBJS.I.00705
8. Chiche A, Le Roux I, von Joest M, Sakai H, Aguin SB, Cazin C, et al. Injury-induced senescence enables *in vivo* reprogramming in skeletal muscle. *Cell Stem Cell*. (2017) 20:407–14.e4. doi: 10.1016/j.stem.2016.11.020
9. Mosteiro L, Pantoja C, Alcazar N, Marion RM, Chondronasiou D, Rovira M, et al. Tissue damage and senescence provide critical signals for cellular reprogramming *in vivo*. *Science*. (2016) 354:aaf4445. doi: 10.1126/science.aaf4445
10. Pignolo RJ, Shore EM, Kaplan FS. Fibrodysplasia ossificans progressiva: clinical and genetic aspects. *Orphanet J Rare Dis*. (2011) 6:80. doi: 10.1186/1750-1172-6-80
11. Pignolo RJ, Shore EM, Kaplan FS. Fibrodysplasia ossificans progressiva: diagnosis, management, and therapeutic horizons. *Pediatr Endocrinol Rev*. (2013) 10(Suppl 2):437–48.
12. Kirkland JL, Tchkonia T. Cellular senescence: a translational perspective. *EBioMedicine*. (2017) 21:21–8. doi: 10.1016/j.ebiom.2017.04.013
13. Xu M, Palmer AK, Ding H, Weivoda MM, Pirtskhalava T, White TA, et al. Targeting senescent cells enhances adipogenesis and metabolic function in old age. *Elife*. (2015) 4:e12997. doi: 10.7554/eLife.12997.028
14. Wang H, Shore EM, Pignolo RJ, Kaplan FS. Activin A amplifies dysregulated BMP signaling and induces chondro-osseous differentiation of primary connective tissue progenitor cells in patients with fibrodysplasia ossificans progressiva (FOP). *Bone*. (2018) 109:218–24. doi: 10.1016/j.bone.2017.11.014
15. Baujat G, Choquet R, Bouee S, Jeanbat V, Courouve L, Ruel A, et al. Prevalence of fibrodysplasia ossificans progressiva (FOP) in France: an estimate based on a record linkage of two national databases. *Orphanet J Rare Dis*. (2017) 12:123. doi: 10.1186/s13023-017-0674-5
16. Connor JM, Evans DA. Genetic aspects of fibrodysplasia ossificans progressiva. *J Med Genet*. (1982) 19:35–9. doi: 10.1136/jmg.19.1.35
17. Pignolo RJ, Bedford-Gay C, Liljesthrom M, Durbin-Johnson BP, Shore EM, Rocke DM, et al. The natural history of flare-ups in fibrodysplasia ossificans progressiva (FOP): a comprehensive global assessment. *J Bone Miner Res*. (2016) 31:650–6. doi: 10.1002/jbmr.2728
18. Pignolo RJ, Kaplan FS. Clinical staging of Fibrodysplasia Ossificans Progressiva (FOP). *Bone*. (2018) 109:111–4. doi: 10.1016/j.bone.2017.09.014
19. Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, et al. Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. *Hum Mutat*. (2009) 30:379–90. doi: 10.1002/humu.20868
20. Kaplan FS, Pignolo RJ, Al Mukaddam MM, Shore EM. Hard targets for a second skeleton: therapeutic horizons for fibrodysplasia ossificans progressiva (FOP). *Expert Opin Orphan Drugs*. (2017) 5:291–4. doi: 10.1080/21678707.2017.1304211
21. Luther J, Driessler F, Megges M, Hess A, Herbort B, Mandic V, et al. Elevated Fra-1 expression causes severe lipodystrophy. *J Cell Sci*. (2011) 124(Pt 9):1465–76. doi: 10.1242/jcs.079855
22. Lai CF, Cheng SL. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. *J Biol Chem*. (2002) 277:15514–22. doi: 10.1074/jbc.M200794200
23. Aksentijevich I, Zhou Q. NF-κB pathway in autoinflammatory diseases: dysregulation of protein modifications by ubiquitin defines a new category of autoinflammatory diseases. *Front Immunol*. (2017) 8:399. doi: 10.3389/fimmu.2017.00399
24. Zhang Q, Lenardo MJ, Baltimore D. 30 years of NF-κB: a blossoming of relevance to human pathobiology. *Cell*. (2017) 168:37–57. doi: 10.1016/j.cell.2016.12.012
25. Rishikaysh P, Dev K, Diaz D, Qureshi WM, Filip S, Mokry J. Signaling involved in hair follicle morphogenesis and development. *Int J Mol Sci*. (2014) 15:1647–70. doi: 10.3390/ijms15011647
26. Botchkarev VA, Botchkareva NV, Roth W, Nakamura M, Chen LH, Herzog W, et al. Noggin is a mesenchymally derived stimulator of hair-follicle induction. *Nat Cell Biol*. (1999) 1:158–64. doi: 10.1038/11078
27. Nesterova A, Yuryev A. Androgenic alopecia: cross-talk between cell signal transduction pathways. In: *Hair and Scalp Disorders*. Intech (2017), p. 141–74. doi: 10.5772/67845
28. Levy CE, Lash AT, Janoff HB, Kaplan FS. Conductive hearing loss in individuals with fibrodysplasia ossificans progressiva. *Am J Audiol*. (1999) 8:29–33. doi: 10.1044/1059-0889(1999/011)
29. Hwang CH, Wu DK. Noggin heterozygous mice: an animal model for congenital conductive hearing loss in humans. *Hum Mol Genet*. (2008) 17:844–53. doi: 10.1093/hmg/ddm356
30. Ohyama T, Basch ML, Mishina Y, Lyons KM, Segil N, Groves AK. BMP signaling is necessary for patterning the sensory and nonsensory regions of the developing mammalian cochlea. *J Neurosci*. (2010) 30:15044–51. doi: 10.1523/JNEUROSCI.3547-10.2010
31. Kan L, Kitterman JA, Procissi D, Chakkalakal S, Peng CY, McGuire TL, et al. CNS demyelination in fibrodysplasia ossificans progressiva. *J Neurol*. (2012) 259:2644–55. doi: 10.1007/s00415-012-6563-x
32. Sabo JK, Aumann TD, Merlo D, Kilpatrick TJ, Cate HS. Remyelination is altered by bone morphogenic protein signaling in demyelinated lesions. *J Neurosci*. (2011) 31:4504–10. doi: 10.1523/JNEUROSCI.5859-10.2011
33. Shields SA, Gilson JM, Blakemore WF, Franklin RJ. Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. *Glia*. (1999) 28:77–83. doi: 10.1002/(SICI)1098-1136(199910)28:1<77::AID-GLIA9>3.0.CO;2-F
34. Kussmaul WG, Esmail AN, Sagar Y, Ross J, Gregory S, Kaplan FS. Pulmonary and cardiac function in advanced fibrodysplasia ossificans progressiva. *Clin Orthop Relat Res*. (1998) 104–9. doi: 10.1097/00003086-199801000-00015
35. Kaplan FS, Strear CM, Zasloff MA. Radiographic and scintigraphic features of modeling and remodeling in the heterotopic skeleton of patients who have fibrodysplasia ossificans progressiva. *Clin Orthop Relat Res*. 1994:238–47. doi: 10.1097/00003086-199407000-00037
36. Harridge SD, Lazarus NR. Physical activity, aging, and physiological function. *Physiology*. (2017) 32:152–61. doi: 10.1152/physiol.00029.2016
37. Ding H, Zhang G, Sin KW, Liu Z, Lin RK, Li M, et al. Activin A induces skeletal muscle catabolism via p38beta mitogen-activated protein kinase. *J Cachexia Sarcopenia Muscle*. (2017) 8:202–12. doi: 10.1002/jcsm.12145
38. Trendelenburg AU, Meyer A, Jacobi C, Feige JN, Glass DJ. TAK-1/p38/nNFκB signaling inhibits myoblast differentiation by increasing levels of Activin A. *Skelet Muscle*. (2012) 2:3. doi: 10.1186/2044-5040-2-3
39. Bauge C, Girard N, Lhuissier E, Bazille C, Boumediene K. Regulation and role of TGFβ signaling pathway in aging and osteoarthritis joints. *Aging Dis*. (2014) 5:394–405. doi: 10.14336/AD.2014.0500394
40. Albilia JB, Tenenbaum HC, Clokie CM, Walt DR, Baker GI, Psutka DJ, et al. Serum levels of BMP-2, 4, 7 and AHSG in patients with degenerative joint disease requiring total arthroplasty of the hip and temporomandibular joints. *J Orthop Res*. (2013) 31:44–52. doi: 10.1002/jor.22182
41. Convente MR, Wang H, Pignolo RJ, Kaplan FS, Shore EM. The immunological contribution to heterotopic ossification disorders. *Curr Osteoporos Rep*. (2015) 13:116–24. doi: 10.1007/s11914-015-0258-z
42. Barruet E, Morales BM, Cain CJ, Ton AN, Wentworth KL, Chan TV, et al. NF-κB/MAPK activation underlies ACVR1-mediated inflammation in human heterotopic ossification. *JCI Insight*. (2018) 3:122958. doi: 10.1172/jci.insight.122958
43. Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: a new immune-metabolic viewpoint for age-related diseases. *Nat Rev Endocrinol*. (2018) 14:576–90. doi: 10.1038/s41574-018-0059-4
44. Hedger MP, Winnall WR, Phillips DJ, de Kretser DM. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm*. (2011) 85:255–97. doi: 10.1016/B978-0-12-385961-7.00013-5
45. Jones KL, Mansell A, Patella S, Scott BJ, Hedger MP, de Kretser DM, et al. Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia. *Proc Natl Acad Sci USA*. (2007) 104:16239–44. doi: 10.1073/pnas.0705971104
46. Kaitu'u-Lino TJ, Phillips DJ, Morison NB, Salamonsen LA. A new role for activin in endometrial repair after menses. *Endocrinology*. (2009) 150:1904–11. doi: 10.1210/en.2008-0738
47. Lockwood GM, Muttukrishna S, Ledger WL. Inhibins and activins in human ovulation, conception and pregnancy. *Hum Reprod Update*. (1998) 4:284–95. doi: 10.1093/humupd/4.3.284
48. Maeshima A, Miya M, Mishima K, Yamashita S, Kojima I, Nojima Y. Activin A: autocrine regulator of kidney development and repair. *Endocr J*. (2008) 55:1–9. doi: 10.1507/endocrj.KR-113

49. Gupta RR, Delai PLR, Glaser DL, Rocke DM, Al Mukaddam M, Pignolo RJ, et al. Prevalence and risk factors for kidney stones in fibrodysplasia ossificans progressiva. *Bone*. (2018) 109:120–3. doi: 10.1016/j.bone.2017.12.010
50. Sim FJ, Zhao C, Penderis J, Franklin RJ. The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *J Neurosci*. (2002) 22:2451–9. doi: 10.1523/JNEUROSCI.22-07-02451.2002
51. Meyers EA, Gobeske KT, Bond AM, Jarrett JC, Peng CY, Kessler JA. Increased bone morphogenetic protein signaling contributes to age-related declines in neurogenesis and cognition. *Neurobiol Aging*. (2016) 38:164–75. doi: 10.1016/j.neurobiolaging.2015.10.035
52. Brady JJ, Li M, Suthram S, Jiang H, Wong WH, Blau HM. Early role for IL-6 signalling during generation of induced pluripotent stem cells revealed by heterokaryon RNA-Seq. *Nat Cell Biol*. (2013) 15:1244–52. doi: 10.1038/ncb2835
53. Mosteiro L, Pantoja C, de Martino A, Serrano M. Senescence promotes *in vivo* reprogramming through p16(INK)(4a) and IL-6. *Aging Cell*. (2018) 17:e12711. doi: 10.1111/acel.12711
54. Wang H, Behrens EM, Pignolo RJ, Kaplan FS. ECSIT links TLR and BMP signaling in FOP connective tissue progenitor cells. *Bone*. (2018) 109:201–9. doi: 10.1016/j.bone.2017.12.024
55. Wang H, Lindborg C, Lounev V, Kim JH, McCarrick-Walmsley R, Xu M, et al. Cellular hypoxia promotes heterotopic ossification by amplifying BMP signaling. *J Bone Miner Res*. (2016) 31:1652–65. doi: 10.1002/jbmr.2848
56. Freund A, Orjalo AV, Desprez PY, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol Med*. (2010) 16:238–46. doi: 10.1016/j.molmed.2010.03.003
57. Munoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol*. (2014) 15:482–96. doi: 10.1038/nrm3823
58. Hayashi Y, Hsiao EC, Sami S, Lancero M, Schlieve CR, Nguyen T, et al. BMP-SMAD-ID promotes reprogramming to pluripotency by inhibiting p16/INK4A-dependent senescence. *Proc Natl Acad Sci USA*. (2016) 113:13057–62. doi: 10.1073/pnas.1603668113
59. Ramirez DM, Ramirez MR, Reginato AM, Medici D. Molecular and cellular mechanisms of heterotopic ossification. *Histol Histopathol*. (2014) 29:1281–5. doi: 10.14670/HH-29.1281
60. Zhu Y, Tchkonia T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, et al. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. *Aging Cell*. (2015) 14:644–58. doi: 10.1111/acel.12344
61. Xu M, Tchkonia T, Ding H, Ogrodnik M, Lubbers ER, Pirtskhalava T, et al. JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age. *Proc Natl Acad Sci USA*. (2015) 112:E6301–10. doi: 10.1073/pnas.1515386112
62. Hino K, Horigome K, Nishio M, Komura S, Nagata S, Zhao C, et al. Activin-A enhances mTOR signaling to promote aberrant chondrogenesis in fibrodysplasia ossificans progressiva. *J Clin Invest*. (2017) 127:3339–52. doi: 10.1172/JCI93521
63. Kirkland JL, Tchkonia T, Zhu Y, Niedernhofer LJ, Robbins PD. The clinical potential of senolytic drugs. *J Am Geriatr Soc*. (2017) 65:2297–301. doi: 10.1111/jgs.14969
64. Tchkonia T, Kirkland JL. Aging, cell senescence, and chronic disease: emerging therapeutic strategies. *JAMA*. (2018) 320:1319–20. doi: 10.1001/jama.2018.12440

Conflict of Interest: 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 Endocrinology

REVIEW
published: 04 February 2020
doi: 10.3389/fendo.2019.00934

High Fidelity of Mouse Models Mimicking Human Genetic Skeletal Disorders

Robert Brommage[1]* *and Claes Ohlsson*[1,2]

[1] Department of Internal Medicine and Clinical Nutrition, Centre for Bone and Arthritis Research, Institute of Medicine, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden, [2] Department of Drug Treatment, Sahlgrenska University Hospital, Gothenburg, Sweden

The 2019 International Skeletal Dysplasia Society nosology update lists 441 genes for which mutations result in rare human skeletal disorders. These genes code for enzymes (33%), scaffolding proteins (18%), signal transduction proteins (16%), transcription factors (14%), cilia proteins (8%), extracellular matrix proteins (5%), and membrane transporters (4%). Skeletal disorders include aggrecanopathies, channelopathies, ciliopathies, cohesinopathies, laminopathies, linkeropathies, lysosomal storage diseases, protein-folding and RNA splicing defects, and ribosomopathies. With the goal of evaluating the ability of mouse models to mimic these human genetic skeletal disorders, a PubMed literature search identified 260 genes for which mutant mice were examined for skeletal phenotypes. These mouse models included spontaneous and ENU-induced mutants, global and conditional gene knockouts, and transgenic mice with gene over-expression or specific base-pair substitutions. The human X-linked gene *ARSE* and small nuclear RNA *U4ATAC*, a component of the minor spliceosome, do not have mouse homologs. Mouse skeletal phenotypes mimicking human skeletal disorders were observed in 249 of the 260 genes (96%) for which comparisons are possible. A supplemental table in spreadsheet format provides PubMed weblinks to representative publications of mutant mouse skeletal phenotypes. Mutations in 11 mouse genes (*Ccn6, Cyp2r1, Flna, Galns, Gna13, Lemd3, Manba, Mnx1, Nsd1, Plod1, Smarcal1*) do not result in similar skeletal phenotypes observed with mutations of the homologous human genes. These discrepancies can result from failure of mouse models to mimic the exact human gene mutations. There are no obvious commonalities among these 11 genes. Body BMD and/or radiologic dysmorphology phenotypes were successfully identified for 28 genes by the International Mouse Phenotyping Consortium (IMPC). Forward genetics using ENU mouse mutagenesis successfully identified 37 nosology gene phenotypes. Since many human genetic disorders involve hypomorphic, gain-of-function, dominant-negative and intronic mutations, future studies will undoubtedly utilize CRISPR/Cas9 technology to examine transgenic mice having genes modified to exactly mimic variant human sequences. Mutant mice will increasingly be employed for drug development studies designed to treat human genetic skeletal disorders.

SIGNIFICANCE

Great progress is being made identifying mutant genes responsible for human rare genetic skeletal disorders and mouse models for genes affecting bone mass,

OPEN ACCESS

Edited by:
Elisabeth Marelise W. Eekhoff, VU University Medical Center, Netherlands

Reviewed by:
David M. Findlay, University of Adelaide, Australia
Jawed Akhtar Siddiqui, University of Nebraska Medical Center, United States

***Correspondence:**
Robert Brommage
brommage@outlook.com

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *02 September 2019*
Accepted: *23 December 2019*
Published: *04 February 2020*

Citation:
Brommage R and Ohlsson C (2020) High Fidelity of Mouse Models Mimicking Human Genetic Skeletal Disorders. Front. Endocrinol. 10:934. doi: 10.3389/fendo.2019.00934

architecture, mineralization and strength. This review organizes data for 441 human genetic bone disorders with regard to heredity, gene function, molecular pathways, and fidelity of relevant mouse models to mimic the human skeletal disorders. PubMed weblinks to citations of 249 successful mouse models are provided.

Keywords: skeletal dysplasia, skeletome, mouse models, genetic disease, nosology

INTRODUCTION

Rare human genetic diseases cumulatively affect about 1 in 200 individuals and involve an estimated 7,000 genes. Major research efforts are underway to identify these mutant genes and characterize their disease phenotypes. Knowledge gained can guide therapies and provide hypotheses to develop future treatments. As recently summarized (1), "Genome sequencing has revolutionized the diagnosis of genetic diseases. Close collaborations between basic scientists and clinical genomicists are now needed to link genetic variants with disease causation. To facilitate such collaborations, we recommend prioritizing clinically relevant genes for functional studies, developing reference variant-phenotype databases, adopting phenotype description standards, and promoting data sharing."

Rare human genetic skeletal dysplasias affect about 1 in 5,000 individuals (2) and account for 5% of all birth defects (3). The International Skeletal Dysplasia Society (ISDS, https://www.isds.ch), promotes scientific progress in the field of skeletal dysplasias and dysostoses, meets every second year, and published skeletal nosology summaries during 2001 (4), 2006 (5), 2010 (6), 2015 (7), and 2019 (8). There are presently 441 skeletal nosology genes, with an average of 20 new genes identified yearly (**Figure 1**). The classification aims to (i) identify metabolic pathways active in cartilage and bone, and their regulatory mechanisms; (ii) identify cellular signaling networks and gene expression sequences implicated in skeletal development; (iii) identify candidate genes for genetic disorders; (iv) facilitate integration of data coming from spontaneous and genetically engineered mouse mutants; (v) help in developing diagnostic strategies; (vi) stimulate the design and exploration of new therapeutic possibilities; and (vii) provide a knowledge framework accessible to physicians as well as to basic scientists and thus to facilitate communication between clinical genetics and pediatrics and the basic sciences (4).

The objectives of the present review include further characterizations of these 441 skeletal nosology genes and evaluating the reliability of mutant mouse models to mimic these human skeletal disorders.

HISTORICAL HIGHLIGHTS

Short stature and other visually obvious skeletal dysplasias were apparent throughout human history (9). The discovery of X-rays by Wilhelm Röntgen (10) was quickly followed by the description of osteopetrosis by Albers-Schönberg (11) and many skeletal dysplasias during the following decades (12). Dual-energy X-ray absorptiometry (DXA) technology, developed during the 1980s (13), permitting quantitation of bone mineral density (BMD), and continued advances in computed tomography (CT), providing 3 dimensional images, lead to increasing sophisticated understanding of bone dysmorphology. The first nosology gene identified was *CA2* (carbonic anhydrase 2, osteopetrosis), initially in 1983 using electrophoretic, enzymatic and immunologic techniques on red blood cell extracts (14), and subsequently by genetic mutation analysis in 1991 (15). The first genetic mutation for any human disease to be identified by WES was *DHODH* (dihydroorotate dehydrogenase), responsible for postaxial acrofacial dysostosis, in 2010 (16).

NOSOLOGY

Nosology is the classification of diseases, which in its simplest form involves symptoms and pathogenic mechanisms. No classification system is perfect and there are often multiple ways to classify a given disorder. At the extremes, "lumpers" and "splitters" prefer few and many categories, respectively (17). Heredity can be X-linked, autosomal dominant, or autosomal recessive. Skeletal dysplasias can affect the skeleton only, or be part of pleiotropic syndromes affecting multiple organs. Mutations of various genes within a molecular pathway can each produce similar phenotypes. Loss-of function (LoF) mutations completely disrupt the activities of their encoded proteins but hypomorphic mutations allowing reduced protein activities occur. Gain-of-function (GoF) mutations increase the activities of enzymes and receptors and produce different phenotypes than LoF mutations. Dominant-negative mutations adversely affect functions of wild-type proteins. Mutations can occur within the protein-coding region of the genome (exome), within introns, or between gene coding regions. Mutations include deletions, duplications, and inversions.

The 2019 edition of the ISDS Nosology and Classification of Skeletal Disorders database organizes mutant human skeletal phenotypes into 42 groups, based on clinical observations and known gene/phenotype relationships (8). A total of 461 disorders and 441 genes are provided, when all 10 genes listed within the Notes sections of the tables (**Table 1**) are included. Updated HGNU gene symbols for 11 genes (**Table 2**) are employed. **Supplemental Table 1** provides an alphabetical list in spreadsheet format of all 441 genes, with information on heredity, gene function and mouse model status. Genetic disorders are not listed, as mutations in many genes result in multiple phenotypes. Inheritance patterns are 242 autosomal recessive, 135 autosomal dominant, 34 autosomal recessive or autosomal dominant depending upon the exact mutation in the gene, 21 X-linked and 11 non-inherited, somatic mutations. Three genes can have either germline or somatic mutations.

RMRP encodes an RNA regulating DNA transcription, *RNU4ATAC* encodes an RNA that is a component of an enzyme

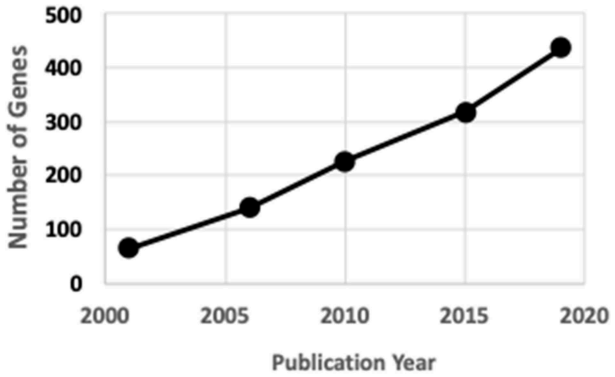


FIGURE 1 | ISDS Nosology gene identification.

TABLE 1 | Genes identified in 2019 Nosology notes section.

Gene	Model status	Nosology notes comments
AFF3 (LAF4 in notes)	Mouse model	Microdeletion on Chr 2
C2CD3	Mouse model	OFD phenotypes
COG1	No data	CDG type 2G
EED	Mouse	Weaver syndrome
LMBR1	Mouse model	Deletion affecting SHH ZRS
MACROH2A1 (H2AFY in notes)	Mouse model	Deletion—PITX1 ectopic activation
RASGRP2	Mouse	Osteopetrosis—leukocyte adhesion
SDC2	Mouse	Chr 8q22.1 duplication
SUZ12	Mouse model	Weaver syndrome
VANGL1 (STB2)	Mouse	Caudal regression—OMIM 600145

TABLE 2 | Gene symbol nomenclature.

Nosology gene symbol	HGNC gene symbol
CIAS1	NLRP3
CDC45L	CDC45
PPGB	CTSA
DHPAT	GNPAT
EVC1	EVC
FAM58A	CCNQ
HSGNAT	HGSNAT
LEPRE1	P3H1
PCNT2	PCNT
WISP3	CCN6
ZAK	MAP3K20

HGNC, Human genome organization gene nomenclature committee.

complex, and *MIR140* is a microRNA. Proteins (and the 3 RNAs) function as enzymes (146, 33%), scaffold components (79, 18%), ligand/receptor signaling molecules (72, 16%), transcription factors (62, 14%), cilia components (36, 8%), matrix proteins (23, 5%), membrane transporters (19, 4%), and cohesionopathy proteins (4, 1%). These eight gene function categories are informative but arbitrary, and other categories can be envisioned. For example, 23 enzymes are involved in the synthesis, processing, and degradation of protein and glycosaminoglycan matrix components. Skeletal disorders include malfunctions of lysosomal function. Signaling genes can be assigned to BMP, FGF, WNT, and other pathways.

There are no orthologous mouse genes for human *ARSE* (arylsulfatase E) and *RNU4ATAC* (RNA, U4atac small nuclear, U12-dependent splicing). **Supplemental Table 1** summarizes published data on the availability and fidelity of mouse models for the 439 human rare bone disease genes. Mutant mice with bone phenotypic data exist for 260 of the 439 genes (59%) with similar bone phenotypes observed for 249 (96%) genes. **Supplemental Table 2** contains PubMed hyperlinks to publications for all 249 genes provided in **Supplement Table 1** having mutant mouse bone phenotypes. These two supplemental tables should provide a major resource for the bone research community.

Mutant mouse bone data are inconsistent with human skeletal phenotypes for 11 genes (*Ccn6, Cyp2r1, Flna, Galns, Gna13, Lemd3, Manba, Mnx1, Nsd1, Plod1, Smarcal1*). There are no obvious explanations for or commonalities among these human-mouse phenotype inconsistencies. For 97 genes (22%) mutant mice have been generated and examined, but no skeletal data were reported. Mutant mice do not appear to have been examined for 82 genes (19%) and 36 (8%) of these genes belong to the understudied Ignorome/Dark Genome (18–20). Individual laboratories and/or consortia are encouraged to examine these genes, now known to contribute to poorly understood human rare bone diseases.

The number of bone nosology genes continues to increase as novel genes affecting skeletal metabolism are identified in human subjects. The genes described in this report form an arbitrary "snapshot" taken during August 2019 and will undoubtedly increase. Skeletal disorders for which mutant genes have not been identified include CDAGS syndrome (OMIM 603116), cherubism with gingival fibromatosis (OMIM 266270), chondrodysplasia punctata tibial-metacarpal type (OMIM 118651), dysplasia epiphysealis hemimelica (OMIM 127800), femur fibula ulna syndrome (OMIM 228200), hemifacial microsomia (OMIM 1642100, genochondromtosis (OMIM 1373600, Moreno–Nishimura–Schmidt syndrome (OMIM 608811), pachydermoperiostosis (OMIM 167100), and thoracolaryngopelvic dysplasia (OMIM 187760).

Formation of a normal skeleton involves BMP, FGF, and WNT signaling pathways and mutations in multiple genes within these pathways often produce skeletal dysplasias. Bone cells respond to parathyroid hormone, the active vitamin D metabolite calcitriol, and circulating FGF23 as part of the calcium-phosphate homeostatic system and disruptions in these hormones produce skeletal endocrinopathies. Skeletal disorders involving aggrecanopathies (13), channelopathies (21), ciliopathies (22, 23), cohesinopathies (24), lamiopathies (25), linkeropathies (26), protein-folding defects (27), ribosomopathies (28), spliceosomopathies (29), and transcription factors (30) show the importance of pathways not often thought to be involved in bone development.

SKELETAL DISORDER VIGNETTES

This section briefly summarizes selected skeletal disorders resulting from various mutations, highlighting the wide range of transcription and translation events that can be disrupted.

- Mutations can be benign with healthy nutrition but produce disease when key nutrients are lacking. All humans have an inactivating mutation in GULO, encoding an enzyme involved in the synthesis of ascorbic acid, and develop scurvy without sufficient dietary intake of vitamin C. The ascorbate synthetic pathway, involving aldehyde and aldose reductases, was only fully characterized in 2010 (31). Ascorbic acid is a required cofactor for the hydroxylation of proline and lysine residues in collagen and disruption of the mouse gulonolactone oxidase gene results in spontaneous bone fractures (32). Similarly, human and mouse *HAAO* and *KYNU* genes are involved in the synthesis of the enzymatic cofactor NAD and inactivating mutations in these human and mouse genes can result in congenital malformations (33).
- X-linked human mutations comprise 6% of the total skeletal disorders. X-inactivation of one of the two X chromosomes in women by long non-coding RNA specific transcript *XIST* occurs, but about 20% of X chromosome genes escape this inactivation (34). *AMER1* and *PORCN* are X-linked genes that code for components of the WNT signaling pathway, with dominant mutations in women causing osteopathia striata with cranial sclerosis and focal dermal hypoplasia (including osteopathia striata), respectively. Due to developmental lethality male patients are extremely rare, but a few males having post-zygotic mosaic mutations have been identified (35, 36). *Amer1* mutations in mice disrupt bone architecture (37) and treating adult mice with inhibitors of the PORCN enzyme reduces bone mass (38).
- Somatic gene mutations in 11 genes (*AKT1, FLBN, GNAS, GREM1, HRAS, IDH1, IDH2, MAP2K1, NOTCH2, NRAS, PIK3CA*) arise in the developing zygote and are not transmitted genetically. Loeys-Dietz syndrome includes several skeletal dysplasias and can result from mutations in *SMAD2*, *SMAD3*, *TGFB2, TGFB3*, *TGFBR1,* or *TGFBR2* and 75% of affected subjects have somatic mutations (39). Melorheostotic, dense hyperostotic bone lesions are caused by somatic mosaic mutations in *KRAS* (40) and *MAP2K1* (41). *MAP2K1* mutations are thought to arise after the formation of dorso-ventral plane (42). *KRAS* and *MAP2K1* are not included among the 441 Nosology disorders. Mutations in *COL11A*1, *EZH2*, and *MET* can be either germline or somatic.
- Deleterious mutations can occur at multiple sites within genes. For example, there are 1053 *COL1A1* DNA variants in the *Osteogenesis Imperfecta Variant Database* as of September 2019 (https://oi.gene.le.ac.uk/home.php?select_db=COL1A1, accessed 13 December, 2019).
- Splicing mutations that disrupt normal exon transcription within the spliceosome are estimated to contribute to 15% of human genetic diseases (43, 44). Acrofacial and mandibulofacial dysostosis often involve spliceosome defects and mutations in *EFTUD2, EIF4A3,* and *SF3B4* genes each result in distinct craniofacial phenotypes. Splice site mutations in *AIFM1* (45), *SERPINF1* (46), and *TRAPPC2* (47) result in skeletal dysplasias.
- MicroRNAs are non-protein coding single-stranded RNAs (48) that regulate gene expression in bone and other tissues. Mouse studies show that microRNA-140 is involved in growth plate development (49, 50). A gain-of function mutation in microRNA-140 results in human skeletal dysplasia (51).
- Subjects with intragenic duplications of *IFT81* (tandem duplication of exons 9 and 10) and *MATN3* (tandem duplication of exons 2–5), detected by WGS, have skeletal dysplasias similar to subjects with LoF mutations in these genes (52).
- Autosomal-dominant syndactyly, synpolydactyly, and brachydactyly types D and E can result from dominant-negative mutations in the homeobox gene *HOXD13*. Duplications of the *HOXD* gene cluster locus produce mesomelic dysplasia with shortened limbs (53, 54). Similar *Hoxd* locus GoF alterations in *ulnaless* mutant mice, generated by X-irradiation, produce similar bone phenotypes (55, 56).
- ISDS nosology includes skeletal disorders resulting from disruptions of calcium-phosphate homeostasis, including various endocrinopathies. Regulation of calcium and phosphorus homeostasis involves *ALPL, CASR, DMP1, ENPP1, FAM20C, FGF23, GALANT3, HRAS, KL, NRAS* and *TRPV6* genes. Parathyroid hormone synthesis and action involve *CDC73, FAM111A, GCM and PTH1R*. Vitamin D synthesis and actions involve *CYP2R1, CYP27B1* and *VDR*. Normal Ca and P homeostasis occurs in humans (57) and mice (58) with deletions of the GC gene and thereby lacking the circulating vitamin D-binding protein (DBP) that binds serum 25-OH-D. Multiple neonatal bone fractures were observed due to maternal hypoparathyroidism and vitamin D deficiency (59).

HEREDITY OF BONE MASS WITHOUT SKELETAL DYSPLASIA

Osteoporosis is a common skeletal disease in which reduced amounts of otherwise normal bone lead to fragility and fractures. Adult bone mass, even within the normal range, has a strong heredity influence (60, 61) and identifying the genes involved in bone mass accumulation during growth and loss during aging has received great interest within the context of the etiology and treatment of osteoporosis. GWAS studies over the past decade described an increasing number of genes affecting BMD, with 518 loci identified in the 2019 UK Biobank analysis (62). Juvenile osteoporosis, although not a true dysplasia as bone architecture is normal, usually has genetic causes (63, 64). There are healthy subjects with unexplained high bone mass (65, 66) and attempts are underway to identify the genes responsible. Recent discoveries of such genes include *LRP6* (67) and *SMAD9* (68).

MOUSE MODELS

All models are wrong, but some are less imperfect than others, and many are useful - George Box

Mouse models make important contributions to understanding and treating human diseases (69–72), including skeletal disorders (73, 74). Mutant mice that model human phenotypes also model successful drugs (75), help identify genes responsible for human genetic disorders and can provide insights for osteoporosis drug development (76). Bone mass and architecture vary in healthy humans and among laboratory mouse strains, with the most commonly studied C57BL/6 mouse strain an outlier having limb bones with high diameters and low cortical thickness (77–81).

The majority of mouse data summarized in this review involve individual investigator-initiated studies examining possible skeletal phenotypes in transgenic mice with specific alterations in genes chosen by the investigator. This approach, known as reverse genetics, utilizes the expertise of the laboratories involved.

In contrast, human studies involve forward genetics, with genes responsible for known skeletal phenotypes identified. Forward genetics is also employed in mouse studies, as genes responsible for spontaneous and mutagen-induced skeletal malformations are identified. The Jackson Laboratories (JAX), with a long history of studying mouse strains, recently employed WES to identify 14 genes having spontaneous mutations causing bone phenotypes (82, 83). Several laboratories employed N-ethyl-N-nitrosourea (ENU) in chemical mutagenesis campaigns to produce mouse lines having a wide-range of phenotypes. This approach yielded 41 genes having mutations causing bone phenotypes similar to the corresponding human skeletal disorders. These 41 genes with relevant citations are provided in **Supplemental Table 3**.

Two high-throughput mouse reverse genetics gene knockout phenotyping campaigns have been undertaken (84). The International Mouse Phenotyping Consortium (IMPC, www.mousephenotype.org) aims to characterize knockout mouse phenotypes for all 20,000 genes (74, 85). Lexicon Pharmaceuticals' Genome5000™ effort examining the druggable genome confirmed known bone phenotypes for 23 genes and identified 11 genes, including *Notum* (86), for which bone phenotypes were not previously characterized (87). Importantly, skeletal phenotypes were described for *Fam20c* (non-lethal Raine syndrome), *Lrrk1* (osteosclerotic metaphyseal dysplasia), *Pappa2* (short stature), *Sfrp4* (Pyle's disease), and *Slc10a7* (skeletal dysplasia) prior to knowledge of the human skeletal dysplasias when mutated in humans (84). For the 439 mouse genes discussed in this review, 149 genes have been examined by the IMPC, yielding 63 viable adult homozygous mouse mutants. Skeletal phenotypes (either body BMD or radiological dysmorphology) were observed for 28 genes. Results from the IMPC phenotyping campaign are summarized in **Table 3**.

TABLE 3 | Summary of International Mouse Phenotyping Campaign (IMPC) models.

Category	Number of genes
Total mouse protein-coding genes[a]	437 (100%)
Genes not assigned for IMPC analyses	52 (12% of total)
Genes with ES cells generated, but no mice	183 (42% of total)
Mice generated without phenotyping	56 (13% of total)
Mouse phenotyping completed	149 (34% of total)
Embryonic and preweaning lethality	86 (58% of 149 phenotypes)
Subviable (Few surviving homozygous mice)[b]	7 (5% of 149 phenotypes)
Lack of bone data[c]	5 (3% of 149 phenotypes)
No observed bone phenotypes[d]	23 (15% of 149 phenotypes)
Bone phenotypes[e]	28 (19% of 149 phenotypes)

[a] *No mouse genes for human ARSE and RNU4ATAC; Mir140 and Rmrp are RNA-coding genes.*
[b] *Cant1, Chst14, Dnajc21, Dnmt3a, Dock6, Egot, and Zswim6.*
[c] *Skeleton not tested for Dmp1, Map3k20, Snx10 and Sulf1; no BMD data for Ltbp2.*
[d] *Bgn, Bhlha9, Cc2d2a, Cfap410, Cyp2r1, Gpc6, Haao, Ick, Idh1, Idh2, Knyu, Npr3, Orc4, Picb4, Ptdss1, Pycr1, Serpinf1, Smarcal1, Tctex1d2, Thpo, Tmem165, and Trappc2.*
[e] *Low BMD for Hdac8, Lpin2, Nek1, P3h1, Phex, Plod1, Pls3, Setd2, Sparc and Wnt10b; high BMD for Col1a2, Fuca1, Gnas, Hgsnat, Lrrk1, and Sgsh; skeletal dysmorphology for Col9a2, Creb3l1, Ctsk, Ift80, Mmp9, Plekhm1, Sh3bp2, Suz12; low BMD and dysmorphology for Cyp27b1; homozygous lethality with adult heterozygous dysmorphology for Pitx1 and Pthlp; and homozygous lethality with fetal dysmorphology and adult heterozygous dysmorphology for Nxn.*

Mouse models of human genetic disorders are employed to evaluate potentially beneficial skeletal actions of therapies approved for other disease indications. Teriparatide treatment increases bone mass in *Lrp5* KO mice mimicking humans with *osteoporosis pseudoglioma syndrome* from loss of function *LRP5* mutations (88, 89). Similarly, anti-sclerostin antibody treatment increases bone mass in mutant mouse models with low bone mass from gene disruptions (90) of *Col1a1* (91, 92), *Col1a2* (93, 94), *Crtap* (95), *Dmp1* (96), *Lrp5* (97), and *Zmpste24* (98). Mechanistic hypotheses can be tested, such as periostin treatment retarding skull suture fusion in heterozygous *Twist1* mice with craniosynostosis (99).

MOUSE STUDY PRECAUTIONS

Several experimental pitfalls should be avoided when performing mouse studies (100).

- Knockout of individual genes can disrupt the functions of neighboring genes (101). Examples include the presence of orofacial defects resulting from a hypomorphic *Pax9* allele during knockout of the neighboring *Slc25a21* gene (102) and glycosaminoglycan accumulation resulting from reduced expression of the *Naglu* gene during knockout of the neighboring *Hsd17b1* gene (103).
- Transgenic Cre mouse lines are invaluable for conditionally activating or inactivating genes of interest. Several reporter genes are available for visualizing bone cells at different stages of development (104). But not all Cre lines are as specific as

originally believed (105–107). Understanding these limitations is critical for experimental design and interpretation.

- Quantitative PCR methods are often not optimized and MIQE (Minimum Information for the publication of qPCR Experiments) guidelines have been established (108, 109). Selection of the appropriate reference gene(s) is important (110–112).
- Many antibodies suffer from a lack of specificity resulting from cross-reactivity to similar epitopes present on multiple proteins. Clifford Saper in 2005, as Editor-in-Chief of *The Journal of Comparative Neurology,* repeatedly received "… distressed communications from authors … to withdraw papers because an antibody against a novel marker was found to stain tissue in knockout animals …" (113). Excellent reviews (not cited here) provide guidelines for successful antibody validation and the purposeful joviality in their titles ("*Antibody Can Get It Right … Antibody Anarchy … Antibody Crimes … A Guide to the Perplexed … Garbage In, Garbage Out … Hitchhiker Antigens … Not for the Faint-Hearted … The Dark Side of the Immunohistochemical Moon … The Good, Bad, and Really Ugly*") emphasizes the seriousness of the problem. Antibodies claimed to be specific for particular proteins should not react against tissues from KO mice missing the gene of interest and validation of antibody specificity using tissues from KO cells or mice is strongly encouraged.
- Established cell lines employed in conjunction with mouse studies can become contaminated and replaced by more robust, faster growing cells (114). Cell line authentication methods exist and should be employed (115, 116). MC3T3-E1 cell subclones vary as models of osteoblast biology (117).

LARGE ANIMAL AND ZEBRAFISH MODELS

Large animals can have advantages over rodents for understanding human genetic disease and drug development. Hypophosphatasia occurs in sheep (118) and dogs (119) having mutations in *ALPL*. Canine genetic skeletal disorders include mutations in *ADAMTSL2*—geleophysic dysplasia (120), *COL1A2*—osteogenesis imperfecta (121), *DVL2*—Robinow syndrome (122), *HES7*—spondylocostal dysostosis (123), and *SERPINH1*—osteogenesis imperfecta (124). Spontaneous mutations in chicken *KIAA0586* (125) *and LMBR1* (126) genes result in the expected bone phenotypes.

Zebrafish are increasing contributing to our knowledge of skeletal genomics (127, 128) and advantages over mouse models include acquiring data more rapidly. Zebrafish mutants have been described for several of the 441 genes in this review. One complication of zebrafish studies is that zebrafish underwent a teleost-specific whole genome duplication and have more than 26,000 protein-coding genes (129). There is a one-to-one relationship between 47% of human genes and a zebrafish ortholog. There are multiple zebrafish genes associated to a single human gene, and vice versa.

DRUG DEVELOPMENT

Exciting advances are being made in developing drug treatments for patients with genetic skeletal disorders (130, 131) and mouse models invariably contribute to this progress. These advances are best reviewed by the laboratories involved, but three examples are illustrative. An antibody to NOTCH2 reverses osteopenia in a mouse model of Hajdu-Cheney syndrome (132). Cinacalcet corrects hypercalcemia in a mouse model of familial hypercalcemia type 2 (133). ENPP1 enzyme replacement therapy improves blood pressure and cardiovascular function in a mouse model of generalized arterial calcification of infancy (134).

Understanding genetic skeletal disorders provides key knowledge for developing osteoporosis therapies (76, 135). Disruptions in genes coding for proteins in the RANK—RANKL—osteoprotegerin signaling pathway involved in osteoclast generation cause human skeletal disorders. The RANKL neutralizing antibody denosumab is a successful osteoporosis therapy. The recently approved anabolic osteoporosis treatment romosozumab, a sclerostin neutralizing antibody, was developed with knowledge gained from subjects with osteosclerosis resulting from *SOST* gene mutations. Subjects with pinocytosis have mutations in the cathepsin K coding gene *CTSK*. Treatment with odanacatib, an inhibitor of cathepsin K in osteoclasts, reduced bone fractures in postmenopausal women but cardiovascular side effects precluded regulatory approval.

FUTURE DIRECTIONS

Since many human disorders involve hypomorphic, gain-of-function, dominant-negative and intronic mutations, future studies will undoubtedly utilize CRISPR/Cas9 technology and other evolving techniques to examine transgenic mice having genes modified to exactly mimic variant human sequences (72, 136). RNA sequencing will increasingly be employed for diagnosis and mechanistic understanding of genetic diseases (137–141).

The IFMRS (International Federation of Musculoskeletal Research Societies), in collaboration with the Broad Institute, is establishing a Musculoskeletal Genomics Knowledge Portal (MGKP) to integrate, interpret and present human data linked to musculoskeletal traits and diseases (http://www.kp4cd.org/about/bone).

AUTHOR CONTRIBUTIONS

RB performed the literature search, data analyses, and prepared the manuscript. CO provided helpful suggestions and reviewed the manuscript.

FUNDING

This study was supported by funding from the Swedish Research Council (Grant 2016-01001), ALF/LUA research grant from the Sahlgrenska University Hospital, Lundberg

Foundation, the Torsten Söderberg Foundation, The Knut and Alice Wallenberg's Foundation and the Novo Nordisk Foundation.

ACKNOWLEDGMENTS

Matt Warman (Boston Children's Hospital) encouraged this project. Scott Youlten (Garvan Institute of Medical Research) independently extracted gene IDs from the 2019 ISDS Nosology paper and provided Ensembl gene IDs.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00934/full#supplementary-material

REFERENCES

1. Manolio TA, Fowler DM, Starita LM, Haendel MA, MacArthur DG, Biesecker LG, et al. Bedside back to bench: building bridges between basic and clinical genomic research. *Cell.* (2017) 169:6–12. doi: 10.1016/j.cell.2017.03.005
2. Geister KA, Camper SA. Advances in skeletal dysplasia genetics. *Annu Rev Genom Hum Genet.* (2015) 16:199–227. doi: 10.1146/annurev-genom-090314-045904
3. Tosi LL, Warman ML. Mechanistic and therapeutic insights gained from studying rare skeletal diseases. *Bone.* (2015) 76:67–75. doi: 10.1016/j.bone.2015.03.016
4. Superti-Furga A, Bonafé L, Rimoin DL. Molecular-pathogenetic classification of genetic disorders of the skeleton. *Am J Med Genet.* (2001) 106:282–93. doi: 10.1002/ajmg.10233
5. Superti-Furga A, Unger S. Nosology and classification of genetic skeletal disorders: 2006 revision. *Am J Med Genet A.* (2007) 143A:1–18. doi: 10.1002/ajmg.a.31483
6. Warman ML, Cormier-Daire V, Hall C, Krakow D, Lachman R, LeMerrer M, et al. Nosology and classification of genetic skeletal disorders: 2010 revision. *Am J Med Genet A.* (2011) 155A:943–68. doi: 10.1002/ajmg.a.33909
7. Bonafé L, Cormier-Daire V, Hall C, Lachman R, Mortier G, Mundlos S, et al. Nosology and classification of genetic skeletal disorders: 2015 revision. *Am J Med Genet A.* (2015) 167A:2869–92. doi: 10.1002/ajmg.a.37365
8. Mortier GR, Cohn DH, Cormier-Daire V, Hall C, Krakow D, Mundlos S, et al. Nosology and classification of genetic skeletal disorders: 2019 revision. *Am J Med Genet A.* (2019) 179:2393–419. doi: 10.1002/ajmg.a.61366
9. Kozma C. Skeletal dysplasia in ancient Egypt. *Am J Med Genet A.* (2008) 146A:3104–12. doi: 10.1002/ajmg.a.32501
10. Röntgen WG. On a new kind of rays. *Science.* (1896) 3:227–31. doi: 10.1126/science.3.59.227
11. Albers-Schönberg HE. Röntgenbilder einer seltenen Knochenerkrankung. *Münchener Medizinische Wochenschrift.* (1904) 51:365–76.
12. Superti-Furga A, Unger S. Genetic disorders of bone - An historical perspective. *Bone.* (2017) 102:1–4. doi: 10.1016/j.bone.2017.07.025
13. Lewiecki EM, Binkley N. DXA: 30 years and counting: Introduction to the 30th anniversary issue. *Bone.* (2017) 104:1–3. doi: 10.1016/j.bone.2016.12.013
14. Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, Tashian RE. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc Natl Acad Sci USA.* (1983) 80:2752–6. doi: 10.1073/pnas.80.9.2752
15. Venta PJ, Welty RJ, Johnson TM, Sly WS, Tashian RE. Carbonic anhydrase II deficiency syndrome in a Belgian family is caused by a point mutation at an invariant histidine residue (107 His-Tyr): complete structure of the normal human CA II gene. *Am J Hum Genet.* (1991) 49:1082–90.
16. Ng SB, Buckingham KJ, Lee C, Bigham AW, Tabor HK, Dent KM, Huff CD, et al. Exome sequencing identifies the cause of a mendelian disorder. *Nat Genet.* (2010) 42:30–5. doi: 10.1038/ng.499
17. McKusick VA. On lumpers and splitters, or the nosology of genetic disease. *Perspect Biol Med.* (1969) 12:298–312. doi: 10.1353/pbm.1969.0039
18. Pandey AK, Lu L, Wang X, Homayouni R, Williams RW. Functionally enigmatic genes: a case study of the brain ignorome. *PLoS ONE.* 9:e88889. doi: 10.1371/journal.pone.0088889
19. Oprea TI, Bologa CG, Brunak S, Campbell A, Gan GN, Gaulton A, et al. Unexplored therapeutic opportunities in the human genome. *Nat Rev Drug Discov.* (2018) 17:317–32. doi: 10.1038/nrd.2018.14
20. Stoeger T, Gerlach M, Morimoto RI, Nunes Amaral LA. Large-scale investigation of the reasons why potentially important genes are ignored. *PLoS Biol.* (2018) 16:e2006643. doi: 10.1371/journal.pbio.2006643
21. Gibson BG, Briggs MD. The aggrecanopathies; an evolving phenotypic spectrum of human genetic skeletal diseases. *Orphanet J Rare Dis.* (2016) 11:86. doi: 10.1186/s13023-016-0459-2
22. Huber C, Cormier-Daire V. Ciliary disorder of the skeleton. *Am J Med Genet C Semin Med Genet.* (2012) 160C:165–74. doi: 10.1002/ajmg.c.31336
23. Yuan X, Serra RA, Yang S. Function and regulation of primary cilia and intraflagellar transport proteins in the skeleton. *Ann NY Acad Sci.* (2015) 1335:78–99. doi: 10.1111/nyas.12463
24. Cohen-Zinder M, Karasik D, Onn I. Structural maintenance of chromosome complexes and bone development: the beginning of a wonderful relationship? *Bonekey Rep.* (2013) 2:388. doi: 10.1038/bonekey.2013.122
25. Zhang H, Kieckhaefer JE, Cao K. Mouse models of laminopathies. *Aging Cell.* (2013) 12:2–10. doi: 10.1111/acel.12021
26. Ritelli M, Cinquina V, Giacopuzzi E, Venturini M, Chiarelli N, Colombi M. Further defining the phenotypic spectrum of B3GAT3 mutations and literature review on linkeropathy syndromes. *Genes.* (2019) 10:E631. doi: 10.3390/genes10090631
27. Makareeva E, Aviles NA, Leikin S. Chaperoning osteogenesis: new protein-folding disease paradigms. *Trends Cell Biol.* (2011) 21:168–76. doi: 10.1016/j.tcb.2010.11.007
28. Trainor PA, Merrill AE. Ribosome biogenesis in skeletal development and the pathogenesis of skeletal disorders. *Biochim Biophys Acta.* (2014) 1842:769–78. doi: 10.1016/j.bbadis.2013.11.010
29. Lehalle D, Wieczorek D, Zechi-Ceide RM, Passos-Bueno MR, Lyonnet S, Amiel J, Gordon CT. A review of craniofacial disorders caused by spliceosomal defects. *Clin Genet.* (2015) 88:405–15. doi: 10.1111/cge.12596
30. Chatterjee S, Sivakamasundari V, Lee WJ, Chan HY, Lufkin T. Making no bones about it: transcription factors in vertebrate skeletogenesis and disease. *Trends Dev Biol.* (2012) 6:45–52.
31. Gabbay KH, Bohren KM, Morello R, Bertin T, Liu J, Vogel P. Ascorbate synthesis pathway: dual role of ascorbate in bone homeostasis. *J Biol Chem.* (2010) 285:19510–20. doi: 10.1074/jbc.M110.110247
32. Aghajanian P, Hall S, Wongworawat MD, Mohan S. The roles and mechanisms of actions of vitamin C in bone: new developments. *J Bone Miner Res.* (2015) 30:1945–55. doi: 10.1002/jbmr.2709
33. Shi H, Enriquez A, Rapadas M, Martin EMMA, Wang R, Moreau J, et al. NAD deficiency, congenital malformations, and niacin upplementation. *N Engl J Med.* (2017) 377:544–52. doi: 10.1056/NEJMoa1616361
34. Tukiainen T, Villani AC, Yen A, Rivas MA, Marshall JL, Satija R, et al. Landscape of X chromosome inactivation across human tissues. *Nature.* (2017) 550:244–8. doi: 10.1038/nature24265
35. Joseph DJ, Ichikawa S, Econs MJ. Mosaicism in osteopathia striata with cranial sclerosis. *J Clin Endocrinol Metab.* (2010) 95:1506–7. doi: 10.1210/jc.2009-2343
36. Vreeburg M, van Geel M, van den Heuij LG, Steijlen PM, van Steensel MA. Focal dermal hypoplasia in a male patient due to mosaicism for a novel PORCN single nucleotide deletion. *J Eur Acad Dermatol Venereol.* (2011) 25:592–5. doi: 10.1111/j.1468-3083.2010.03782.x

37. Comai G, Boutet A, Tanneberger K, Massa F, Rocha AS, Charlet A, Panzolini C, et al. Genetic and molecular insights into genotype-phenotype relationships in Osteopathia Striata with Cranial Sclerosis (OSCS) through the analysis of novel mouse Wtx mutant alleles. *J Bone Miner Res.* (2018) 33:875–87. doi: 10.1002/jbmr.3387
38. Funck-Brentano T, Nilsson KH, Brommage R, Henning P, Lerner UH, Koskela A, et al. Porcupine inhibitors impair trabecular and cortical bone mass and strength in mice. *J Endocrinol.* (2018) 238:13–23. doi: 10.1530/JOE-18-0153
39. Loeys BL, Dietz HC. Loeys-Dietz Syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, Amemiya A, editors. *GeneReviews®*. Seattle, WA: University of Washington (1993–2019).
40. Whyte MP, Griffith M, Trani L, Mumm S, Gottesman GS, McAlister WH, et al. Melorheostosis: exome sequencing of an associated dermatosis implicates postzygotic mosaicism of mutated KRAS. *Bone*. (2017) 101:145–55. doi: 10.1016/j.bone.2017.04.010
41. Fratzl-Zelman N, Roschger P, Kang H, Jha S, Roschger A, Blouin S, et al. Melorheostotic bone lesions caused by somatic mutations in MAP2K1 have deteriorated microarchitecture and periosteal reaction. *J Bone Miner Res.* (2019) 34:883–95. doi: 10.1002/jbmr.3656
42. Jha S, Laucis N, Kim L, Malayeri A, Dasgupta A, Papadakis GZ, et al. CT analysis of anatomical distribution of melorheostosis challenges the sclerotome hypothesis. *Bone*. (2018) 117:31–6. doi: 10.1016/j.bone.2018.09.005
43. Park E, Pan Z, Zhang Z, Lin L, Xing Y. The expanding landscape of alternative splicing variation in human populations. *Am J Hum Genet.* (2018) 102:11–26. doi: 10.1016/j.ajhg.2017.11.002
44. Scotti MM, Swanson MS. RNA mis-splicing in disease. *Nat Rev Genet.* (2016) 17:19–32. doi: 10.1038/nrg.2015.3
45. Miyake N, Wolf NI, Cayami FK, Crawford J, Bley A, Bulas D, et al. Neurogenetics X-linked hypomyelination with spondylometaphyseal dysplasia (H-SMD) associated with mutations in AIFM1. *Neurogenetics.* (2017) 18:185–94. doi: 10.1007/s10048-017-0520-x
46. Jin Z, Burrage LC, Jiang MM, Lee YC, Bertin T, Chen Y, et al. Whole-exome sequencing identifies an intronic cryptic splice site in SERPINF1 causing osteogenesis imperfecta Type VI. *JBMR Plus*. (2018) 2:235–9. doi: 10.1002/jbm4.10044
47. Fukuma M, Takagi M, Shimazu T, Imamura H, Yagi H, Nishimura G, et al. A familial case of spondyloepiphyseal dysplasia tarda caused by a novel splice site mutation in TRAPPC2. *Clin Pediatr Endocrinol.* (2018) 27:193–6. doi: 10.1297/cpe.27.193
48. Cheng VK, Au PC, Tan KC, Cheung CL. MicroRNA and human bone health. *JBMR Plus*. (2018) 3:2–13. doi: 10.1002/jbm4.10115
49. Nakamura Y, Inloes JB, Katagiri T, Kobayashi T. Chondrocyte-specific microRNA-140 regulates endochondral bone development and targets Dnpep to modulate bone morphogenetic protein signaling. *Mol Cell Biol.* (2011) 31:3019–28. doi: 10.1128/MCB.05178-11
50. Papaioannou G, Mirzamohammadi F, Lisse TS, Nishimori S, Wein MN, Kobayashi T. MicroRNA-140 provides robustness to the regulation of hypertrophic chondrocyte differentiation by the PTHrP-HDAC4 pathway. *J Bone Miner Res.* (2015) 30:1044–52. doi: 10.1002/jbmr.2438
51. Grigelioniene G, Suzuki HI, Taylan F, Mirzamohammadi F, Borochowitz ZU, Ayturk UM, et al. Gain-of-function mutation of microRNA-140 in human skeletal dysplasia. *Nat Med.* (2019) 25:583–90. doi: 10.1038/s41591-019-0353-2
52. Pettersson M, Vaz R, Hammarsjö A, Eisfeldt J, Carvalho CMB, Hofmeister W, et al. Alu-Alu mediated intragenic duplications in IFT81 and MATN3 are associated with skeletal dysplasias. *Hum Mutat.* (2018) 39:1456–67. doi: 10.1002/humu.23605
53. Kantaputra PN, Klopocki E, Hennig BP, Praphanphoj V, Le Caignec C, Isidor B, et al. Mesomelic dysplasia Kantaputra type is associated with duplications of the HOXD locus on chromosome 2q. *Eur J Hum Genet.* (2010) 18:1310–14. doi: 10.1038/ejhg.2010.116
54. Le Caignec C, Pichon O, Briand A, de Courtivron B, Bonnard C, Lindenbaum P, et al. Fryns type mesomelic dysplasia of the upper limbs caused by inverted duplications of the HOXD gene cluster. *Eur J Hum Genet.* (2019). doi: 10.1038/s41431-019-0522-2. [Epub ahead of print].
55. Peichel CL, Prabhakaran B, Vogt TF. The mouse Ulnaless mutation deregulates posterior HoxD gene expression and alters appendicular patterning. *Development*. (1997) 124:3481–92.
56. Hérault Y, Fraudeau N, Zákány J, Duboule D. Ulnaless (Ul), a regulatory mutation inducing both loss-of-function and gain-of-function of posterior Hoxd genes. *Development*. (1997) 124:3493–500.
57. Henderson CM, Fink SL, Bassyouni H, Argiropoulos B, Brown L, Laha TJ, et al. Vitamin D-binding protein deficiency and homozygous deletion of the GC gene. *N Engl J Med.* (2019) 380:1150–7. doi: 10.1056/NEJMoa1807841
58. Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, et al. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. *J Clin Invest.* (1999) 103:239–51. doi: 10.1172/JCI5244
59. Alikasifoglu A, Gonc EN, Yalcin E, Dogru D, Yordam N. Neonatal hyperparathyroidism due to maternal hypoparathyroidism and vitamin D deficiency: a cause of multiple bone fractures. *Clin Pediatr*. (2005) 44:267–9. doi: 10.1177/000992280504400312
60. Bjørnerem Å, Bui M, Wang X, Ghasem-Zadeh A, Hopper JL, Zebaze R, et al. Genetic and environmental variances of bone microarchitecture and bone remodeling markers: a twin study. *J Bone Miner Res.* (2015) 30:519–27. doi: 10.1002/jbmr.2365
61. Karasik D, Demissie S, Zhou Y, Lu D, Broe KE, Bouxsein ML, et al. Heritability and genetic correlations for bone microarchitecture: the framingham study families. *J Bone Miner Res.* (2017) 32:106–14. doi: 10.1002/jbmr.2915
62. Morris JA, Kemp JP, Youlten SE, Laurent L, Logan JG, Chai RC, et al. An atlas of genetic influences on osteoporosis in humans and mice. *Nat Genet.* (2019) 51:258–66. doi: 10.1038/s41588-018-0302-x
63. Collet C, Ostertag A, Ricquebourg M, Delecourt M, Tueur G, Isidor B, et al. Primary osteoporosis in young adults: Genetic basis and identification of novel variants in causal genes. *JBMR Plus.* (2017) 2:12–21. doi: 10.1002/jbm4.10020
64. Mäkitie RE, Costantini A, Kämpe A, Alm JJ, Mäkitie O. New insights into monogenic causes of osteoporosis. *Front Endocrinol*. (2019) 10:70. doi: 10.3389/fendo.2019.00070
65. Gregson CL, Wheeler L, Hardcastle SA, Appleton LH, Addison KA, Brugmans M, et al. Mutations in known monogenic high bone mass loci only explain a small proportion of high bone mass cases. *J Bone Miner Res*. (2016) 31:640–9. doi: 10.1002/jbmr.2706
66. Gregson CL, Newell F, Leo PJ, Clark GR, Paternoster L, Marshall M, et al. Genome-wide association study of extreme high bone mass: contribution of common genetic variation to extreme BMD phenotypes and potential novel BMD-associated genes. *Bone.* (2018) 114:62–71. doi: 10.1016/j.bone.2018.06.001
67. Whyte MP, McAlister WH, Zhang F, Bijanki VN, Nenninger A, Gottesman GS, et al. New explanation for autosomal dominant high bone mass: mutation of low-density lipoprotein receptor-related protein 6. *Bone*. (2019) 127:228–43. doi: 10.1016/j.bone.2019.05.003
68. Gregson CL, Bergen DJM, Leo P, Sessions RB, Wheeler L, Hartley A, et al. A rare mutation in SMAD9 associated with high bone mass identifies the SMAD-dependent BMP signaling pathway as a potential anabolic target for osteoporosis. *J Bone Miner Res.* (2019). doi: 10.1002/jbmr.3875. [Epub ahead of print].
69. Gurumurthy CB, Lloyd KCK. Generating mouse models for biomedical research: technological advances. *Dis Model Mech.* (2019) 12:1. doi: 10.1242/dmm.029462
70. Hmeljak J, Justice MJ. From gene to treatment: supporting rare disease translational research through model systems. *Dis Model Mech.* (2019) 12:dmm039271. doi: 10.1242/dmm.039271
71. Spector JM, Harrison RS, Fishman MC. Fundamental science behind today's important medicines. *Sci Transl Med.* (2018) 10:438. doi: 10.1126/scitranslmed.aaq1787
72. Wangler MF, Yamamoto S, Chao HT, Posey JE, Westerfield M, Postlethwait J, et al. Model organisms facilitate rare disease diagnosis and therapeutic research. *Genetics.* (2017) 207:9–27. doi: 10.1534/genetics.117.203067
73. Kimmel DB. Animal models in bone research. In: Smith SY, Varela A, Samadfam R, editors. *Bone Toxicology.* Cham: Springer Nature (2017). p. 129–171. doi: 10.1007/978-3-319-56192-9_4

74. Maynard RD, Ackert-Bicknell CL. Mouse models and online resources for functional analysis of osteoporosis genome-wide association studies. *Front Endocrinol*. (2019) 10:277. doi: 10.3389/fendo.2019.00277
75. Zambrowicz BP, Sands AT. Knockouts model the 100 best-selling drugs–will they model the next 100? *Nat Rev Drug Discov*. (2003) 2:38–51. doi: 10.1038/nrd987
76. Brommage R. Genetic approaches to identifying novel osteoporosis drug targets. *J Cell Biochem*. (2015) 116:2139–45. doi: 10.1002/jcb.25179
77. Bouxsein ML, Myers KS, Shultz KL, Donahue LR, Rosen CJ, Beamer WG. Ovariectomy-induced bone loss varies among inbred strains of mice. *J Bone Miner Res*. (2005) 20:1085–92. doi: 10.1359/JBMR.050307
78. Li X, Mohan S, Gu W, Wergedal J, Baylink DJ. Quantitative assessment of forearm muscle size, forelimb grip strength, forearm bone mineral density, and forearm bone size in determining humerus breaking strength in 10 inbred strains of mice. *Calcif Tissue Int*. (2001) 68:365–9. doi: 10.1007/s00223-001-0004-7
79. Lodberg A, Vegger JB, Jensen MV, Larsen CM, Thomsen JS, Brüel A. Immobilization induced osteopenia is strain specific in mice. *Bone Rep*. (2015) 2:59–67. doi: 10.1016/j.bonr.2015.04.001
80. Lovell DP, Johnson FM, Willis DB. Quantitative genetic variation in the skeleton of the mouse: II. Description of variation within and between inbred strains. *Am J Anat*. (1986) 176:287–303. doi: 10.1002/aja.1001760304
81. Sabsovich I, Clark JD, Liao G, Peltz G, Lindsey DP, Jacobs CR, et al. Bone microstructure and its associated genetic variability in 12 inbred mouse strains: microCT study and in silico genome scan. *Bone*. (2008) 42:439–51. doi: 10.1016/j.bone.2007.09.041
82. Fairfield H, Srivastava A, Ananda G, Liu R, Kircher M, Lakshminarayana A, et al. Exome sequencing reveals pathogenic mutations in 91 strains of mice with Mendelian disorders. *Genome Res*. (2015) 25:948–57. doi: 10.1101/gr.186882.114
83. Palmer K, Fairfield H, Borgeia S, Curtain M, Hassan MG, Dionne L, Yong Karst S, et al. Discovery and characterization of spontaneous mouse models of craniofacial dysmorphology. *Dev Biol*. (2016) 415:216–27. doi: 10.1016/j.ydbio.2015.07.023
84. Brommage R, Powell DR, Vogel P. Predicting human disease mutations and identifying drug targets from mouse gene knockout phenotyping campaigns. *Dis Model Mech*. (2019) 12:5. doi: 10.1242/dmm.038224
85. Cacheiro P, Haendel MA, Smedley D, International Mouse Phenotyping Consortium and the Monarch Initiative. New models for human disease from the international mouse phenotyping consortium. *Mamm Genome*. (2019) 30:143–50. doi: 10.1007/s00335-019-09804-5
86. Brommage R, Liu J, Vogel P, Mseeh F, Thompson AY, Potter DG, et al. NOTUM inhibition increases endocortical bone formation and bone strength. *Bone Res*. (2019) 7:2. doi: 10.1038/s41413-018-0038-3
87. Brommage R, Liu J, Hansen GM, Kirkpatrick LL, Potter DG, Sands AT, et al. High-throughput screening of mouse gene knockouts identifies established and novel skeletal phenotypes. *Bone Res*. (2014) 2:14034. doi: 10.1038/boneres.2014.34
88. Iwaniec UT, Wronski TJ, Liu J, Rivera MF, Arzaga RR, Hansen G, et al. PTH stimulates bone formation in mice deficient in Lrp5. *J Bone Miner Res*. (2007) 22:394–402. doi: 10.1359/jbmr.061118
89. Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, et al. The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. *J Biol Chem*. (2006) 281:23698–711. doi: 10.1074/jbc.M601000200
90. Jacobsen CM. Application of anti-sclerostin therapy in non-osteoporosis disease models. *Bone*. (2017) 96:18–23. doi: 10.1016/j.bone.2016.10.018
91. Roschger A, Roschger P, Keplingter P, Klaushofer K, Abdullah S, Kneissel M, et al. Effect of sclerostin antibody treatment in a mouse model of severe osteogenesis imperfecta. *Bone*. (2014) 66:182–8. doi: 10.1016/j.bone.2014.06.015
92. Sinder BP, Eddy MM, Ominsky MS, Caird MS, Marini JC, Kozloff KM. Sclerostin antibody improves skeletal parameters in a Brtl/+ mouse model of osteogenesis imperfecta. *J Bone Miner Res*. (2013) 28:73–80. doi: 10.1002/jbmr.1717
93. Jacobsen CM, Barber LA, Ayturk UM, Roberts HJ, Deal LE, Schwartz MA, et al. Targeting the LRP5 pathway improves bone properties in a mouse model of osteogenesis imperfecta. *J Bone Miner Res*. (2014) 29:2297–306. doi: 10.1002/jbmr.2198
94. Little DG, Peacock L, Mikulec K, Kneissel M, Kramer I, Cheng TL, et al. Combination sclerostin antibody and zoledronic acid treatment outperforms either treatment alone in a mouse model of osteogenesis imperfecta. *Bone*. (2017) 101:96–103. doi: 10.1016/j.bone.2017.04.016
95. Grafe I, Alexander S, Yang T, Lietman C, Homan EP, Munivez E, et al. Sclerostin antibody treatment improves the bone phenotype of Crtap(-/-) mice, a model of recessive osteogenesis imperfecta. *J Bone Miner Res*. (2016) 31:1030–40. doi: 10.1002/jbmr.2776
96. Ren Y, Han X, Jing Y, Yuan B, Ke H, Liu M, et al. Sclerostin antibody (Scl-Ab) improves osteomalacia phenotype in dentin matrix protein 1 (Dmp1) knockout mice with little impact on serum levels of phosphorus and FGF23. *Matrix Biol*. (2016) 52–4:151–61. doi: 10.1016/j.matbio.2015.12.009
97. Kedlaya R, Veera S, Horan DJ, Moss RE, Ayturk UM, Jacobsen CM, et al. Sclerostin inhibition reverses skeletal fragility in an Lrp5-deficient mouse model of OPPG syndrome. *Sci Transl Med*. (2013) 5:211ra158. doi: 10.1126/scitranslmed.3006627
98. Choi JY, Lai JK, Xiong ZM, Ren M, Moorer MC, Stains JP, et al. Diminished canonical β-catenin signaling during osteoblast differentiation contributes to osteopenia in progeria. *J Bone Miner Res*. (2018) 33:2059–70. doi: 10.1002/jbmr.3549
99. Bai S, Li D, Xu L, Duan H, Yuan J, Wei M. Recombinant mouse periostin ameliorates coronal sutures fusion in Twist1+/- mice. *J Transl Med*. (2018) 16:103. doi: 10.1186/s12967-018-1454-2
100. He Y, Yuan C, Chen L, Liu Y, Zhou H, Xu N, et al. While it is not deliberate, much of today's biomedical research contains logical and technical flaws, showing a need for corrective action. *Int J Med Sci*. (2018) 15:309–22. doi: 10.7150/ijms.23215
101. West DB, Engelhard EK, Adkisson M, Nava AJ, Kirov JV, Cipollone A, et al. Transcriptome analysis of targeted mouse mutations reveals the topography of local changes in gene expression. *PLoS Genet*. (2016) 12:e1005691. doi: 10.1371/journal.pgen.1005691
102. Maguire S, Estabel J, Ingham N, Pearson S, Ryder E, Carragher DM, et al. Targeting of Slc25a21 is associated with orofacial defects and otitis media due to disrupted expression of a neighbouring gene. *PLoS ONE*. (2014) 9:e91807. doi: 10.1371/journal.pone.0091807
103. Jokela H, Hakkarainen J, Kätkänaho L, Pakarinen P, Ruohonen ST, Tena-Sempere M, et al. Deleting the mouse Hsd17b1 gene results in a hypomorphic Naglu allele and a phenotype mimicking a lysosomal storage disease. *Sci Rep*. (2017) 7:16406. doi: 10.1038/s41598-017-16618-5
104. Roeder E, Matthews BG, Kalajzic I. Visual reporters for study of the osteoblast lineage. *Bone*. (2016) 92:189–95. doi: 10.1016/j.bone.2016.09.004
105. Dallas SL, Xie Y, Shiflett LA, Ueki Y. Mouse Cre models for the study of bone diseases. *Curr Osteoporos Rep*. (2018) 16:466–77. doi: 10.1007/s11914-018-0455-7
106. Heffner CS, Herbert Pratt C, Babiuk RP, Sharma Y, Rockwood SF, Donahue LR, et al. Supporting conditional mouse mutagenesis with a comprehensive Cre characterization resource. *Nat Commun*. (2012) 3:1218. doi: 10.1038/ncomms2186
107. Huang W, Olsen BR. Skeletal defects in Osterix-Cre transgenic mice. *Transgenic Res*. (2015) 24:167–72. doi: 10.1007/s11248-014-9828-6
108. Bustin SA, Wittwer CT. MIQE: a step toward more robust and reproducible quantitative PCR. *Clin Chem*. (2017) 63:1537–8. doi: 10.1373/clinchem.2016.268953
109. Bustin S, Nolan T. Talking the talk, but not walking the walk: RT-qPCR as a paradigm for the lack of reproducibility in molecular research. *Eur J Clin Invest*. (2017) 47:756–74. doi: 10.1111/eci.12801
110. Abuna RPF, Oliveira FS, Ramos JIR, Lopes HB, Freitas GP, Souza ATP, et al. Selection of reference genes for quantitative real-time polymerase chain reaction studies in rat osteoblasts. *J Cell Physiol*. (2018) 234:749–56. doi: 10.1002/jcp.26886
111. Yang X, Hatfield JT, Hinze SJ, Mu X, Anderson PJ, Powell BC. Bone to pick: the importance of evaluating reference genes for RT-qPCR quantification of gene expression in craniosynostosis and bone-related tissues and cells. *BMC Res Notes*. (2012) 5:222. doi: 10.1186/1756-0500-5-222

112. Couasnay G, Frey C, Elefteriou F. Promoter cre-specific genotyping assays for authentication of Cre-driver mouse lines. *JBMR Plus.* (2019) 3:e10128. doi: 10.1002/jbm4.10128
113. Saper CB. Editorial: an open letter to our readers on the use of antibodies. *J Comp Neurol*. (2005) 493:477–8. doi: 10.1002/cne.20839
114. Neimark J. Line of attack. *Science*. (2015) 347:938–40. doi: 10.1126/science.347.6225.938
115. Almeida JL, Cole KD, Plant AL. Standards for cell line authentication and beyond. *PLoS Biol.* (2016) 14:e1002476. doi: 10.1371/journal.pbio.1002476
116. Bairoch A. The cellosaurus, a cell-line knowledge resource. *J Biomol Tech.* (2018) 29:25–38. doi: 10.7171/jbt.18-2902-002
117. Hwang PW, Horton JA. Variable osteogenic performance of MC3T3-E1 subclones impacts their utility as models of osteoblast biology. *Sci Rep.* (2019) 9:8299. doi: 10.1038/s41598-019-44575-8
118. Williams DK, Pinzón C, Huggins S, Pryor JH, Falck A, Herman F, et al. Genetic engineering a large animal model of human hypophosphatasia in sheep. *Sci Rep*. (2018) 8:16945. doi: 10.1038/s41598-018-35079-y
119. Kyöstilä K, Syrjä P, Lappalainen AK, Arumilli M, Hundi S, Karkamo V, et al. A homozygous missense variant in the alkaline phosphatase gene ALPL is associated with a severe form of canine hypophosphatasia. *Sci Rep.* (2019) 9:973. doi: 10.1038/s41598-018-37801-2
120. Packer RA, Logan MA, Guo LT, Apte SS, Bader H, O'Brien DP, et al. Clinical phenotype of Musladin-Lueke syndrome in 2 beagles. *J Vet Intern Med.* (2017) 31:532–8. doi: 10.1111/jvim.14654
121. Quist EM, Doan R, Pool RR, Porter BF, Bannasch DL, Dindot SV. Identification of a candidate mutation in the COL1A2 gene of a chow with osteogenesis imperfecta. *J Hered.* (2018) 109:308–14. doi: 10.1093/jhered/esx074
122. Mansour TA, Lucot K, Konopelski SE, Dickinson PJ, Sturges BK, Vernau KL, et al. Whole genome variant association across 100 dogs identifies a frame shift mutation in DISHEVELLED 2 which contributes to Robinow-like syndrome in Bulldogs and related screw tail dog breeds. *PLoS Genet.* (2018) 14:e1007850. doi: 10.1371/journal.pgen.1007850
123. Willet CE, Makara M, Reppas G, Tsoukalas G, Malik R, Haase B, et al. Canine disorder mirrors human disease: exonic deletion in HES7 causes autosomal recessive spondylocostal dysostosis in miniature Schnauzer dogs. *PLoS ONE.* (2015) 10:e0117055. doi: 10.1371/journal.pone.0117055
124. Lindert U, Weis MA, Rai J, Seeliger F, Hausser I, Leeb T, et al. Molecular consequences of the SERPINH1/HSP47 mutation in the dachshund natural model of osteogenesis imperfecta. *J Biol Chem.* (2015) 290:17679–89. doi: 10.1074/jbc.M115.661025
125. Yin Y, Bangs F, Paton IR, Prescott A, James J, Davey MG, et al. The Talpid3 gene (KIAA0586) encodes a centrosomal protein that is essential for primary cilia formation. *Development*. (2009) 136:655–64. doi: 10.1242/dev.028464
126. Maas SA, Suzuki T, Fallon JF. Identification of spontaneous mutations within the long-range limb-specific Sonic hedgehog enhancer (ZRS) that alter Sonic hedgehog expression in the chicken limb mutants oligozeugodactyly and silkie breed. *Dev Dyn.* (2011) 240:1212–22. doi: 10.1002/dvdy.22634
127. Kwon RY, Watson CJ, Karasik D. Using zebrafish to study skeletal genomics. *Bone*. (2019) 126:37–50. doi: 10.1016/j.bone.2019.02.009
128. Lleras-Forero L, Winkler C, Schulte-Merker S. Zebrafish and medaka as models for biomedical research of bone diseases. *Dev Biol*. (2019). doi: 10.1016/j.ydbio.2019.07.009. [Epub ahead of print].
129. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* (2013) 496:498–503. doi: 10.1038/nature12111
130. Briggs MD, Bell PA, Wright MJ, Pirog KA. New therapeutic targets in rare genetic skeletal diseases. *Exp Opin Orphan Drugs.* (2015) 3:1137–1154. doi: 10.1517/21678707.2015.1083853
131. Semler O, Rehberg M, Mehdiani N, Jackels M, Hoyer-Kuhn H. Current and emerging therapeutic options for the management of rare skeletal diseases. *Paediatr Drugs*. (2019) 21:95–106. doi: 10.1007/s40272-019-00330-0
132. Canalis E, Sanjay A, Yu J, Zanotti S. An antibody to Notch2 reverses the osteopenic phenotype of Hajdu-Cheney mutant male mice. *Endocrinology*. (2017) 158:730–42. doi: 10.1210/en.2016-1787
133. Howles SA, Hannan FM, Gorvin CM, Piret SE, Paudyal A, Stewart M, et al. Cinacalcet corrects hypercalcemia in mice with an inactivating Gα11 mutation. *JCI Insight*. (2017) 2:20. doi: 10.1172/jci.insight.96540
134. Khan T, Sinkevicius KW, Vong S, Avakian A, Leavitt MC, Malanson H, et al. ENPP1 enzyme replacement therapy improves blood pressure and cardiovascular function in a mouse model of generalized arterial calcification of infancy. *Dis Model Mech*. (2018) 11:dmm035691. doi: 10.1242/dmm.035691
135. Brommage R. New targets and emergent therapies for osteoporosis. *Handb Exp Pharmacol*. (2019). doi: 10.1007/164_2019_329. [Epub ahead of print].
136. Wu N, Liu B, Du H, Zhao S, Li Y, Cheng X, et al. The progress of CRISPR/Cas9-mediated gene editing in generating mouse/zebrafish models of human skeletal diseases. *Comput Struct Biotechnol J.* (2019) 17:954–62. doi: 10.1016/j.csbj.2019.06.006
137. Ayturk U. RNA-seq in skeletal biology. *Curr Osteoporos Rep*. (2019) 17:178–85. doi: 10.1007/s11914-019-00517-x
138. Gonorazky HD, Naumenko S, Ramani AK, Nelakuditi V, Mashouri P, Wang P, et al. Expanding the boundaries of RNA sequencing as a diagnostic tool for rare Mendelian disease. *Am J Hum Genet.* (2019) 104:466–83. doi: 10.1016/j.ajhg.2019.01.012
139. Khayal LA, Grünhagen J, Provazník I, Mundlos S, Kornak U, Robinson PN, et al. Transcriptional profiling of murine osteoblast differentiation based on RNA-seq expression analyses. *Bone*. (2018) 113:29–40. doi: 10.1016/j.bone.2018.04.006
140. Sebastian A, Hum NR, Morfin C, Murugesh DK, Loots GG. Global gene expression analysis identifies Mef2c as a potential player in Wnt16-mediated transcriptional regulation. *Gene*. (2018) 675:312–21. doi: 10.1016/j.gene.2018.06.079
141. Stark R, Grzelak M, Hadfield J. RNA sequencing: the teenage years. *Nat Rev Genet.* (2019) 20:631–56. doi: 10.1038/s41576-019-0150-2

Conflict of Interest: 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 Endocrinology

REVIEW
published: 09 April 2020
doi: 10.3389/fendo.2020.00165

WNT Signaling and Bone: Lessons From Skeletal Dysplasias and Disorders

Yentl Huybrechts, Geert Mortier, Eveline Boudin† and Wim Van Hul†*

Center of Medical Genetics, Antwerp University Hospital, University of Antwerp, Antwerp, Belgium

Skeletal dysplasias are a diverse group of heritable diseases affecting bone and cartilage growth. Throughout the years, the molecular defect underlying many of the diseases has been identified. These identifications led to novel insights in the mechanisms regulating bone and cartilage growth and homeostasis. One of the pathways that is clearly important during skeletal development and bone homeostasis is the Wingless and int-1 (WNT) signaling pathway. So far, three different WNT signaling pathways have been described, which are all activated by binding of the WNT ligands to the Frizzled (FZD) receptors. In this review, we discuss the skeletal disorders that are included in the latest nosology of skeletal disorders and that are caused by genetic defects involving the WNT signaling pathway. The number of skeletal disorders caused by defects in WNT signaling genes and the clinical phenotype associated with these disorders illustrate the importance of the WNT signaling pathway during skeletal development as well as later on in life to maintain bone mass. The knowledge gained through the identification of the genes underlying these monogenic conditions is used for the identification of novel therapeutic targets. For example, the genes underlying disorders with altered bone mass are all involved in the canonical WNT signaling pathway. Consequently, targeting this pathway is one of the major strategies to increase bone mass in patients with osteoporosis. In addition to increasing the insights in the pathways regulating skeletal development and bone homeostasis, knowledge of rare skeletal dysplasias can also be used to predict possible adverse effects of these novel drug targets. Therefore, this review gives an overview of the skeletal and extra-skeletal phenotype of the different skeletal disorders linked to the WNT signaling pathway.

Keywords: skeletal dysplasias, Wingless and Int-1 (WNT)/planar cell polarity (PCP) pathway, Wingless and int-1 (WNT)/β-catenin pathway, Wingless and int-1 (WNT)/Ca^{2+} pathway, osteoporosis

OPEN ACCESS

Edited by:
Katherine A. Staines, University of Brighton, United Kingdom

Reviewed by:
Thomas Funck-Brentano, Université de Paris, France
Maria Felicia Faienza, University of Bari Aldo Moro, Italy
Elena Tsourdi, Dresden University of Technology, Germany

***Correspondence:**
Wim Van Hul
wim.vanhul@uantwerpen.be

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *20 December 2019*
Accepted: *09 March 2020*
Published: *09 April 2020*

Citation:
Huybrechts Y, Mortier G, Boudin E and Van Hul W (2020) WNT Signaling and Bone: Lessons From Skeletal Dysplasias and Disorders. Front. Endocrinol. 11:165. doi: 10.3389/fendo.2020.00165

INTRODUCTION

In the last decades, plenty of examples have been generated where the identification of causal genes for rare, monogenic diseases resulted in novel insights into the role and functioning of already known pathways. A textbook example of this is Wingless and int-1 (WNT; pronounced "wint") signaling and its role in bone formation and homeostasis. First indications of this involvement were generated by studying rare monogenic conditions with abnormal bone mass, delivering novel targets for drug development for osteoporosis, a common disorder characterized by decreased bone mass in late life. Recently, an antibody against a WNT signaling inhibitor was approved as an anabolic agent for the treatment of osteoporosis.

The name WNT was introduced almost 30 years ago referring to the wingless-type mouse mammary tumor virus integration site (int-1) which was the first member from this gene family being described (1, 2). Meanwhile, three different pathways have been described, one canonical pathway also known as the WNT/β-catenin pathway and two non-canonical pathways, the WNT/Ca^{2+}-dependent pathway and the WNT/planar cell polarity (PCP) pathway (**Figure 1**). All three are initiated by the binding of an extracellular WNT ligand to the N-terminal domain of one of the seven transmembrane-span FZD receptors. Depending on the pathway, also co-receptors are involved with the low-density lipoprotein (LDL)-related receptor (LRP)-5 and−6 being specific for canonical WNT signaling.

In mammalians, 19 different WNT ligands have been identified and 10 FZD receptors (3), already illustrating the involvement of this pathway in a broad range of cellular processes. All three pathways have a number of functions both during embryonic development and in adult life. These include cell fate specification, cell proliferation and migration, as well as body axis patterning. Furthermore, they are also important for cell functioning as well as processes of cell death. For some processes, only one of the three pathways is involved, but for others, evidence was generated indicating convergence of some of them (4).

At the beginning of this century, an additional role of WNT signaling was discovered following new gene identifications in some rare monogenic skeletal dysplasias. As explained in detail below, the study of conditions with either decreased or increased bone mass resulted in the identification of mutations in several genes involved in especially canonical WNT signaling (5). The most recent revision of the nosology and classification of genetic skeletal disorders (6) includes 461 different diseases. These disorders are interesting experiments of nature to gain insights into the regulatory mechanisms of bone formation, resorption, and homeostasis both during development and during adult life. In this review, we aim to discuss those skeletal disorders in which abnormal WNT signaling contributes to their pathogenesis. Furthermore, the implications of the novel insights toward more common bone disorders such as osteoporosis are highlighted.

EXTRACELLULAR MODULATORS

As previously mentioned, the WNT signaling pathway is activated by the binding of WNT ligands. Because of the broad functions of this pathway, additional regulation mechanisms are required to ensure proper timely and spatially functioning of the pathway. Extracellular modulators, including WNT inhibitors and activators, contribute to this complex regulation. Not surprisingly, mutations in various components of this pathway have been described in skeletal dysplasias.

WNT Ligands

WNT ligands are secreted glycoproteins with a length of 350–400 amino acids. In humans, 19 different ligands have been identified, all containing 23–24 conserved cysteine residues (7, 8). A distinction between canonical (e.g., WNT1 and WNT3) and non-canonical (e.g., WNT5A) WNTs can be made, although overlap between the different pathways has been suggested. Various WNT ligands are associated with skeletal disorders, as described below.

WNT1

WNT1 is of major importance for the regulation of bone homeostasis, through binding with the co-receptor LRP5. Mutations in the *WNT1* gene are found in families with osteogenesis imperfecta (OI) type XV and early-onset osteoporosis (9–11).

OI is a hereditary connective tissue disorder, characterized by bone fragility, hearing loss, and dentinogenesis imperfecta. In the majority of patients, the disease is caused by heterozygous mutations in *COL1A1* and *COL1A2*, which encode the type I collagen pro α-chains. However, also recessive forms of OI do exist. One example is OI type XV caused by bi-allelic loss-of-function mutations in the *WNT1* gene (11–15). Moreover, the importance of the WNT signaling pathway in OI is emphasized by the observation of increased serum levels of Dickkopf1 (DKK1), an antagonist of the canonical WNT pathway, in OI patients. However, so far, no disease-causing OI mutations have been described in DKK1 (16).

Osteoporosis is a common skeletal disorder characterized by low bone mass, impaired bone quality, and increased fracture risk (14, 17). Whereas, most patients show symptoms later in life, early-onset osteoporosis can already present in adolescence. Some of this early-onset forms of osteoporosis are caused by heterozygous *WNT1* mutations (10, 12–15). These mutations result in disturbed bone remodeling and subsequent imbalance in bone homeostasis (13).

WNT3

WNT3 is critical for axis formation and limb growth in vertebrates (18, 19), and a homozygous nonsense mutation (p.Glu83*) in the *WNT3* gene has been reported in tetra-amelia syndrome type 1 (19). Tetra-amelia is an autosomal recessive disorder, characterized by the absence of all four limbs. It is extremely rare, with an incidence of 1.5–4 per 100,000 births (19–21).

WNT5A

Unlike the other WNT ligands discussed in this review, WNT5A belongs to the non-canonical WNT/PCP pathway. In order to exert its functions, such as promoting osteoblast differentiation, WNT5A binds to the cysteine-rich domain of receptor tyrosine kinase-like orphan receptor (ROR) 1/2 (22–24). Heterozygous loss-of-function mutations in the *WNT5A* gene are associated with autosomal dominant Robinow syndrome (RS) type 1 (ADRS1) (24–27). RS is characterized by skeletal abnormalities (short stature, brachydactyly, and mesomelic limb shortening predominantly of the upper limbs), genital hypoplasia, and typical facial dysmorphisms. The disorder is genetically and clinically heterogeneous, showing both dominant and recessive inheritance patterns. Additional phenotypic features of RS include hypertelorism, dental problems, and kidney abnormalities.

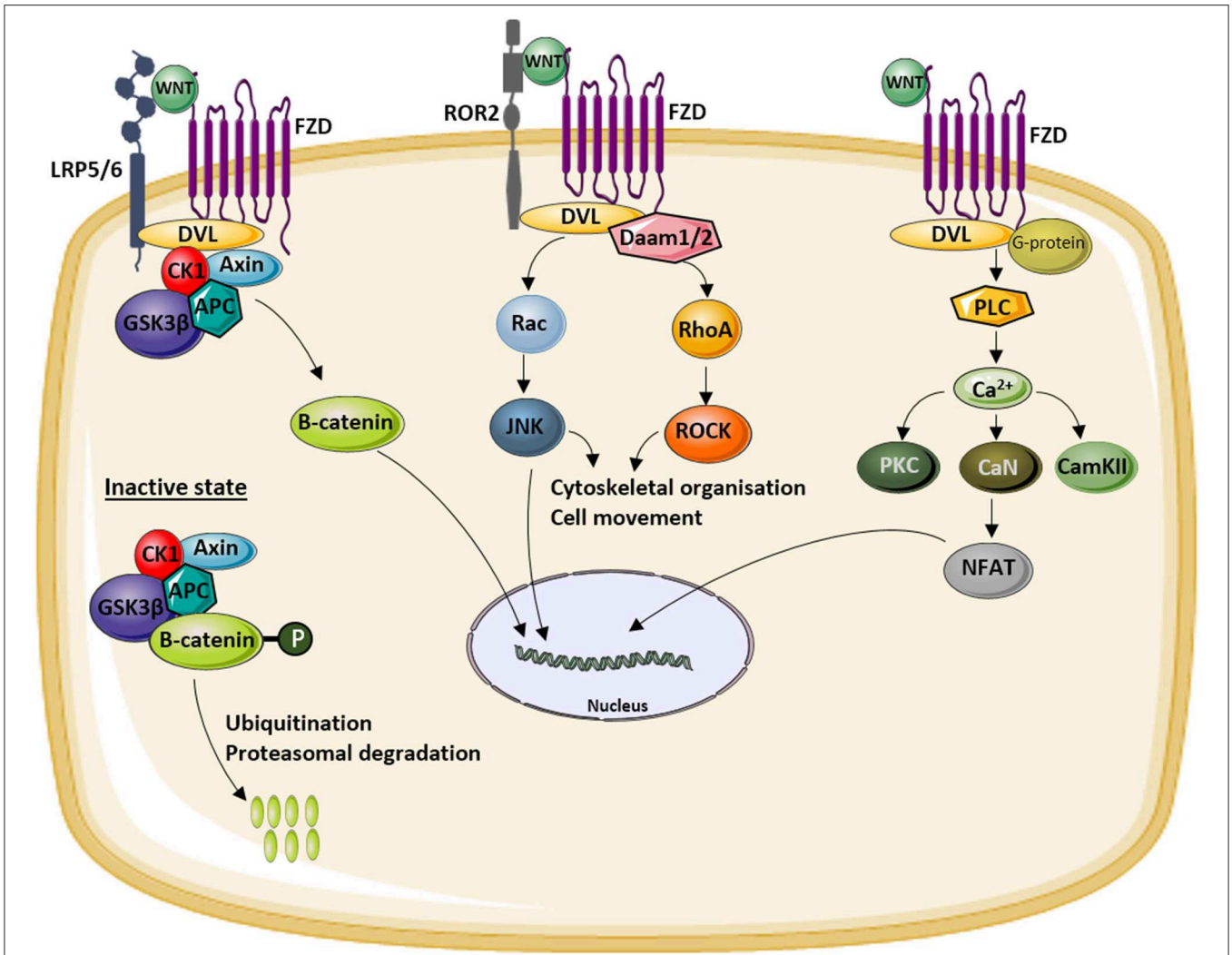


FIGURE 1 | Overview of the different WNT signaling pathways. WNT ligands can activate the canonical or WNT/β-catenin signaling pathway (left) by binding to a receptor complex formed by low-density lipoprotein (LDL)-related receptor (LRP)5/6 and Frizzled (FZD). This activation results in the translocation of hypophosphorylated β-catenin to the nucleus. In the inactive state, β-catenin is degraded by the proteasome after phosphorylation by a glycogen synthase kinase 3β (GSK3β)-Axin-casein kinase 1 (CK1)–adenomatous polyposis coli (APC) complex and subsequent ubiquitination. The activation of the non-canonical WNT/planar cell polarity (PCP) pathway (middle) results in the activation of c-jun NH2-terminal kinase (JNK) *via* disheveled (DVL) and the Rac and Rho small GTPases. Activation of the non-canonical WNT/Ca^{2+} pathway (right) by binding of WNT to an FZD receptor results in intracellular Ca^{2+} release which activates a number of calcium-sensitive enzymes [protein kinase C (PKC), calcineurin (CaN), calmodulin-dependent protein kinase II (CamKII)]. More downstream nuclear factor of activated T cells (NF-AT) is activated and translocates to the nucleus to induce the expression of target genes.

WNT6

Acro-pectoro-vertebral dysplasia (F-syndrome) is a rare skeletal disorder with a dominant inheritance pattern (28, 29). Symptoms include carpal and tarsal synostosis, malformations of the first and second fingers with frequent syndactyly, and spina bifida occulta (28–30). Although the molecular basis remains unknown, Thiele et al. (29) mapped the disease to a 6.5 cM region on chromosome 2q36, where the *WNT6* gene is located. As this gene is expressed during limb development, it is a promising candidate gene for harboring disease-causing mutations for F-syndrome (6, 29).

WNT7A

WNT7A forms a receptor complex with LRP6 and FZD5 in order to activate the canonical WNT signaling pathway (31, 32). It is expressed in the dorsal ectoderm and involved in the formation of the dorsoventral axis in limb development (32–34). Moreover, it also contributes to anteroposterior patterning by maintaining sonic hedgehog expression (32–34). Consistently, mutations in *WNT7A* cause limb malformations in Al-Awadi–Raas–Rothschild syndrome (AARRS) and Fuhrmann syndrome (FS) (31–37). Patients with AARRS display short upper limbs, severe malformations of the lower limbs, hypoplastic pelvis, anteriorly displaced genitalia, underdeveloped nails, and absence

of uterus. The phenotype observed in FS is milder (e.g., solely ulnar deficiency and oligodactyly) since this is due to only a reduced function of the protein compared to complete loss-of-function mutations as seen in AARRS (32, 34–37). Although both are rare autosomal recessive disorders, different mutations contribute to the highly variable disease phenotypes (31, 34–36, 38, 39).

WNT10B

The WNT10B ligand is a key regulator for osteogenesis and is involved in all stages of limb development (24, 40–42). WNT10B plays an important role in the development of hands and feet, and bi-allelic mutations can cause split-hand/foot malformation type 6 (SHFM6). Generally, SHFMs are complex limb malformations that affect the central rays of hands and feet (42–44). From a clinical and genetic point of view, SHFM is highly heterogeneous. Both isolated and syndromic forms can be distinguished, as well as reduced penetrance has been reported (42–44). While most types of SHFM are autosomal dominantly inherited, SHFM6 follows an autosomal recessive pattern of inheritance (43, 44).

WNT Inhibitors

SOST

Sclerostin, encoded by the *SOST* gene, is an osteocyte-derived secreted glycoprotein with a cysteine-knot motif (45). It acts as an extracellular antagonist of the canonical WNT signaling pathway by binding to the first β-propeller domain of LRP5/6 (46–49). Hence, sclerostin functions as a key regulator in bone metabolism and, particularly, bone formation (46, 50). Moreover, an interaction with the extracellular domain of LRP4 has been described, and studies demonstrated that this interaction is essential for normal sclerostin functioning (51, 52).

SOST was identified as the disease-causing gene for several high bone mass disorders, including sclerosteosis type 1. This rare skeletal dysplasia, caused by bi-allelic loss-of-function mutations in the *SOST* gene, is characterized by hyperostosis, tall stature, and syndactyly. The bone overgrowth affects predominantly the skull, mandible, and tubular bones in the limbs. Involvement of the skull base may cause compression of the cranial nerves and subsequent facial palsy, hearing loss, and blindness (50, 53–55). In 1998, Van Buchem disease, another autosomal recessive high-bone mass disorder, was mapped to the same chromosomal region as sclerosteosis (56). The molecular cause was a bi-allelic 52-kb deletion 35-kb downstream of *SOST* (23, 57). Since the deleted region contains a myocyte-enhancer factor 2C (Mef2c) binding site, imperative for *SOST* expression, the deletion results in reduced transcription of *SOST* (23). Although there is phenotypic overlap between sclerosteosis and Van Buchem disease, the phenotype of the latter is usually milder, as the patients have normal stature and do not exhibit syndactyly (54, 55). Another very rare sclerotic bone disorder is craniodiaphyseal dysplasia (CDD), inherited in an autosomal dominant manner. Clinical manifestations include sclerosis and hyperostosis, especially of the skull and facial bones, resulting in facial dysmorphism or "leontiasis ossea" (49, 58). Mutations causing CDD are located in the signal peptide of sclerostin and affect the secretion of sclerostin in a dominant negative manner.

GREM1

Gremlin has been characterized as a bone morphogenetic protein (BMP) inhibitor that regulates limb and kidney formation during development (59). However, WNT antagonistic activity has also been described since luciferase assays showed reduced stimulatory effects of WNT3A in the presence of gremlin (60, 61). In 2010, Dimitrov et al. (62) described a monoallelic 1.7 Mb duplication containing both the *GREM1* and the *FMN1* locus in a patient with Cenani–Lenz-like non-syndromic bilateral oligosyndactyly. *FMN1* contains a *cis*-regulatory region that is essential for the activation of gremlin in the limb bud. Hence, genomic rearrangements can lead to an altered function of gremlin and subsequent phenotypic abnormalities (39, 62–64). Interestingly, the phenotype of the patient is similar to that of the transgenic chick with overexpression of *Grem1* (65, 66). Furthermore, *GREM1* has been suggested to decrease the metastatic potential of osteosarcoma. Besides reduced gremlin levels in osteosarcoma cells, downregulation of *GREM1* was also associated with an increased degree of proliferation and angiogenesis (67).

SFRP4

Secreted FZD-related protein (sFRP) 4 is one of the five members of the sFRP family, known for antagonizing the WNT signaling pathway (68–70). sFRPs contain a C-terminal netrin-like domain (NTR) and an N-terminal cysteine-rich domain, sharing up to 50% sequence homology with the cysteine-rich domain of FZD receptors (24, 68, 70, 71). Consequently, sFRPs serve as decoy receptors for WNT ligands, thereby preventing activation of both canonical and non-canonical WNT signaling (24, 68–70). sFRP4 is a 346-amino acid protein, expressed in the majority of tissues (70). Regarding skeletal tissue, it contributes to maintaining normal osteoblast and osteoclast function during bone development and in the adult skeleton (68–70). A disrupted function of sFRP4 was discovered in Pyle disease, a recessive disorder affecting long bones with metaphyseal widening, cortex thinning, increased trabecular bone, decreased bone mineral density, and bone fragility (24, 72–74). Since 2016, various bi-allelic truncating mutations in *sFRP4* have been confirmed to cause Pyle disease (70, 72, 73). These nonsense mutations prohibit appropriate WNT and BMP signaling interaction, which is crucial to maintain appropriate stability of cortical bone (70, 71, 73).

WNT Activators

Glypicans

Glypicans (GPCs), a subfamily of heparin sulfate proteoglycans, are linked to the cell surface *via* a glycosyl-phosphatidylinositol (GPI) anchor (75–77). The main function of the GPCs is regulating the activity of growth factors, such as hedgehogs and (non-)canonical WNTs (77–80). Six highly conserved GPCs have been identified in mammals, and pathogenic variants in *GPC4* and *GPC6* have been found in patients clinically diagnosed with skeletal dysplasia.

GPC4

GPC4 is located on chromosome Xq26 and is a regulator of the WNT/PCP pathway (79, 81). Only recently, hemizygous truncating and frameshift mutations were found in families with Keipert syndrome (82). These mutations are recognized as loss of function due to the subsequent loss of essential domains, such as the GPI anchor. Keipert syndrome is characterized by craniofacial and digital abnormalities, accompanied by learning difficulties and deafness (82, 83). Furthermore, there are indications that hemizygous pathological variants could result in an X-linked form of RS. White et al. (84) described a non-synonymous missense variant (p.Arg412Lys) in a male patient with an RS-like phenotype, including brachydactyly, mesomelia, and facial dysmorphisms.

GPC6

GPC6 is the smallest protein of the GPC family and is widely expressed in the growth plate during skeletal development (76, 77, 85). Genetic null mutations in *GPC6* prevent anchoring to the membrane, thereby disrupting the action of GPC6 in developmental processes (75–77). Such mutations were found in omodysplasia type 1 (OMOD1), an autosomal recessive skeletal dysplasia. Both nonsense mutations and genomic rearrangements have been described to contribute to the development of OMOD1 (76). This rare syndrome is characterized by cranial dysmorphisms, short stature of prenatal onset with severe shortening of the humeri ("omo" is the Greek term for humerus) and femora, and restricted mobility in elbows, hips, and knees. In addition, extra-skeletal manifestations, including congenital heart defects and cognitive delay, can also be observed (76, 77). There is also an autosomal dominant form of OMOD (OMOD2), which is milder with normal stature and is caused by heterozygous mutations in the *FZD2* gene (see below).

RSPO2

R-spondins (RSPO) are secreted proteins and activators of the canonical WNT signaling pathway (86–88). The WNT agonistic activity is a direct result of the formation of a ternary complex with leucine-rich repeat-containing G protein-coupled receptor 4, 5, or 6 (LGR4/5/6) and Zinc and ring finger 3 (Znrf3) or Ring finger protein 43 (Rnf43), as this complex prevents ubiquitination of the WNT receptors (86, 89–91). RSPOs are key regulators in bone development and bone homeostasis, partly mediated by RSPO2, which has high expression levels in the apical ectodermal ridge of limb buds, but also in the postnatal skeleton, lungs, and brain tissue (86). Similar to *WNT3* alterations (see above), an impaired RSPO2 function contributes to the development of tetra-amelia. However, patients affected with *RSPO2* mutations exhibit additional hypoplasia or aplasia of the lungs (91). Bi-allelic nonsense mutations, deletions, and frameshift mutations were described in families with tetra-amelia type 2 (91). Furthermore, a homozygous missense mutation (p.Arg69Cys) was reported in humerofemoral hypoplasia with radiotibial ray deficiency, albeit in only one consanguineous family. The affected family members showed severe dysostosis with malformation of all four limbs, absence of tibiae, and hypoplasia of the pelvis (91).

RECEPTORS AND CO-RECEPTORS

Both the canonical and non-canonical WNT signaling pathways are activated by the binding of WNT ligands to the FZD receptors alone or in combination with specific co-receptors. Identification of single disease mutations in LRP5, LRP6, and ROR2 highlights the importance of these co-receptors in the regulation of canonical and non-canonical WNT signaling during skeletal development.

FZD2

FZD2 is a member of the FZD receptor family that includes nine G protein-coupled receptors. These FZD receptors interact with disheveled (DVL) upon activation by one of the WNT ligands which results in the initiation of one of the different WNT pathways. In 2015, Saal et al. (92) demonstrated that mutations in *FZD2* can cause autosomal dominant OMOD, also referred to as OMOD2. OMOD2 is a rare skeletal dysplasia which is marked by severe rhizomelic shortening of the upper limbs, mild facial dysmorphism (frontal bossing, depressed nasal bridge, and short nose), and genital hypoplasia (92). Clinically, OMOD2 can be distinguished from OMOD1 (recessive form—discussed previously) by the presence of short first metacarpals and normal stature (93).

As mentioned, heterozygous mutations in *FZD2* can cause OMOD2. To date, one missense mutation (p.Gly434Val) and two nonsense mutations involving adjacent amino acids (p.Ser547* and p.Trp548*) have been reported (92–95). The nonsense mutations are located in the intracellular domain of FZD2, more precisely in the conserved DVL binding motif (KTxxW). Subsequent functional studies have demonstrated that in the presence of p.Trp548* mutant FZD2, the localization of DVL at the membrane and the activation of the canonical WNT signaling pathway upon WNT treatment are lost (92). The effect of the identified mutations on the non-canonical WNT signaling pathways was not investigated. Several of the clinical features that are reported in patients with OMOD2 are also present in patients with RS (described previously). Therefore, it is likely that the causal genes for these disorders are involved in the same pathway(s). As it is shown that WNT5A/ROR2 interaction can induce WNT/PCP signaling (see below), it is likely that FZD2 is also involved in this pathway.

ROR2

ROR2 is a member of the ROR kinase family of orphan receptors with tyrosine kinase activity which is involved in the WNT signaling pathway as co-receptor of the non-canonical PCP pathway. The extracellular domain of ROR2 which is important for protein–protein interactions contains several conserved domains such as an immunoglobin-like domain, an FZD-like cysteine-rich domain (CRD), and a Kringle domain (KD). The intracellular region contains a tyrosine kinase (TK) domain followed by serine/threonine-rich and proline-rich structures. Mutations in *ROR2* can cause either autosomal dominant brachydactyly type B1 (BDB1) or autosomal recessive RS type 1 (ARRS1) (96, 97).

ARRS1 is characterized by short stature, mesomelic limb shortening, genital hypoplasia, and typical facial dysmorphisms (including hypertelorism). In addition, patients with ARRS1 may also have vertebral segmentation defects such as hemivertebrae and rib fusions (98). Since the identification of *ROR2* as a disease-causing gene for ARRS1, more than 25 different mutations have been identified (99). These mutations include missense, nonsense, and frameshift mutations located in both the intra- and extracellular regions of the protein (96, 98–101). Based on the phenotypic overlap between ARRS1 and *Ror2* knockout mouse, it was suggested that the mutations result in loss of function of ROR2 due to decreased protein stability and intracellular entrapment of the mutant protein (98, 101). ROR2 serves as a co-receptor for WNT5A-induced non-canonical WNT signaling of which WNT/PCP signaling is shown to be most important for limb development. Loss of function of ROR2 will most likely result in decreased WNT/PCP signaling and limb deformities (102).

BDB1 is the most severe form of brachydactyly and is one of the first described types of heritable brachydactyly. It is characterized by hypoplasia and/or aplasia of the distal phalanges and nails in hand and feet resulting in an amputation-like phenotype (101, 102). The BDB1 causing mutations in *ROR2* are all nonsense or frameshift mutations that are located in the intracellular region of the protein and co-localize in two domains, either immediately N-terminal or C-terminal of the TK domain (101). Genotype–phenotype correlation demonstrated that the latter group of mutations results in a more severe phenotype (103). Since parents of ARRS1 patients do not show brachydactyly, it is suggested that BDB1 causing mutations are gain-of-function mutations (96).

LRP Receptors

The LRPs are a group of evolutionary conserved receptors regulating a wide range of cellular processes. In order to regulate these processes, LRPs can modulate a variety of pathways, including canonical WNT signaling activity (104). The extracellular region of the majority of the LRP receptors contains at least one ligand-binding domain composed of cysteine-rich ligand-binding-type repeats and one epidermal growth factor (EGF)-precursor homology domain composed of EGF repeats and a YWTD/β-propeller domain. Most LRP receptors have in the intracellular region at least one NPxY-(endocytosis) motif. However, LRP5/6 are lacking this motif (105). Mutations in three members of the LRP family, namely, LRP4, LRP5, and LRP6, are reported to result in rare skeletal disorders (51, 106, 107).

LRP5

Almost 20 years ago, genetic research highlighted the importance of LRP5 in the regulation of bone formation with the identification of disease-causing mutations in patients with abnormal low or high bone mass. Subsequent studies demonstrated that LRP5, as a co-receptor of the canonical WNT signaling pathway, regulates osteoblast proliferation and differentiation as well as osteocyte apoptosis (108). More specifically, homozygous loss-of-function mutations in LRP5 can cause the osteoporosis-pseudoglioma (OPPG) syndrome which is characterized by reduced bone mass, increased bone fragility, and severely reduced visual acuity (109). So far, over 70 different mutations in *LRP5* have been reported to cause OPPG, and these mutations are spread throughout the gene. Mutations affecting splicing or introducing premature stop codons result in the most severe phenotypes (110). Furthermore, reduced bone mass is also reported in heterozygous mutation carriers (111). Besides the severe OPPG phenotype, it is reported that heterozygous OPPG loss-of-function mutations in *LRP5* can cause juvenile-onset osteoporosis without an ocular phenotype in children (112).

As mentioned, mutations in *LRP5* can also result in a skeletal phenotype with increased bone mass (106, 113, 114). When consulting OMIM or the nosology of skeletal disorders (6), mutations in *LRP5* are identified in patients diagnosed with high bone mass (HBM) phenotype, autosomal dominant osteopetrosis type 1, autosomal dominant Van Buchem disease, Worth disease, endosteal hyperostosis, or osteosclerosis. Although these rare disorders have a different name, they represent the same disorder which is characterized by increased bone mass especially affecting the skull and tubular bones and a reduced fracture risk (106). Due to the increased bone mass of the skull, headaches and cranial nerve entrapment are commonly reported in these patients (115). Furthermore, these disorders are all caused by heterozygous gain-of-function mutations in *LRP5*. All mutations are located in the first β-propeller domain of the protein, and functional studies demonstrated that they disrupt the binding of canonical WNT signaling inhibitors sclerostin and DKK1 with the co-receptor. Although mutations in DKK1 are not reported in patients with monogenic skeletal disorders, different studies have shown that DKK1 is an important regulator of the WNT signaling pathway and bone mass *via* its interaction with LRP5 (116). As a result, mutations disrupting the binding of LRP5 with sclerostin and DKK1 result in increased canonical WNT signaling activity which consequently leads to increased bone formation (116–118).

LRP6

LRP6 is the closest homolog of LRP5, and functional studies demonstrated that LRP6, similar to LRP5, is a co-receptor of the canonical WNT signaling pathway (104). However, mutations in *LRP6* are less common compared to *LRP5*, most likely due to a more important role for LRP6 during embryonic development. However, recently, heterozygous mutations in the first β-propeller domain of LRP6 were identified in two families with HBM phenotype. The amino acids mutated in LRP6 are homologs to known LRP5 mutations (107). Although no functional studies were performed, it is highly likely that the mutations, similar to those reported for LRP5, disturb the binding of sclerostin and DKK1 and consequently result in increased WNT signaling activity and increased bone mass. The skeletal phenotype of LRP6 mutant patients was similar to the phenotype reported in patients with LRP5 mutations. However, in the small group of patients with HBM mutations in LRP6, no cranial nerve entrapment was reported which is commonly found in LRP5 HBM mutant patients. In addition, in patients with HBM mutations in LRP6, absence of the adult maxillary lateral incisors was reported. Since the latter is never reported in LRP5 HBM mutant patients, this finding can maybe be used to distinguish between both HBM types (107). Besides the high

bone mass phenotype, heterozygous loss-of-function mutations in *LRP6* are previously shown to cause coronary artery disease and tooth agenesis (119, 120). Remarkably, in the family with coronary artery disease due to a mutation in *LRP6* described by Mani et al. (119), osteoporosis was also reported. Based on the role of LRP6 in WNT signaling, it was suggested that the observed osteoporosis phenotype was also caused by the mutation in *LRP6*.

LRP4

Another LRP receptor which is implicated in the regulation of bone formation is LRP4. In contrast to LRP5/6, LRP4 is involved in the inhibition of the canonical WNT signaling pathway through its interaction with sclerostin. The identification of disease-causing *LRP4* mutations in syndromic disorders demonstrated that LRP4 is not only important in the regulation of bone mass but also regulates limb and kidney development among others (51, 121, 122). Mutations in *LRP4* can cause sclerosteosis type 2, Cenani–Lenz syndrome (CLS), isolated syndactyly, and congenital myasthenia gravis depending on type and location of the mutations (51, 122–124). As myasthenia gravis patients have no skeletal phenotype and the mutations do not affect WNT signaling activity (125), it is not further discussed in this review.

The clinical phenotype of sclerosteosis type 2 is highly similar to the sclerosteosis type 1 phenotype previously described. Sclerosteosis type 2 causing mutations in *LRP4* are located in the third β-propeller domain and result in decreased binding of sclerostin (51, 124). Due to the impaired LRP4–sclerostin binding, serum levels of sclerostin are elevated in these patients and the activity of the canonical WNT signaling pathway is increased in osteoblasts, leading to increased bone formation (51, 124).

As mentioned, bi-allelic mutations in *LRP4* can also cause CLS. CLS is marked by syndactyly, synostosis, and renal abnormalities, similar to what has been discussed above for patients with a mutation in *GREM1* that interacts with LRP4. CLS-causing mutations are spread throughout LRP4, except for the third β-propeller domain (122, 123). Functional studies indicated CLS-causing mutations result in a decreased expression of the receptor at the cell membrane. This indicates that CLS-causing mutations have an effect not only on the inhibition of the canonical WNT signaling pathway by sclerostin but also on the regulation of other pathways such as MUSK-agrin signaling. In addition, in an individual with isolated syndactyly, compound heterozygous missense mutations within the fourth β-propeller domain have been described (126). *In vitro* studies demonstrated that the presence of the mutations results in decreased inhibition of the WNT signaling activity by LRP4 similar as reported for CLS mutations. Therefore, it is suggested that these patients might suffer from a mild type of CLS.

INTRACELLULAR REGULATORS OF WNT SIGNALING

Activation of the different FZD receptors and co-receptors results intracellularly in a complex signaling cascade which ultimately leads to transcription of target genes and regulation of a wide range of cellular processes. There are many proteins involved in this intracellular signaling; however, only a few underlie the development of rare inherited skeletal dysplasias.

Disheveled

The disheveled protein family consists of three members (DVL1-3) which all act downstream of FZD receptors in both the canonical and non-canonical WNT signaling pathway. The DVL proteins share highly conserved regions, namely, the amino-terminal DIX (N-terminus) domain, a PDZ (central) domain, a carboxyl-terminal DEP (C-terminus) domain, and two regions with positively charged amino acid residues (127). These conserved regions are important to mediate protein–protein interactions and define the further propagation of the signal through either the canonical or non-canonical pathway. Furthermore, DVL can move from the cytoplasm to the nucleus, and this is also required for proper propagation of the WNT signaling. To ensure the movement of DVL to the nucleus, DVL proteins contain a nuclear export sequence (NES) and nuclear localization sequence (NLS) (128).

DVL proteins are highly important for normal functioning of the different WNT signaling pathways. Genetic defects can consequently affect the skeleton. Heterozygous mutations in both *DVL1* and *DVL3* have been identified in individuals with autosomal dominant RS (ADRS) types 2 and 3, respectively (129–131). As mentioned, ADRS is marked by skeletal abnormalities, genital abnormalities, and dysmorphic facial features (132).

DVL1

In 2015, two independent research groups reported the identification of *de novo* frameshift mutations in exon 14 of *DVL1* in patients with ADRS without mutations in *WNT5A* (129, 130). In total, nine different frameshift mutations, all located in the penultimate exon of *DVL1,* have been reported so far. All mutations result in the same premature stop codon in the last exon and escape nonsense-mediated mRNA decay. The mutations are located downstream of the abovementioned conserved domains, and all mutations result in a novel shared, highly basic C-terminal tail sequence that most likely exerts novel signaling functions (129–131). More in detail, *in vitro* luciferase reporter assays demonstrated that co-transfection of wild-type and mutant DVL1, representing the heterozygous mutations, results in significantly increased canonical WNT signaling activity compared to the effect of wild-type DVL1 alone. More interestingly, several patients with mutations in *DVL1* show increased bone mass which is not seen in the other autosomal dominant or recessive forms of RS (129). Most likely, the increased bone mass in ADRS2 patients is caused by the effect of *DVL1* mutations on canonical WNT signaling. In addition, ADRS2 patients often have macrocephaly and normal stature (>10% percentile) (129, 130).

DVL3

In a subsequent study, it was shown that not only mutations in *DVL1* but also *DVL3* can cause ADRS (131). Similar as for DVL1, the mutations result in a frameshift to the−1-reading frame and a shared premature stop codon in the last exon. The DVL3 mutants are shown to escape nonsense-mediated mRNA

decay. All identified mutations have 83 C-terminal amino acids in common (131, 133). The functional effect of this C-terminal tail on regulation of canonical and/or non-canonical WNT signaling pathway is still unknown. Patients with mutations in *DVL3* (ADRS3) can be distinguished from ADRS2 patients based on the presence of short stature and macrocephaly. Furthermore, congenital heart defects and cleft lip and/or cleft palate were reported in the majority of the patients (131).

NXN

Nucleoredoxin (NXN) is an oxidative stress response protein which is highly expressed during murine limb bud development and can directly bind DVL *via* the PDZ domain. By binding to DVL, NXN can inhibit DVL signaling activity and consequently different WNT signaling pathways (134–136). It is suggested that the interaction of NXN with DVL is a key regulatory mechanism to maintain spatial and temporal balance between canonical and non-canonical WNT signaling during development (84). Recently, *NXN* was identified as the causative gene for autosomal recessive RS type 2 (ARRS2). In two families without mutations in the known causative gene *ROR2*, White et al. (84) identified bi-allelic variants which segregated with the ARRS2 phenotype consisting of short stature, mesomelic shortening, genital hypoplasia, and typical facial dysmorphisms. Mice lacking Nxn also show craniofacial defects, suggesting that the mutations reported in the patients are loss-of-function mutations (136, 137).

RAC3

RAC3 is a member of the Rac subfamily of the Rho family of GTPases. Upon activation by WNT ligands, RAC3 can interact with DVL to activate downstream signaling including c-jun NH2-terminal kinase (JNK)/c-jun phosphorylation which is necessary for cytoskeletal organization. White et al. (84) reported in 2018 the identification of a rare variant in RAC3 in a patient with a Robinow-like phenotype including facial dysmorphism and developmental delay, seizures, an abnormal electroencephalogram, and a thin corpus callosum. Based on the role of RAC3 in the WNT/PCP pathway (**Figure 3**), they suggest that the identified *de novo* variant can cause the Robinow-like phenotype in the patient (84). However, additional functional studies or identification of additional affected individuals with possible pathogenic variants is necessary. In a more recent study, Costain et al. (138) also identified heterozygous missense variants in *RAC3* in patients with neurodevelopmental disorder with structural brain anomalies and dysmorphic facial features. Since there is some overlap between the clinical phenotype of the patients described in both reports, most likely the patients described by both research groups suffer from the same disease.

APC2

Adenomatous polyposis coli (*APC*) is a tumor suppressor gene that can regulate canonical WNT signaling as a part of the multiprotein destruction complex that targets β-catenin for phosphorylation and degradation (139). APC2 or APC-like protein is a homolog of APC which is mostly expressed in the postmitotic neurons during development (140). Both proteins share a 20-amino acid repeat motif that is capable of binding β-catenin. However, in addition to this shared motif, APC also contains a second motif which is lacking in APC2 and can bind β-catenin with a higher affinity. Nevertheless, APC2 is still capable of depleting intracellular β-catenin, although less efficient than APC (141).

Homozygous loss-of-function mutations in *APC2* have recently been described in patients with a Sotos-like phenotype (142). Sotos syndrome is an overgrowth syndrome, also known as cerebral gigantism. Individuals with APC2 mutations resemble patients with Sotos syndrome because they show intellectual disability and relative macrocephaly with a long face and prominent chin (142). The role of the canonical WNT signaling pathway in the development of this disorder is not yet investigated.

AMER1

APC Membrane Recruitment Protein 1 (AMER1), also known as FAM123B or Wilms tumor on the X chromosome (WTX), is an intracellular inhibitor of the canonical WNT signaling pathway. AMER1 can interact with the AXIN/APC/GSK3β/β-catenin multiprotein complex and enhances the ubiquitination and degradation of β-catenin (143, 144). *AMER1* is located on the X-chromosome, and loss-of-function mutations or gene deletions can cause the X-linked dominant disorder osteopathia striata with cranial sclerosis (OSCS) (145). Radiographs of patients with OSCS show cranial sclerosis and dense linear striations in the submetaphyseal regions of the long bones and pelvis. Besides these radiographic signs, patients with OSCS often also present with macrocephaly, broad nasal bridge, frontal bossing, ocular hypertelorism, hearing loss, and palate abnormalities. Less common symptoms of OSCS are cardiac malformations and cognitive defects. In males, OSCS can be lethal due to severe heart defects and/or gastrointestinal malformations (143, 145, 146).

PORCN

Porcupine O-acyltransferase (PORCN) is a member of the evolutionarily conserved porcupine (PORC) gene family of endoplasmic reticulum transmembrane proteins that are involved in the processing and secretion of WNT ligands. *PORCN* is located on the X-chromosome, and heterozygous loss-of-function mutations can cause focal dermal hypoplasia (FDH) also known as Goltz syndrome in female patients. Goltz syndrome caused by mutations in *PORCN* is rarely reported in males which leads to the suspicion that non-mosaic Goltz syndrome is embryonically lethal for males (147, 148). Goltz syndrome is a multisystem syndrome that is characterized by dermal abnormalities including focal dermal hypoplasia among others, ophthalmologic features, facial anomalies, and skeletal abnormalities. The latter are reported in ∼80% of the patients and include syndactyly or polydactyly, osteopathic striae, hypoplasia or absence of digits, scoliosis, and/or facial asymmetry (148, 149).

DISCUSSION

The identification of novel disease-causing genes for rare skeletal dysplasias accelerated significantly in the last decades,

TABLE 1 | Overview of the main skeletal dysplasias caused by mutations in genes involved in WNT signaling.

Gene	Phenotype	OMIM	Skeletal	Other symptoms	LOF/GOF	Genetic associations
EXTRACELLULAR MODULATORS						
WNT1	Osteogenesis imperfecta type XV (AR)	615220	Recurrent bone fractures Bone deformity Short stature Low bone mass	Blue sclerae (not all patients) Brain malformations (not all patients) Hearing loss Dentinogenesis imperfecta	LOF	BMD[a,b]
	Early-onset osteoporosis (AD)	615221	Recurrent bone fractures Low bone mass Impaired bone quality (trabecular and cortical)		LOF	
WNT3	Tetra-amelia syndrome type 1 (AR)	273395	Absence of all limbs Pelvis hypoplasia	Cleft lip/palate Pulmonary abnormalities Urogenital defects Kidney/spleen/adrenal glands hypoplasia	LOF	/
WNT5A	Robinow syndrome type 1 (AD)	180700	Short stature Mesomelic limb shortening Hypertelorism Mandibular hypoplasia Dental problems	Genital hypoplasia Kidney abnormalities	LOF	/
WNT6	Acro-pectoro-vertebral dysplasia (F-syndrome, AD)	102510	Carpal/tarsal synostoses Skeletal malformations Syndactyly/polydactyly	Craniofacial anomalies Spina bifida occulta	LOF	/
WNT7A	Fuhrmann syndrome (AR)	228930	Limb shortening Fibular/ulnar hypoplasia Oligosyndactyly		Partial LOF	/
	Al-Awadi–Raas–Rothschild syndrome (AR)	276820	Limb shortening Absence of ulna/fibula Oligosyndactyly Pelvis hypoplasia	Kidney agenesis Genital hypoplasia (females: absence of uterus, anteriorly displaced genitalia) Hypoplasia/absence of nails	LOF	
WNT10B	Split-hand/foot malformation type 6 (AR)	225300	Malformations of hands/feet		LOF	**B**MD[c]
SOST	Sclerosteosis type 1 (AR)	269500	Progressive skeletal overgrowth (tubular bones, skull, mandible) Tall stature Syndactyly	Cranial nerve compression due to increased bone mass of the skull	LOF	BMD[a,b]/fractures[a,d]
	Van Buchem disease (AR)	239100	Increased thickness of bones (tubular bones, skull, mandible)	Cranial nerve compression due to increased bone mass of the skull	Partial LOF	
	Craniodiaphyseal dysplasia (AD)	122860	Sclerosis Hyperostosis (skull/facial bones) Facial distortion (= leontiasis ossea)	Severe neurologic impairment	LOF (dominant negative)	
Grem1	Cenani–Lenz-like non-syndromic oligosyndactyly (SP/AD)	NA	Bilateral oligosyndactyly		LOF	/
sFRP4	Pyle disease (AR)	265900	Metaphyseal widening (long bones) Cortex thinning Increased trabecular bone mass Decreased bone mineral density Fractures Genu valgum Dental abnormalities		LOF	BMD[e]/fractures[a]
GPC4	Keipert syndrome (X-linked)	301026	Craniofacial abnormalities Digits abnormalities	Learning difficulties Deafness	LOF	/
	Robinow-like phenotype (X-linked)		Brachydactyly Mesomelia Facial dysmorphism			

(Continued)

TABLE 1 | Continued

Gene	Phenotype	OMIM	Skeletal	Other symptoms	LOF/GOF	Genetic associations
GPC6	Omodysplasia type 1 (AR)	258315	Short stature Severe limb malformations (shortening of humeri/femora) Restricted mobility in elbows/hip/knees Craniofacial dysmorphism	Cryptorchidism Hernias Congenital heart defects Cognitive delay	LOF	BMD[a,b]
RSPO2	Tetra-amelia syndrome type 2 (AR)	618021	Absence of all limbs Pelvis hypoplasia	Lung hypo/aplasia Cleft lip/palate Dysmorphic features	LOF	BMD[a]
	Humerofemoral dysplasia (AR)	618022	Severe dysostosis Malformation of all limbs Absence of tibiae Femoral deficiency (not all patients) Absence of digits (preaxial side) Pelvis hypoplasia		LOF	
(CO-)RECEPTORS						
FZD2	Omodysplasia type 2 (AD)	164745	Craniofacial dysmorphism Limb shortening	Genital hypoplasia	LOF	/
ROR2	Robinow syndrome type 1 (AR)	268310	Short stature Limb shortening Facial dysmorphisms	Genital hypoplasia	LOF	BMD[f]
	Brachydactyly type B1 (AD)	113000	Hypoplastic/aplastic distal phalanges and nails in hands and feet Hypoplastic middle phalanges		GOF	
LRP5	Osteoporosis-pseudoglioma syndrome (AR)	259770	Reduced bone mass and strength	Blindness due to abnormal blood vessel development in the eye	LOF	BMD[a,b]/fractures[d]
	(Juvenile) osteoporosis (AD)		Reduced bone mass and strength		LOF	
	Endosteal hyperostosis (AD)	144750	Cortical thickening of the long bones Generalized sclerosis Increased thickness of the skull Increased bone strength Torus palatinus (some patients)	Cranial nerve compression due to increased bone mass of the skull	GOF	
	Osteopetrosis type 1 (AD)	607634				
	Van Buchem disease type 2 (AD)	607636				
	High bone mass phenotype (AD)	601884				
LRP6	High bone mass phenotype (AD)		Cortical thickening of the long bones Generalized sclerosis Increased thickness of the skull Increased bone strength	absence of the adult maxillary lateral incisors	GOF	BMD[a,b]
LRP4	Sclerosteosis type 2 (AR)	614305	Progressive skeletal overgrowth Cortical thickening of the tubular bones and skull Facial asymmetry Syndactyly	Cranial nerve compression due to increased bone mass of the skull	Partial LOF	BMD[a,b]
	Cenani–Lenz syndrome (AR)	212780	Distal bone malformations Syndactyly Mild facial dysmorphism	Kidney anomalies	LOF	
INTRACELLULAR REGULATORS						
DVL1	Robinow syndrome type 2 (AD)	616331	Limb shortening Facial dysmorphisms Osteosclerosis Short stature (rare)	Genital hypoplasia	LOF/GOF*	/

(Continued)

TABLE 1 | Continued

Gene	Phenotype	OMIM	Skeletal	Other symptoms	LOF/GOF	Genetic associations
DVL3	Robinow syndrome type 3 (AD)	616894	Limb shortening Facial dysmorphisms Short stature Macrocephaly (not all patients)	Genital hypoplasia	LOF	/
NXN	Robinow syndrome type 2 (AR)	618529	Limb shortening Facial dysmorphisms Short stature Macrocephaly Brachydactyly	Congenital anomalies (omphalocele, ventral hernia, and cardiac anomalies)	LOF	BMD[a,b]
RAC3	Robinow-like phenotype		Facial dysmorphism	Developmental delay Seizures Abnormal electroencephalogram Thin corpus callosum	LOF	/
APC2	Sotos syndrome type 3 (AR)	617169	Macrocephaly Long face Prominent chin and nose	Severe receptive and expressive language disorder, learning disabilities, and hyperactive behavior	?	/
AMER1	Osteopathia striata with cranial sclerosis (X-linked)	300373	Sclerosis of the long bones and skull Longitudinal striations in the long bones, pelvis, and scapulae Macrocephaly	Cardiac, intestinal, and genitourinary malformations (males) Cleft palate	LOF	/
PORCN	Goltz syndrome (X-linked)	305600	Syndactyly, ectrodactyly, polydactyly Osteopathic striae Hypoplasia or absence of digits Scoliosis Facial asymmetry	Dermal abnormalities (focal dermal hypoplasia, subepidermal subcutaneous fat deposits, …) Ocular, urinary, gastrointestinal, cardiovascular, neurologic, and oral abnormalities	LOF	/

**Combined expression of WT and mutant DVL1 results in increased canonical WNT signaling; however, the effect of DVL1 on the WNT/PCP pathway is most likely LOF based on the LOF mutations identified in ROR2 and WNT5A.*

AD, autosomal dominant; AR, autosomal recessive; BMD, bone mineral density; DVL, disheveled; GOF, gain of function; LOF, loss of function; PCP, planar cell polarity; WT, wild type.
[a]Morris et al. (151); [b]Kichaev et al. (152); [c]Van Camp et al. (153); [d]Trajanoska et al. (154); [e]Wang et al. (155); [f]Mullin et al. (156).

initially by positional cloning efforts and more recently by the availability of next-generation sequencing technology. This resulted in the identification of the disease-causing gene for 92% of the skeletal disorders (6). The increased knowledge on monogenic diseases resulted in a better understanding of the pathological mechanisms and highlighted which pathways regulate specific cellular processes. This information is also relevant for understanding more common multifactorial diseases. Furthermore, it has been shown that therapeutic targets which are based on genetic evidence from Mendelian traits as well as genome-wide association studies (GWASs) are more likely to be successful in clinical studies for multifactorial diseases (150). Here, we focused on skeletal dysplasias caused by mutations in genes that encode proteins that are directly involved in one of the WNT signaling pathways. As shown in **Table 1**, mutations in these genes can result in a variety of skeletal dysplasias, each with specific clinical features. The broad spectrum of clinical observations reflect the cellular and spatial functions of WNT signaling, some of them associated with embryonal development, others with bone mass and homeostasis in adult life. For example, the clinical features of RS and OMOD are similar which led to the hypothesis that all causative genes are involved in the WNT/PCP pathway which is previously shown to be important during limb development (**Figure 2**) (102). On the other hand, the influence of canonical WNT signaling on bone mass was highlighted by unraveling the underlying pathogenic mechanisms of disorders with a progressively increasing bone mass such as sclerosteosis, Van Buchem disease, and high bone mass phenotypes (osteosclerosis) (51, 53, 57, 107, 113). The genes causing these disorders, *SOST, LRP4, LRP5*, and *LRP6*, are all involved in the canonical WNT signaling pathway (**Figure 3**), and all mutations reported result in an increased canonical WNT signaling (**Table 1**). In addition to the conditions with increased bone mass mentioned, mutations in *WNT1*, a ligand inducing canonical WNT signaling, and *LRP5* can also result in decreased canonical WNT signaling activity and consequently decreased bone mass (**Table 1; Figure 3**) (12–15, 109). All these data show that the canonical WNT signaling pathway is an important regulator of bone mass as is also corroborated by the observation in GWASs that many of these genes harbor genetic polymorphisms associated with bone mineral density or fractures (151, 152, 154) (**Table 1**). This is in line with a general observation that there is an enrichment in genes for Mendelian disorders near regions associated in GWASs with a similar phenotype (157).

The evidence for an important role for WNT signaling in regulating bone mass makes it an obvious candidate for drug development for the treatment of osteoporosis. The incidental

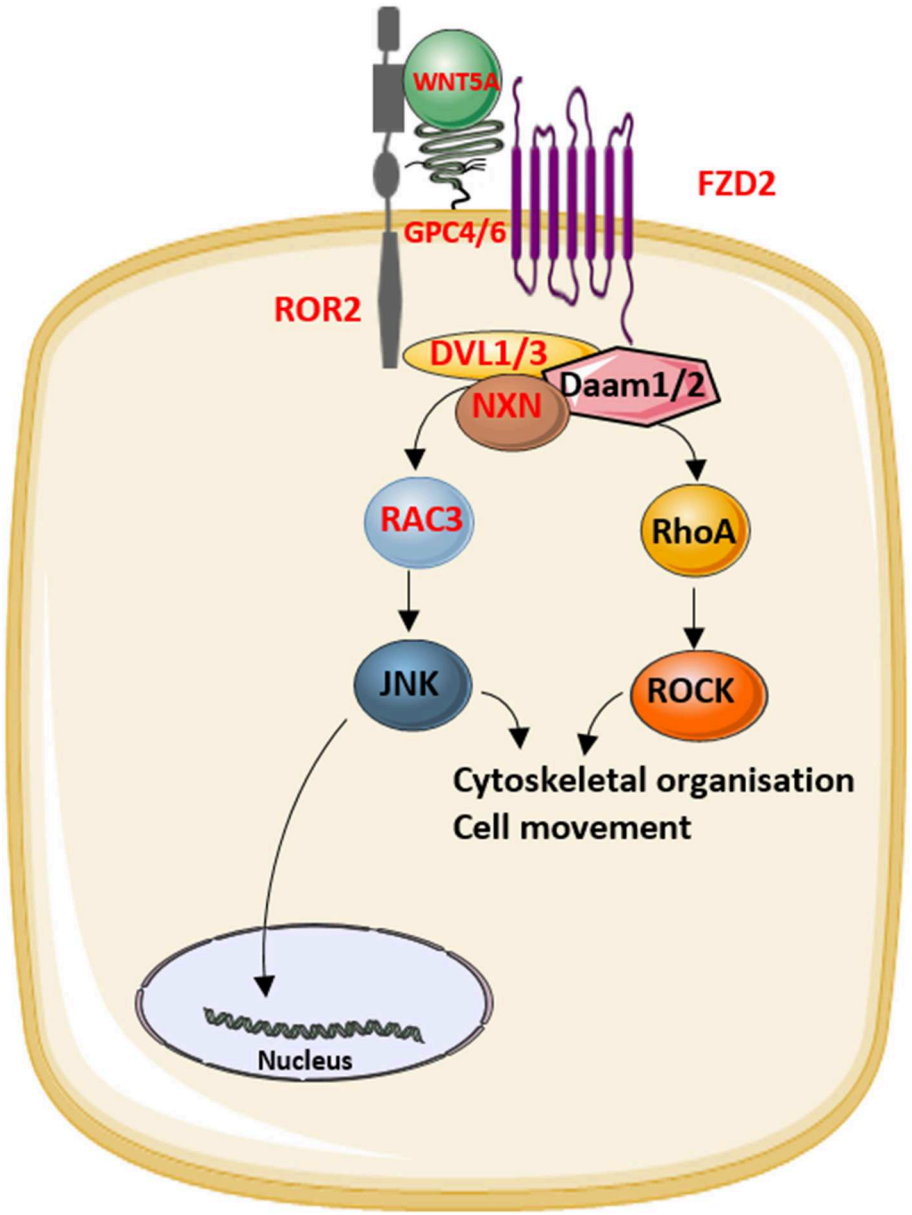


FIGURE 2 | The role of the proteins that underlie Robinow syndrome or omodysplasia (indicated in red) in the WNT/planar cell polarity (PCP) pathway.

finding that lithium chloride (LiCl), a first-line treatment for bipolar disorders, increases canonical WNT signaling activity and increases bone formation already confirms the therapeutic potential of the pathway (158, 159). But at the same time, phenotypic features of single mutation diseases can give insights into possible adverse effects of interfering with the pathway. For example, a rare nonsense mutation (c.376C>T) was identified in the *LGR4* gene, which encodes a receptor for RSPO1–4, activators of WNT signaling. The mutation is specific for the Icelandic population, as described by Styrkarsdottir et al. (160), and shows strong association with osteoporotic fractures and low bone mineral density (BMD). But at the same time, the variant is associated with an increased risk of squamous cell carcinoma of the skin and biliary tract cancer (160). Also for other types of cancer, abnormal WNT signaling has been reported. These cancers can be caused by single mutations (germline or mosaic) in, for example, *APC* or *WTX*, but also common genetic variation in *WNT3*, *DVL1*, and *NXN* is previously associated with increased cancer risk (161–166). However, increased cancer risk is not reported for any of the rare skeletal dysplasias described in this review.

Currently, the treatment of osteoporosis is mainly based on the use of antiresorptive agents, such as bisphosphonates, without the capacity to rebuild the lost bone. Consequently,

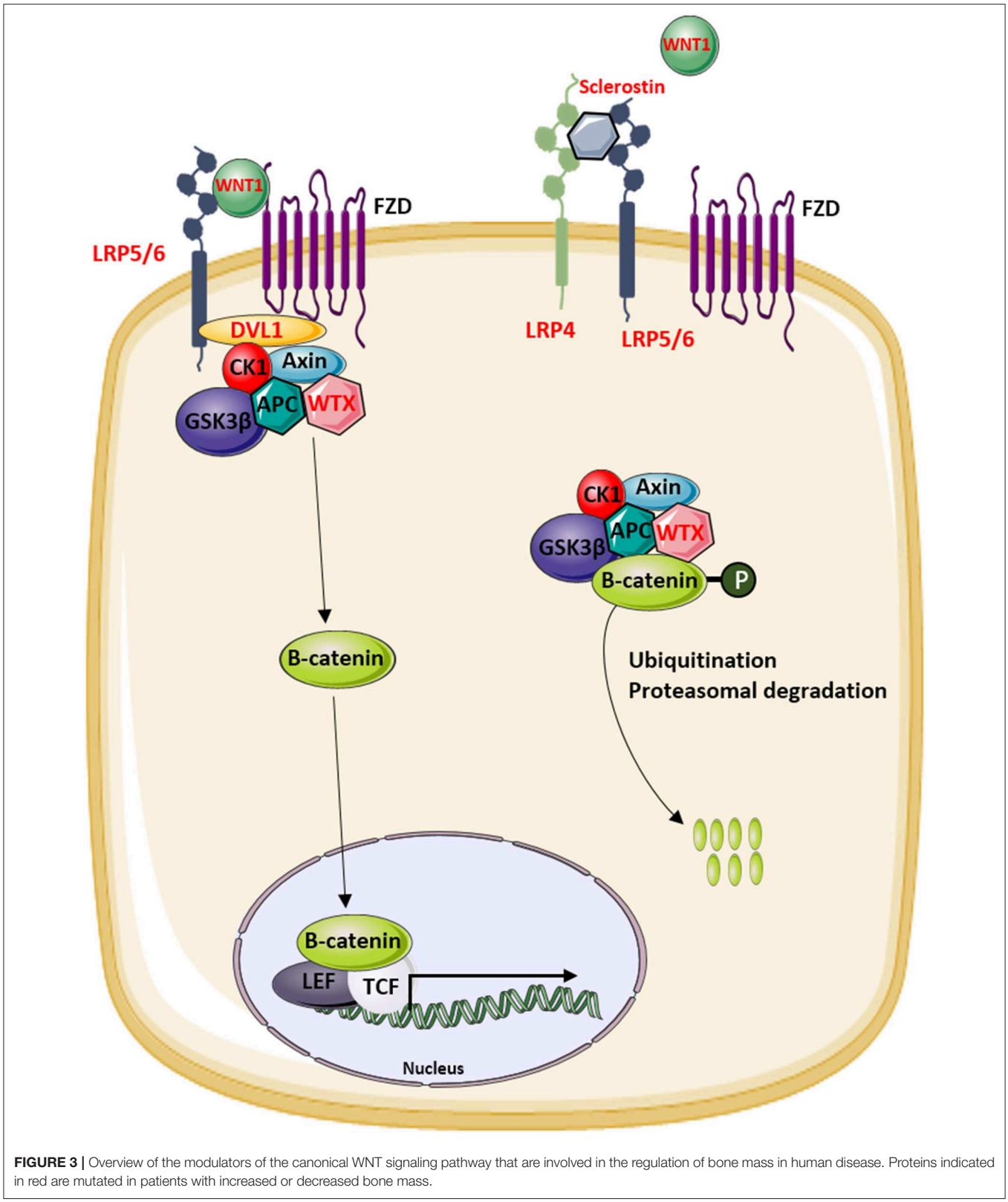


FIGURE 3 | Overview of the modulators of the canonical WNT signaling pathway that are involved in the regulation of bone mass in human disease. Proteins indicated in red are mutated in patients with increased or decreased bone mass.

there is an urgent need for more specific, anabolic therapies for osteoporosis, making canonical WNT signaling of major interest. The pharmaceutical industry focused on sclerostin as a therapeutic target based on the expression of sclerostin being almost exclusive in bone tissue, the progressive increase in bone mass in adults with sclerosteosis and Van Buchem

disease and on the absence of non-skeletal clinical features in these patients (167). Clinical trials demonstrated that treatment with romosozumab (Evenity™, Amgen/UCB), a monoclonal antibody for sclerostin, results in increased bone mass and decreased fracture risk (168, 169). However, a phase III clinical trial demonstrated that although romosozumab is clearly beneficial for bone health, treatment was also associated with slightly increased incidence of cardiovascular events (169). Increased incidence of cardiovascular events was observed in men treated with romosozumab compared to placebo (170) and in postmenopausal women treated with romosozumab compared to women treated with alendronate (169). When compared to postmenopausal women treated with placebo, romosozumab-treated individuals have a similar incidence of cardiovascular events (168). In addition, in patients pretreated with alendronate, the incidence of cardiovascular events was lower (171). Furthermore, in postmenopausal women who were treated with alendronate after romosozumab treatment, the difference in cardiovascular events remained stable compared to patients treated with alendronate alone (169). Therefore, additional studies are needed to investigate whether combined treatment of alendronate and romosozumab can reduce the cardiovascular events. Clinical data from sclerosteosis and Van Buchem patients who lack sclerostin do not report an increased incidence of cardiovascular events in these patients (172) which suggests that targeting sclerostin does not cause cardiovascular disease. However, for now, the use of romosozumab is only approved for the treatment of severe osteoporosis in postmenopausal women but is contraindicated in patients with an increased cardiovascular risk. Therefore, the identification of new therapeutic targets potentially interfering with WNT signaling remains imperative.

In conclusion, mutations in the WNT signaling pathway can lead to a broad range of skeletal dysplasias. Especially those characterized by an increased bone mass have been instrumental in highlighting the role of WNT signaling in bone formation and homeostasis with therapeutic applications for the treatment of osteoporosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

EB holds a postdoctoral Grant (12A3814N) with the research foundation Flanders (FWO Vlaanderen). This work was supported by a Methusalem grant to the Genomed consortium (FFB190208).

REFERENCES

1. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell*. (1982) 31:99–109. doi: 10.1016/0092-8674(82)90409-3
2. Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, Mcmahon A, et al. A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell*. (1991) 64:231. doi: 10.1016/0092-8674(91)90633-A
3. Katoh M. WNT and FGF gene clusters (review). *Int J Oncol.* (2002) 21:1269–73. doi: 10.3892/ijo.21.6.1269
4. Van Amerongen R, Nusse R. Towards an integrated view of Wnt signaling in development. *Development*. (2009) 136:3205–14. doi: 10.1242/dev.033910
5. Boudin E, Van Hul W. Mechanisms in endocrinology: genetics of human bone formation. *Eur J Endocrinol.* (2017) 177:R69–83. doi: 10.1530/EJE-16-0990
6. Mortier GR, Cohn DH, Cormier-Daire V, Hall C, Krakow D, Mundlos S, et al. Nosology and classification of genetic skeletal disorders: 2019 revision. *Am J Med Genet A*. (2019) 179:2393–419. doi: 10.1002/ajmg.a.61366
7. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. *Genes Dev.* (1997) 11:3286–305. doi: 10.1101/gad.11.24.3286
8. Miller JR. The Wnts. *Genome Biol.* (2002) 3:3001-15. doi: 10.1186/gb-2002-3-10-reviews1028
9. Joeng KS, Lee YC, Jiang MM, Bertin TK, Chen Y, Abraham AM, et al. The swaying mouse as a model of osteogenesis imperfecta caused by WNT1 mutations. *Hum Mol Genet.* (2014) 23:4035–42. doi: 10.1093/hmg/ddu117
10. Joeng KS, Lee YC, Lim J, Chen Y, Jiang MM, Munivez E, et al. Osteocyte-specific WNT1 regulates osteoblast function during bone homeostasis. *J Clin Invest.* (2017) 127:2678–88. doi: 10.1172/JCI92617
11. Cao YJ, Zhang H, Zhang ZL. Novel Mutations In The Wnt1, Tmem38b, P4hb, And Pls3 Genes in four unrelated Chinese families with osteogenesis imperfecta. *Endocr Pract.* (2019) 25:230–41. doi: 10.4158/EP-2018-0443
12. Fahiminiya S, Majewski J, Mort J, Moffatt P, Glorieux FH, Rauch F. Mutations in WNT1 are a cause of osteogenesis imperfecta. *J Med Genet.* (2013) 50:345–8. doi: 10.1136/jmedgenet-2013-101567
13. Keupp K, Beleggia F, Kayserili H, Barnes AM, Steiner M, Semler O, et al. Mutations in WNT1 cause different forms of bone fragility. *Am J Hum Genet.* (2013) 92:565–74. doi: 10.1016/j.ajhg.2013.02.010
14. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. *N Engl J Med.* (2013) 368:1809–16. doi: 10.1056/NEJMoa1215458
15. Pyott SM, Tran TT, Leistritz DF, Pepin MG, Mendelsohn NJ, Temme RT, et al. WNT1 mutations in families affected by moderately severe and progressive recessive osteogenesis imperfecta. *Am J Hum Genet.* (2013) 92:590–7. doi: 10.1016/j.ajhg.2013.02.009
16. Brunetti G, Papadia F, Tummolo A, Fischetto R, Nicastro F, Piacente L, et al. Impaired bone remodeling in children with osteogenesis imperfecta treated and untreated with bisphosphonates: the role of DKK1, RANKL, and TNF-alpha. *Osteoporos Int.* (2016) 27:2355–65. doi: 10.1007/s00198-016-3501-2
17. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet.* (2012) 44:491–501. doi: 10.1038/ng.2249
18. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet.* (1999) 22:361–5. doi: 10.1038/11932
19. Niemann S, Zhao C, Pascu F, Stahl U, Aulepp U, Niswander L, et al. Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am J Hum Genet.* (2004) 74:558–63. doi: 10.1086/382196
20. Gupta AM, Jaipal NU. A very rare case of isolated tetra-amelia. *J Evol Med Dent Sci.* (2017) 6:337–9. doi: 10.14260/Jemds/2017/75
21. Kaur L, Singh D, and Kaur, M. P. Diagnosis of tetra-amelia in the first trimester. *J Fetal Med*. (2017) 4:37–9. doi: 10.1007/s40556-016-0111-1
22. Janda CY, Waghray D, Levin AM, Thomas C, Garcia KC. Structural basis of Wnt recognition by Frizzled. *Science*. (2012) 337:59–64. doi: 10.1126/science.1222879
23. Kobayashi Y, Uehara S, Udagawa N, Takahashi N. Regulation of bone metabolism by Wnt signals. *J Biochem.* (2016) 159:387–92. doi: 10.1093/jb/mvv124

24. Maeda K, Kobayashi Y, Koide M, Uehara S, Okamoto M, Ishihara A, et al. The regulation of bone metabolism and disorders by Wnt signaling. *Int J Mol Sci.* (2019) 20:5525. doi: 10.3390/ijms20225525
25. Robinow M, Silverman FN, Smith HD. A newly recognized dwarfing syndrome. *Am J Dis Child*. (1969) 117:645–51. doi: 10.1001/archpedi.1969.02100030647005
26. Person AD, Beiraghi S, Sieben CM, Hermanson S, Neumann AN, Robu ME, et al. WNT5A mutations in patients with autosomal dominant Robinow syndrome. *Dev Dyn.* (2010) 239:327–37. doi: 10.1002/dvdy.22156
27. Roifman M, Marcelis CL, Paton T, Marshall C, Silver R, Lohr JL, et al. De novo WNT5A-associated autosomal dominant Robinow syndrome suggests specificity of genotype and phenotype. *Clin Genet.* (2015) 87:34–41. doi: 10.1111/cge.12401
28. Camera G, Camera A, Pozzolo S, Costa M, Mantero R. F-syndrome (F-form of acro-pectoro-vertebral dysplasia): report on a second family. *Am J Med Genet.* (1995) 57:472–5. doi: 10.1002/ajmg.1320570322
29. Thiele H, Mccann C, Van't Padje S, Schwabe GC, Hennies HC, Camera G, et al. (2004). Acropectorovertebral dysgenesis (F syndrome) maps to chromosome 2q36. *J. Med. Genet.* 41, 213–218. doi: 10.1136/jmg.2003.014894
30. Grosse FH, Opitz, JJM. The F-form of acropectorovertebral dysplasia: the F-syndrome. *Birth Defects Orig Artic Ser*. (1969) 3:48–63.
31. Kantaputra PN, Mundlos S, Sripathomsawat W. A novel homozygous Arg222Trp missense mutation in WNT7A in two sisters with severe Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome. *Am J Med Genet A*. (2010) 152a:2832–7. doi: 10.1002/ajmg.a.33673
32. Lan L, Wang W, Huang Y, Bu X, Zhao C. Roles of Wnt7a in embryo development, tissue homeostasis, and human diseases. *J Cell Biochem.* (2019) 120:18588–98. doi: 10.1002/jcb.29217
33. Woods CG, Stricker S, Seemann P, Stern R, Cox J, Sherridan E, et al. Mutations in WNT7A cause a range of limb malformations, including Fuhrmann syndrome and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome. *Am J Hum Genet.* (2006) 79:402–8. doi: 10.1086/506332
34. Eyaid W, Al-Qattan MM, Al Abdulkareem I, Fetaini N, Al Balwi M. A novel homozygous missense mutation (c.610G>A, p.Gly204Ser) in the WNT7A gene causes tetra-amelia in two Saudi families. *Am J Med Genet A*. (2011) 155a:599–604. doi: 10.1002/ajmg.a.33717
35. Garavelli L, Wischmeijer A, Rosato S, Gelmini C, Reverberi S, Sassi S, et al. Al-Awadi-Raas-Rothschild (limb/pelvis/uterus-hypoplasia/aplasia) syndrome and WNT7A mutations: genetic homogeneity and nosological delineation. *Am J Med Genet A*. (2011) 155a:332–6. doi: 10.1002/ajmg.a.33793
36. Mutlu MB, Cetinkaya A, Koc N, Ceylaner G, Erguner B, Aydin H, et al. A novel missense mutation, p. (R102W) in WNT7A causes Al-Awadi Raas-Rothschild syndrome in a fetus. *Eur J Med Genet.* (2016) 59:604–6. doi: 10.1016/j.ejmg.2016.09.009
37. Sun L, Huang Y, Zhao S, Zhong W, Lin M, Guo Y, et al. Advances in understanding the genetics of syndromes involving congenital upper limb anomalies. (2019) 4:3. doi: 10.21037/aoj.2019.06.03
38. Al-Qattan MM, Al-Balwi M, Eyaid W, Al-Abdulkarim I, Al-Turki S. Congenital duplication of the palm syndrome: gene analysis and the molecular basis of its clinical features. *J Hand Surg Eur.* (2009) 34:247–51. doi: 10.1177/1753193408099828
39. Al-Qattan MM, Alkuraya FS. Cenani-Lenz syndrome and other related syndactyly disorders due to variants in LRP4, GREM1/FMN1, and APC: Insight into the pathogenesis and the relationship to polyposis through the WNT and BMP antagonistic pathways. *Am J Med Genet A*. (2019) 179:266–79. doi: 10.1002/ajmg.a.60694
40. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD, et al. Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci USA.* (2005) 102:3324–9. doi: 10.1073/pnas.0408742102
41. Glass DA II, Karsenty G. Molecular bases of the regulation of bone remodeling by the canonical Wnt signaling pathway. *Curr Top Dev Biol.* (2006) 73:43–84. doi: 10.1016/S0070-2153(05)73002-7
42. Kantaputra PN, Kapoor S, Verma P, Intachai W, Ketudat Cairns JR. Split hand-foot malformation and a novel WNT10B mutation. *Eur J Med Genet.* (2018) 61:372–5. doi: 10.1016/j.ejmg.2018.02.001
43. Ugur SA, Tolun A. Homozygous WNT10b mutation and complex inheritance in Split-Hand/Foot Malformation. *Hum Mol Genet.* (2008) 17:2644–53. doi: 10.1093/hmg/ddn164
44. Blattner A, Huber AR, Rothlisberger B. Homozygous nonsense mutation in WNT10B and sporadic split-hand/foot malformation (SHFM) with autosomal recessive inheritance. *Am J Med Genet A.* (2010) 152a:2053–6. doi: 10.1002/ajmg.a.33504
45. Veverka V, Henry AJ, Slocombe PM, Ventom A, Mulloy B, Muskett FW, et al. Characterization of the structural features and interactions of sclerostin: molecular insight into a key regulator of Wnt-mediated bone formation. *J Biol Chem.* (2009) 284:10890–900. doi: 10.1074/jbc.M807994200
46. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, et al. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J.* (2003) 22:6267–76. doi: 10.1093/emboj/cdg599
47. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, et al. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem.* (2005) 280:19883–7. doi: 10.1074/jbc.M413274200
48. Semenov M, Tamai K, He X. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J Biol Chem.* (2005) 280:26770–5. doi: 10.1074/jbc.M504308200
49. Kim SJ, Bieganski T, Sohn YB, Kozlowski K, Semenov M, Okamoto N, et al. Identification of signal peptide domain SOST mutations in autosomal dominant craniodiaphyseal dysplasia. *Hum Genet.* (2011) 129:497–502. doi: 10.1007/s00439-011-0947-3
50. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, et al. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am J Hum Genet.* (2001) 68:577–89. doi: 10.1086/318811
51. Leupin O, Piters E, Halleux C, Hu S, Kramer I, Morvan F, et al. Bone overgrowth-associated mutations in the LRP4 gene impair sclerostin facilitator function. *J Biol Chem.* (2011) 286:19489–500. doi: 10.1074/jbc.M110.190330
52. Shen C, Xiong WC, Mei L. LRP4 in neuromuscular junction and bone development and diseases. *Bone*. (2015) 80:101–8. doi: 10.1016/j.bone.2015.05.012
53. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, et al. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet.* (2001) 10:537–43. doi: 10.1093/hmg/10.5.537
54. Appelman-Dijkstra NM, Papapoulos SE. From disease to treatment: from rare skeletal disorders to treatments for osteoporosis. *Endocrine*. (2016) 52:414–26. doi: 10.1007/s12020-016-0888-7
55. Sebastian A, Loots GG. Genetics of Sost/SOST in sclerosteosis and van Buchem disease animal models. *Metab Clin Exp.* (2018) 80:38–47. doi: 10.1016/j.metabol.2017.10.005
56. Van Hul W, Balemans W, Van Hul E, Dikkers FG, Obee H, Stokroos RJ, et al. Van Buchem disease (hyperostosis corticalis generalisata) maps to chromosome 17q12-q21. *Am J Hum Genet.* (1998) 62:391–9. doi: 10.1086/301721
57. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, et al. Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *J Med Genet.* (2002) 39:91–7. doi: 10.1136/jmg.39.2.91
58. Bieganski T, Baranska D, Miastkowska I, Kobielski A, Gorska-Chrzastek M, Kozlowski K. A boy with severe craniodiaphyseal dysplasia and apparently normal mother. *Am J Med Genet A.* (2007) 143A:2435–43. doi: 10.1002/ajmg.a.31938
59. Brazil DP, Church RH, Surae S, Godson C, Martin F. BMP signalling: agony and antagony in the family. *Trends Cell Biol.* (2015) 25:249–64. doi: 10.1016/j.tcb.2014.12.004
60. Gazzerro E, Pereira RC, Jorgetti V, Olson S, Economides AN, Canalis E. Skeletal overexpression of gremlin impairs bone formation and causes osteopenia. *Endocrinology*. (2005) 146:655–65. doi: 10.1210/en.2004-0766
61. Gazzerro E, Smerdel-Ramoya A, Zanotti S, Stadmeyer L, Durant D, Economides AN, et al. Conditional deletion of gremlin causes a transient increase in bone formation and bone mass. *J Biol Chem.* (2007) 282:31549–57. doi: 10.1074/jbc.M701317200

62. Dimitrov BI, Voet T, De Smet L, Vermeesch JR, Devriendt K, Fryns JP, et al. Genomic rearrangements of the GREM1-FMN1 locus cause oligosyndactyly, radio-ulnar synostosis, hearing loss, renal defects syndrome and Cenani–Lenz-like non-syndromic oligosyndactyly. *J Med Genet.* (2010) 47:569–74. doi: 10.1136/jmg.2009.073833
63. Zuniga A, Michos O, Spitz F, Haramis AP, Panman L, Galli A, et al. Mouse limb deformity mutations disrupt a global control region within the large regulatory landscape required for Gremlin expression. *Genes Dev.* (2004) 18:1553–64. doi: 10.1101/gad.299904
64. Umair MAF, Bilal M, Abbas S. Syndactyly genes and classification: a mini-review. *JBC Genetics.* (2018) 1:34–47. doi: 10.24911/JBCGenetics/183-1532177257
65. Capdevila J, Tsukui T, Rodriquez Esteban C, Zappavigna V, Izpisua Belmonte JC. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol Cell.* (1999) 4:839–49. doi: 10.1016/S1097-2765(00)80393-7
66. Merino R, Rodriguez-Leon J, Macias D, Ganan Y, Economides AN, Hurle JM. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development.* (1999) 126:5515–22.
67. Gu Q, Luo Y, Chen C, Jiang D, Huang Q, Wang X. GREM1 overexpression inhibits proliferation, migration and angiogenesis of osteosarcoma. *Exp Cell Res.* (2019) 384:111619. doi: 10.1016/j.yexcr.2019.111619
68. Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci.* (2003) 116:2627–34. doi: 10.1242/jcs.00623
69. Haraguchi R, Kitazawa R, Mori K, Tachibana R, Kiyonari H, Imai Y, et al. sFRP4-dependent Wnt signal modulation is critical for bone remodeling during postnatal development and age-related bone loss. *Sci Rep.* (2016) 6:25198. doi: 10.1038/srep25198
70. Pawar NM, Rao P. Secreted frizzled related protein 4 (sFRP4) update: A brief review. *Cell Signal.* (2018) 45:63–70. doi: 10.1016/j.cellsig.2018.01.019
71. Claudel M, Jouzeau JY, Cailotto F. Secreted Frizzled-related proteins (sFRPs) in osteo-articular diseases: much more than simple antagonists of Wnt signaling? *FEBS J.* (2019) 286:4832–51. doi: 10.1111/febs.15119
72. Kiper POS, Saito H, Gori F, Unger S, Hesse E, Yamana K, et al. Cortical-Bone Fragility–Insights from sFRP4 Deficiency in Pyle's Disease. *N Engl J Med.* (2016) 374:2553–62. doi: 10.1056/NEJMoa1509342
73. Galada C, Shah H, Shukla A, Girisha KM. A novel sequence variant in SFRP4 causing Pyle disease. *J Hum Genet.* (2017) 62:575–6. doi: 10.1038/jhg.2016.166
74. Arboleya L, Queiro R, Alperi M, Lorenzo JA, Ballina J. Pyle's disease: a human model of differentiated cortical and trabecular homeostasis. *Reumatol Clin.* (2018) 16:56–8.
75. Veugelers M, De Cat B, Ceulemans H, Bruystens AM, Coomans C, Durr J, et al. Glypican-6, a new member of the glypican family of cell surface heparan sulfate proteoglycans. *J Biol Chem.* (1999) 274:26968–77. doi: 10.1074/jbc.274.38.26968
76. Campos-Xavier AB, Martinet D, Bateman J, Belluoccio D, Rowley L, Tan TY, et al. Mutations in the heparan-sulfate proteoglycan glypican 6 (GPC6) impair endochondral ossification and cause recessive omodysplasia. *Am J Hum Genet.* (2009) 84:760–70. doi: 10.1016/j.ajhg.2009.05.002
77. Capurro M, Izumikawa T, Suarez P, Shi W, Cydzik M, Kaneiwa T, et al. Glypican-6 promotes the growth of developing long bones by stimulating Hedgehog signaling. *J Cell Biol.* (2017) 216:2911–26. doi: 10.1083/jcb.201605119
78. Reichsman F, Smith L, Cumberledge S. Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J Cell Biol.* (1996) 135:819–27. doi: 10.1083/jcb.135.3.819
79. Ohkawara B, Yamamoto TS, Tada M, Ueno N. Role of glypican 4 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. *Development.* (2003) 130:2129–38. doi: 10.1242/dev.00435
80. Song HH, Shi W, Xiang YY, Filmus J. The loss of glypican-3 induces alterations in Wnt signaling. *J Biol Chem.* (2005) 280:2116–25. doi: 10.1074/jbc.M410090200
81. Krakow D. Skeletal dysplasias. *Clin Perinatol.* (2015) 42:301–19. doi: 10.1016/j.clp.2015.03.003
82. Amor DJ, Stephenson SEM, Mustapha M, Mensah MA, Ockeloen CW, Lee WS, et al. Pathogenic variants in GPC4 cause Keipert syndrome. *Am J Hum Genet.* (2019) 104:914–24. doi: 10.1016/j.ajhg.2019.02.026
83. Keipert JA, Fitzgerald MG, Danks DM. A new syndrome of broad terminal phalanges and facial abnormalities. *Aust Paediatr J.* (1973) 9:10–3. doi: 10.1111/j.1440-1754.1973.tb02215.x
84. White JJ, Mazzeu JF, Coban-Akdemir Z, Bayram Y, Bahrambeigi V, Hoischen A, et al. WNT signaling perturbations underlie the genetic heterogeneity of robinow syndrome. *Am J Hum Genet.* (2018) 102:27–43. doi: 10.1016/j.ajhg.2017.10.002
85. Paine-Saunders S, Viviano BL, Saunders S. GPC6, a novel member of the glypican gene family, encodes a product structurally related to GPC4 and is colocalized with GPC5 on human chromosome 13. *Genomics.* (1999) 57:455–8. doi: 10.1006/geno.1999.5793
86. Shi GX, Mao WW, Zheng XF, Jiang LS. The role of R-spondins and their receptors in bone metabolism. *Prog Biophys Mol Biol.* (2016) 122:93–100. doi: 10.1016/j.pbiomolbio.2016.05.012
87. Kim KA, Zhao J, Andarmani S, Kakitani M, Oshima T, Binnerts ME, et al. R-Spondin proteins: a novel link to beta-catenin activation. *Cell Cycle.* (2006) 5:23–6. doi: 10.4161/cc.5.1.2305
88. Jin YR, Turcotte TJ, Crocker AL, Han XH, Yoon JK. The canonical Wnt signaling activator, R-spondin2, regulates craniofacial patterning and morphogenesis within the branchial arch through ectodermal-mesenchymal interaction. *Dev Biol.* (2011) 352:1–13. doi: 10.1016/j.ydbio.2011.01.004
89. Lebensohn AM, Rohatgi R. R-spondins can potentiate WNT signaling without LGRs. *Elife.* (2018) 7:e33126. doi: 10.7554/eLife.33126
90. Park S, Cui J, Yu W, Wu L, Carmon KS, Liu QJ. Differential activities and mechanisms of the four R-spondins in potentiating Wnt/beta-catenin signaling. *J Biol Chem.* (2018) 293:9759–69. doi: 10.1074/jbc.RA118.002743
91. Szenker-Ravi E, Altunoglu U, Leushacke M, Bosso-Lefevre C, Khatoo M, Thi Tran H, et al. RSPO2 inhibition of RNF43 and ZNRF3 governs limb development independently of LGR4/5/6. *Nature.* (2018) 557:564–9. doi: 10.1038/s41586-018-0118-y
92. Saal HM, Prows CA, Guerreiro I, Donlin M, Knudson L, Sund KL, et al. A mutation in FRIZZLED2 impairs Wnt signaling and causes autosomal dominant omodysplasia. *Hum Mol Genet.* (2015) 24:3399–409. doi: 10.1093/hmg/ddv088
93. Nagasaki K, Nishimura G, Kikuchi T, Nyuzuki H, Sasaki S, Ogawa Y, et al. Nonsense mutations in FZD2 cause autosomal-dominant omodysplasia: Robinow syndrome-like phenotypes. *Am J Med Genet A.* (2018) 176:739–42. doi: 10.1002/ajmg.a.38623
94. Turkmen S, Spielmann M, Gunes N, Knaus A, Flottmann R, Mundlos S, et al. A Novel de novo FZD2 mutation in a patient with autosomal dominant omodysplasia. *Mol Syndromol.* (2017) 8:318–24. doi: 10.1159/000479721
95. Warren HE, Louie RJ, Friez MJ, Frias JL, Leroy JG, Spranger JW, et al. Two unrelated patients with autosomal dominant omodysplasia and FRIZZLED2 mutations. *Clin Case Rep.* (2018) 6:2252–5. doi: 10.1002/ccr3.1818
96. Afzal AR, Jeffery S. One gene, two phenotypes: ROR2 mutations in autosomal recessive Robinow syndrome and autosomal dominant brachydactyly type B. *Hum Mutat.* (2003) 22:1–11. doi: 10.1002/humu.10233
97. Habib R, Amin-Ud-Din M, Ahmad W. A nonsense mutation in the gene ROR2 underlying autosomal dominant brachydactyly type B. *Clin Dysmorphol.* (2013) 22:47–50. doi: 10.1097/MCD.0b013e32835c6c8c
98. Van Bokhoven H, Celli J, Kayserili H, Van Beusekom E, Balci S, Brussel W, et al. Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. *Nat Genet.* (2000) 25:423–6. doi: 10.1038/78113
99. Yang K, Zhu J, Tan Y, Sun X, Zhao H, Tang G, et al. Whole-exome sequencing identified compound heterozygous variants in ROR2 gene in a fetus with Robinow syndrome. *J Clin Lab Anal.* (2019) 34:e23074. doi: 10.1002/jcla.23074
100. Afzal AR, Rajab A, Fenske CD, Oldridge M, Elanko N, Ternes-Pereira E, et al. Recessive Robinow syndrome, allelic to dominant brachydactyly type B, is caused by mutation of ROR2. *Nat Genet.* (2000) 25:419–22. doi: 10.1038/78107
101. Schwarzer W, Witte F, Rajab A, Mundlos S, Stricker S. A gradient of ROR2 protein stability and membrane localization confers brachydactyly type B

or Robinow syndrome phenotypes. *Hum Mol Genet.* (2009) 18:4013–21. doi: 10.1093/hmg/ddp345
102. Wang B, Sinha T, Jiao K, Serra R, Wang J. Disruption of PCP signaling causes limb morphogenesis and skeletal defects and may underlie Robinow syndrome and brachydactyly type B. *Hum Mol Genet.* (2011) 20:271–85. doi: 10.1093/hmg/ddq462
103. Schwabe GC, Tinschert S, Buschow C, Meinecke P, Wolff G, Gillessen-Kaesbach G, et al. Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly type B. *Am J Hum Genet.* (2000) 67:822–31. doi: 10.1086/303084
104. Boudin E, Fijalkowski I, Piters E, Van Hul W. The role of extracellular modulators of canonical Wnt signaling in bone metabolism and diseases. *Semin Arthritis Rheum.* (2013) 43:220–40. doi: 10.1016/j.semarthrit.2013.01.004
105. Pohlkamp T, Wasser CR, Herz J. Functional roles of the interaction of APP and lipoprotein receptors. *Front Mol Neurosci.* (2017) 10:54. doi: 10.3389/fnmol.2017.00054
106. Van Wesenbeeck L, Cleiren E, Gram J, Beals RK, Benichou O, Scopelliti D, et al. Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density. *Am J Hum Genet.* (2003) 72:763–71. doi: 10.1086/368277
107. Whyte MP, Mcalister WH, Zhang F, Bijanki VN, Nenninger A, Gottesman GS, et al. New explanation for autosomal dominant high bone mass: Mutation of low-density lipoprotein receptor-related protein 6. *Bone.* (2019) 127:228–43. doi: 10.1016/j.bone.2019.05.003
108. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med.* (2013) 19:179–92. doi: 10.1038/nm.3074
109. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, et al. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell.* (2001) 107:513–23. doi: 10.1016/S0092-8674(01)00571-2
110. Astiazaran MC, Cervantes-Sodi M, Rebolledo-Enriquez E, Chacon-Camacho O, Villegas V, Zenteno JC. Novel homozygous LRP5 mutations in Mexican patients with osteoporosis-pseudoglioma syndrome. *Genet Test Mol Biomarkers.* (2017) 21:742–6. doi: 10.1089/gtmb.2017.0118
111. Laine CM, Chung BD, Susic M, Prescott T, Semler O, Fiskerstrand T, et al. Novel mutations affecting LRP5 splicing in patients with osteoporosis-pseudoglioma syndrome (OPPG). *Eur J Hum Genet.* (2011) 19:875–81. doi: 10.1038/ejhg.2011.42
112. Korvala J, Juppner H, Makitie O, Sochett E, Schnabel D, Mora S, et al. Mutations in LRP5 cause primary osteoporosis without features of OI by reducing Wnt signaling activity. *BMC Med Genet.* (2012) 13:26. doi: 10.1186/1471-2350-13-26
113. Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, et al. High bone density due to a mutation in LDL-receptor-related protein 5. *N Engl J Med.* (2002) 346:1513–21. doi: 10.1056/NEJMoa013444
114. Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, et al. A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am J Hum Genet.* (2002) 70:11–9. doi: 10.1086/338450
115. Kwee ML, Balemans W, Cleiren E, Gille JJ, Van Der Blij F, Sepers JM, et al. An autosomal dominant high bone mass phenotype in association with craniosynostosis in an extended family is caused by an LRP5 missense mutation. *J Bone Miner Res.* (2005) 20:1254–60. doi: 10.1359/JBMR.050303
116. Balemans W, Piters E, Cleiren E, Ai M, Van Wesenbeeck L, Warman ML, et al. The binding between sclerostin and LRP5 is altered by DKK1 and by high-bone mass LRP5 mutations. *Calcif Tissue Int.* (2008) 82:445–53. doi: 10.1007/s00223-008-9130-9
117. Semenov MV, He X. LRP5 mutations linked to high bone mass diseases cause reduced LRP5 binding and inhibition by SOST. *J Biol Chem.* (2006) 281:38276–84. doi: 10.1074/jbc.M609509200
118. Balemans W, Devogelaer JP, Cleiren E, Piters E, Caussin E, Van Hul W. Novel LRP5 missense mutation in a patient with a high bone mass phenotype results in decreased DKK1-mediated inhibition of Wnt signaling. *J Bone Miner Res.* (2007) 22:708–16. doi: 10.1359/jbmr.070211
119. Mani A, Radhakrishnan J, Wang H, Mani A, Mani MA, Nelson-Williams C, et al. LRP6 mutation in a family with early coronary disease and metabolic risk factors. *Science.* (2007) 315:1278–82. doi: 10.1126/science.1136370
120. Ockeloen CW, Khandelwal KD, Dreesen K, Ludwig KU, Sullivan R, Van Rooij I, et al. Novel mutations in LRP6 highlight the role of WNT signaling in tooth agenesis. *Genet Med.* (2016) 18:1158–62. doi: 10.1038/gim.2016.10
121. Karner CM, Dietrich MF, Johnson EB, Kappesser N, Tennert C, Percin F, et al. Lrp4 regulates initiation of ureteric budding and is crucial for kidney formation–a mouse model for Cenani-Lenz syndrome. *PLoS ONE.* (2010) 5:e10418. doi: 10.1371/journal.pone.0010418
122. Kariminejad A, Stollfuss B, Li Y, Bogershausen N, Boss K, Hennekam RC, et al. (2013). Severe Cenani-Lenz syndrome caused by loss of LRP4 function. *Am. J. Med. Genet. A* 161A, 1475–1479. doi: 10.1002/ajmg.a.35920
123. Li Y, Pawlik B, Elcioglu N, Aglan M, Kayserili H, Yigit G, et al. LRP4 mutations alter Wnt/beta-catenin signaling and cause limb and kidney malformations in Cenani-Lenz syndrome. *Am J Hum Genet.* (2010) 86:696–706. doi: 10.1016/j.ajhg.2010.03.004
124. Fijalkowski I, Geets E, Steenackers E, Van Hoof V, Ramos FJ, Mortier G, et al. A Novel Domain-Specific Mutation in a Sclerosteosis Patient Suggests a Role of LRP4 as an Anchor for Sclerostin in Human Bone. *J Bone Miner Res.* (2016) 31:874–81. doi: 10.1002/jbmr.2782
125. Ohkawara B, Cabrera-Serrano M, Nakata T, Milone M, Asai N, Ito K, et al. LRP4 third beta-propeller domain mutations cause novel congenital myasthenia by compromising agrin-mediated MuSK signaling in a position-specific manner. *Hum Mol Genet.* (2014) 23:1856–68. doi: 10.1093/hmg/ddt578
126. Sukenik Halevy R, Chien HC, Heinz B, Bamshad MJ, Nickerson DA, University of Washington Center for Mendelian G, et al. Mutations in the fourth beta-propeller domain of LRP4 are associated with isolated syndactyly with fusion of the third and fourth fingers. *Hum Mutat.* (2018) 39:811–15. doi: 10.1002/humu.23417
127. Sharma M, Castro-Piedras I, Simmons GE Jr, Pruitt K. Dishevelled: A masterful conductor of complex Wnt signals. *Cell Signal.* (2018) 47:52–64. doi: 10.1016/j.cellsig.2018.03.004
128. Itoh K, Brott BK, Bae GU, Ratcliffe MJ, Sokol SY. Nuclear localization is required for Dishevelled function in Wnt/beta-catenin signaling. *J Biol.* (2005) 4:3. doi: 10.1186/jbiol20
129. Bunn KJ, Daniel P, Rosken HS, O'neill AC, Cameron-Christie SR, Morgan T, et al. Mutations in DVL1 cause an osteosclerotic form of Robinow syndrome. *Am J Hum Genet.* (2015) 96:623–30. doi: 10.1016/j.ajhg.2015.02.010
130. White J, Mazzeu JF, Hoischen A, Jhangiani SN, Gambin T, Alcino MC, et al. DVL1 frameshift mutations clustering in the penultimate exon cause autosomal-dominant Robinow syndrome. *Am J Hum Genet.* (2015) 96:612–22. doi: 10.1016/j.ajhg.2015.02.015
131. White JJ, Mazzeu JF, Hoischen A, Bayram Y, Withers M, Gezdirici A, et al. DVL3 Alleles resulting in a−1 frameshift of the last exon mediate autosomal-dominant robinow syndrome. *Am J Hum Genet.* (2016) 98:553–61. doi: 10.1016/j.ajhg.2016.01.005
132. Roifman M, Brunner H, Lohr J, Mazzeu J, Chitayat D. Autosomal dominant robinow syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. *GeneReviews((R)).* Seattle, WA: University of Washington (1993) 1993–2020.
133. Danyel M, Kortum F, Dathe K, Kutsche K, Horn D. Autosomal dominant Robinow syndrome associated with a novel DVL3 splice mutation. *Am J Med Genet A.* (2018) 176:992–6. doi: 10.1002/ajmg.a.38635
134. Funato Y, Michiue T, Asashima M, Miki H. The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-beta-catenin signalling through dishevelled. *Nat Cell Biol.* (2006) 8:501–8. doi: 10.1038/ncb1405
135. Funato Y, Michiue T, Terabayashi T, Yukita A, Danno H, Asashima M, et al. Nucleoredoxin regulates the Wnt/planar cell polarity pathway in Xenopus. *Genes Cells.* (2008) 13:965–75. doi: 10.1111/j.1365-2443.2008.01220.x
136. Funato Y, Terabayashi T, Sakamoto R, Okuzaki D, Ichise H, Nojima H, et al. Nucleoredoxin sustains Wnt/beta-catenin signaling by retaining a pool of inactive dishevelled protein. *Curr Biol.* (2010) 20:1945–52. doi: 10.1016/j.cub.2010.09.065
137. Boles MK, Wilkinson BM, Wilming LG, Liu B, Probst FJ, Harrow J, et al. Discovery of candidate disease genes in ENU-induced mouse mutants by large-scale sequencing, including a splice-site mutation in nucleoredoxin. *PLoS Genet.* (2009) 5:e1000759. doi: 10.1371/journal.pgen.1000759
138. Costain G, Callewaert B, Gabriel H, Tan TY, Walker S, Christodoulou J, et al. *De novo* missense variants in RAC3 cause a novel neurodevelopmental syndrome. *Genet Med.* (2019) 21:1021–6. doi: 10.1038/s41436-018-0323-y

139. Xing Y, Clements WK, Le Trong I, Hinds TR, Stenkamp R, Kimelman D, et al. Crystal structure of a beta-catenin/APC complex reveals a critical role for APC phosphorylation in APC function. *Mol Cell*. (2004) 15:523–33. doi: 10.1016/j.molcel.2004.08.001
140. Shintani T, Ihara M, Tani S, Sakuraba J, Sakuta H, Noda M. APC2 plays an essential role in axonal projections through the regulation of microtubule stability. *J Neurosci*. (2009) 29:11628–40. doi: 10.1523/JNEUROSCI.2394-09.2009
141. Schneikert J, Vijaya Chandra SH, Ruppert JG, Ray S, Wenzel EM, Behrens J. Functional comparison of human adenomatous polyposis coli (APC) and APC-like in targeting beta-catenin for degradation. *PLoS ONE*. (2013) 8:e68072. doi: 10.1371/journal.pone.0068072
142. Almuriekhi M, Shintani T, Fahiminiya S, Fujikawa A, Kuboyama K, Takeuchi Y, et al. Loss-of-function mutation in APC2 causes Sotos syndrome features. *Cell Rep*. (2015) 10:1585–98. doi: 10.1016/j.celrep.2015.02.011
143. Perdu B, De Freitas F, Frints SG, Schouten M, Schrander-Stumpel C, Barbosa M, et al. Osteopathia striata with cranial sclerosis owing to WTX gene defect. *J Bone Miner Res*. (2010) 25:82–90. doi: 10.1359/jbmr.090707
144. Tanneberger K, Pfister AS, Kriz V, Bryja V, Schambony A, Behrens J. Structural and functional characterization of the Wnt inhibitor APC membrane recruitment 1 (Amer1). *J Biol Chem*. (2011) 286:19204–14. doi: 10.1074/jbc.M111.224881
145. Jenkins ZA, Van Kogelenberg M, Morgan T, Jeffs A, Fukuzawa R, Pearl E, et al. Germline mutations in WTX cause a sclerosing skeletal dysplasia but do not predispose to tumorigenesis. *Nat Genet*. (2009) 41:95–100. doi: 10.1038/ng.270
146. Perdu B, Lakeman P, Mortier G, Koenig R, Lachmeijer AM, Van Hul W. Two novel WTX mutations underscore the unpredictability of male survival in osteopathia striata with cranial sclerosis. *Clin Genet*. (2011) 80:383–8. doi: 10.1111/j.1399-0004.2010.01553.x
147. Grzeschik KH, Bornholdt D, Oeffner F, Konig A, Del Carmen Boente M, Enders H, et al. Deficiency of PORCN, a regulator of Wnt signaling, is associated with focal dermal hypoplasia. *Nat Genet*. (2007) 39:833–5. doi: 10.1038/ng2052
148. Stevenson DA, Chirpich M, Contreras Y, Hanson H, Dent K. Goltz syndrome and PORCN mosaicism. *Int J Dermatol*. (2014) 53:1481–4. doi: 10.1111/ijd.12605
149. Bree AF, Grange DK, Hicks MJ, Goltz RW. Dermatologic findings of focal dermal hypoplasia (Goltz syndrome). *Am J Med Genet C Semin Med Genet*. (2016) 172C:44–51. doi: 10.1002/ajmg.c.31472
150. King EA, Davis JW, Degner JF. Are drug targets with genetic support twice as likely to be approved? Revised estimates of the impact of genetic support for drug mechanisms on the probability of drug approval. *PLoS Genet*. (2019) 15:e1008489. doi: 10.1371/journal.pgen.1008489
151. Morris JA, Kemp JP, Youlten SE, Laurent L, Logan JG, Chai RC, et al. An atlas of genetic influences on osteoporosis in humans and mice. *Nat Genet*. (2019) 51:258–66. doi: 10.1038/s41588-018-0302-x
152. Kichaev G, Bhatia G, Loh PR, Gazal S, Burch K, Freund MK, et al. Leveraging Polygenic Functional enrichment to improve GWAS power. *Am J Hum Genet*. (2019) 104:65–75. doi: 10.1016/j.ajhg.2018.11.008
153. Van Camp JK, Beckers S, Zegers D, Boudin E, Nielsen TL, Andersen M, et al. Genetic association study of WNT10B polymorphisms with BMD and adiposity parameters in Danish and Belgian males. *Endocrine*. (2013) 44:247–54. doi: 10.1007/s12020-012-9869-7
154. Trajanoska K, Morris JA, Oei L, Zheng HF, Evans DM, Kiel DP, et al. Assessment of the genetic and clinical determinants of fracture risk: genome wide association and mendelian randomisation study. *BMJ*. (2018) 362:k3225. doi: 10.1136/bmj.k3225
155. Wang H, Zhang F, Zeng J, Wu Y, Kemper KE, Xue A, et al. Genotype-by-environment interactions inferred from genetic effects on phenotypic variability in the UK Biobank. *Sci Adv*. (2019) 5:eaaw3538. doi: 10.1126/sciadv.aaw3538
156. Mullin BH, Walsh JP, Zheng HF, Brown SJ, Surdulescu GL, Curtis C, et al. Genome-wide association study using family-based cohorts identifies the WLS and CCDC170/ESR1 loci as associated with bone mineral density. *BMC Genomics*. (2016) 17:136. doi: 10.1186/s12864-016-2481-0
157. Freund MK, Burch KS, Shi H, Mancuso N, Kichaev G, Garske KM, et al. Phenotype-Specific enrichment of mendelian disorder genes near GWAS regions across 62 complex traits. *Am J Hum Genet*. (2018) 103:535–52. doi: 10.1016/j.ajhg.2018.08.017
158. Clement-Lacroix P, Ai M, Morvan F, Roman-Roman S, Vayssiere B, Belleville C, et al. Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci USA*. (2005) 102:17406–11. doi: 10.1073/pnas.0505259102
159. Vestergaard P. Skeletal effects of central nervous system active drugs: anxiolytics, sedatives, antidepressants, lithium and neuroleptics. *Curr Drug Saf*. (2008) 3:185–9. doi: 10.2174/157488608785699432
160. Styrkarsdottir U, Thorleifsson G, Sulem P, Gudbjartsson DF, Sigurdsson A, Jonasdottir A, et al. Nonsense mutation in the LGR4 gene is associated with several human diseases and other traits. *Nature*. (2013) 497:517–20. doi: 10.1038/nature12124
161. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, et al. APC mutations occur early during colorectal tumorigenesis. *Nature*. (1992) 359:235–7. doi: 10.1038/359235a0
162. Rivera MN, Kim WJ, Wells J, Driscoll DR, Brannigan BW, Han M, et al. An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. *Science*. (2007) 315:642–5. doi: 10.1126/science.1137509
163. Ruteshouser EC, Robinson SM, Huff V. Wilms tumor genetics: mutations in WT1, WTX, and CTNNB1 account for only about one-third of tumors. *Genes Chromosomes Cancer*. (2008) 47:461–70. doi: 10.1002/gcc.20553
164. Fehringer G, Kraft P, Pharoah PD, Eeles RA, Chatterjee N, Schumacher FR, et al. Cross-Cancer genome-wide analysis of lung, ovary, breast, prostate, and colorectal cancer reveals novel pleiotropic associations. *Cancer Res*. (2016) 76:5103–14. doi: 10.1158/0008-5472.CAN-15-2980
165. Wang M, Gu D, Du M, Xu Z, Zhang S, Zhu L, et al. Common genetic variation in ETV6 is associated with colorectal cancer susceptibility. *Nat Commun*. (2016) 7:11478. doi: 10.1038/ncomms11478
166. Michailidou K, Lindstrom S, Dennis J, Beesley J, Hui S, Kar S, et al. Association analysis identifies 65 new breast cancer risk loci. *Nature*. (2017) 551:92–4. doi: 10.1038/nature24284
167. Appelman-Dijkstra NM, Papapoulos SE. Sclerostin inhibition in the management of osteoporosis. *Calcif Tissue Int*. (2016) 98:370–80. doi: 10.1007/s00223-016-0126-6
168. Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E, Ferrari S, et al. Romosozumab treatment in postmenopausal women with osteoporosis. *N Engl J Med*. (2016) 375:1532–43. doi: 10.1056/NEJMoa1607948
169. Saag KG, Petersen J, Brandi ML, Karaplis AC, Lorentzon M, Thomas T, et al. Romosozumab or alendronate for fracture prevention in women with osteoporosis. *N Engl J Med*. (2017) 377:1417–27. doi: 10.1056/NEJMoa1708322
170. Lewiecki EM, Blicharski T, Goemaere S, Lippuner K, Meisner PD, Miller PD, et al. A phase III randomized placebo-controlled trial to evaluate efficacy and safety of romosozumab in men with osteoporosis. *J Clin Endocrinol Metab*. (2018) 103:3183–93. doi: 10.1210/jc.2017-02163
171. Langdahl BL, Libanati C, Crittenden DB, Bolognese MA, Brown JP, Daizadeh NS, et al. Romosozumab (sclerostin monoclonal antibody) versus teriparatide in postmenopausal women with osteoporosis transitioning from oral bisphosphonate therapy: a randomised, open-label, phase 3 trial. *Lancet*. (2017) 390:1585–94. doi: 10.1016/S0140-6736(17)31613-6
172. Van Lierop AH, Appelman-Dijkstra NM, Papapoulos SE. Sclerostin deficiency in humans. *Bone*. (2017) 96:51–62. doi: 10.1016/j.bone.2016.10.010

Conflict of Interest: 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 Endocrinology

MINI REVIEW
published: 19 June 2020
doi: 10.3389/fendo.2020.00394

Human Fibroblasts as a Model for the Study of Bone Disorders

*Lauria Claeys[1], Nathalie Bravenboer[2], Elisabeth M. W. Eekhoff[3] and Dimitra Micha[1]**

[1] Department of Clinical Genetics, Amsterdam Movement Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [2] Department of Clinical Chemistry, Amsterdam Movement Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [3] Department of Internal Medicine Section Endocrinology, Amsterdam Movement Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands

Bone tissue degeneration is an urgent clinical issue, making it a subject of intensive research. Chronic skeletal disease forms can be prevalent, such as the age-related osteoporosis, or rare, in the form of monogenetic bone disorders. A barrier in the understanding of the underlying pathological process is the lack of accessibility to relevant material. For this reason, cells of non-bone tissue are emerging as a suitable alternative for models of bone biology. Fibroblasts are highly suitable for this application; they populate accessible anatomical locations, such as the skin tissue. Reports suggesting their utility in preclinical models for the study of skeletal diseases are increasingly becoming available. The majority of these are based on the generation of an intermediate stem cell type, the induced pluripotent stem cells, which are subsequently directed to the osteogenic cell lineage. This intermediate stage is circumvented in transdifferentiation, the process regulating the direct conversion of fibroblasts to osteogenic cells, which is currently not well-explored. With this mini review, we aimed to give an overview of existing osteogenic transdifferentiation models and to inform about their applications in bone biology models.

Keywords: fibroblast, preclinical model, osteoblast, bone disease, osteogenic transdifferentiation

OPEN ACCESS

Edited by:
Claire Elizabeth Clarkin, University of Southampton, United Kingdom

Reviewed by:
David M. Findlay, University of Adelaide, Australia
Sudip Sen, All India Institute of Medical Sciences, India

***Correspondence:**
Dimitra Micha
d.micha@amsterdamumc.nl

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *30 August 2019*
Accepted: *18 May 2020*
Published: *19 June 2020*

Citation:
Claeys L, Bravenboer N, Eekhoff EMW and Micha D (2020) Human Fibroblasts as a Model for the Study of Bone Disorders. Front. Endocrinol. 11:394. doi: 10.3389/fendo.2020.00394

INTRODUCTION

Bone disorders encompass a wide range of chronic disorders with diverse etiologies, including both genetic and environmental factors. Next generation sequencing has contributed to the identification of the responsible genomic loci for several of the 500 Mendelian bone disorders, which has expanded our understanding of bone biology and its pathology in more frequent conditions, such as fractures at a postmenopausal age (1, 2). Nonetheless, for many of these patients this information remains to be translated into meaningful therapies. This gap between the genetic breakthrough and treatment can be largely attributed to the lack of cell models relevant for the study of bone tissue, in which the (genetic) defect can be examined and interrogated for the exploration of a therapeutic intervention. Such a model also offers the prospect of screening the large number of genetic variants of questionable pathogenicity, which are frequently found in exome analyses (3).

The paucity of bone material, which can be understandably explained by the invasive nature of the biopsy, has prompted efforts for the invention of bone cell models, which can faithfully recapitulate the disease mechanism by making use of more accessible patient materials. In the recent years, the attention toward fibroblasts as a starting point for differentiation to other cell types, including osteoblasts, has been growing. Fibroblasts are a common resident cell type in connective tissue found almost ubiquitously in the human body, including the easily accessible skin. Despite

their recognition as a prominent cell type for about a century (4), their characterization remains obscure due to the lack of suitable markers and their uncharacterized diversity (5). However, their potential in osteogenesis can be demonstrated both in nature, in disorders of pathological ossification, as well as in more artificial systems of *in vitro* osteogenic differentiation. This allows the consideration of fibroblast-based models for the study of bone disorders. Particularly, this review focuses on human fibroblast-based models of osteogenic transdifferentiation for modeling of osteoblast-dependent disorders (**Table 1**).

BONE BIOLOGY

In order to appreciate the value and shortcomings of relevant cell models, an overview of bone biology is required. The bone tissue typically consists of the mineral and organic components, which confer its stiffness and flexibility, respectively, to ensure its competence during continuous exposure to mechanical stress. Particularly, collagen type I constitutes the largest part of the organic mass; in addition to supporting cell growth and function, it also serves as a scaffold for mineral deposition. Bone tissue adapts to environmental cues by constant remodeling, which is primarily orchestrated by three different cell types, the osteoblasts, osteoclasts, and osteocytes. Osteoblasts have an anabolic role in building bone tissue by secreting the collagen-rich extracellular matrix (ECM) whereas osteoclasts perform a catabolic function by degrading the bone tissue. In this setting some of the osteoblasts become buried in the mineralized ECM, which triggers their differentiation to osteocytes. The latter are mechanosensing cells, which coordinate the function and differentiation of osteoblasts and osteoclasts, depending on their exposure to mechanical loading (21). Considering the closely interconnected relation of these cells, it is easy to deduce that defects in osteoblast differentiation or function can cause disease by influencing the net effect on bone mass development (22, 23). Thus, models allowing the study of osteoblast biology can be insightful in delineating the disease mechanism.

THE HUMBLE FIBROBLAST

The fibroblast is generally known as a spindle-shaped adherent cell type and a common resident of mesenchymal stroma. It has been considered as a rather inert cell type for many years with the sole role of producing large amounts of ECM proteins, such as collagen type I, intended for the homeostasis of the connective tissue. However, it is steadily becoming more clear that fibroblasts have a much broader function, which includes the regulation of immune and inflammatory responses, for example during cutaneous wound repair (24), as well as during cell differentiation and behavior of neighboring cell types (25). It is also accepted that they represent a heterogeneous cell population, whose diversity extends not only across different anatomical locations but also within the same tissue, such as in the skin layers (26). Despite their abundance, their precise nature remains poorly characterized since they lack specific defining markers. It is perhaps partially because of this unspecialized character that they exhibit such a great plasticity and the ability to differentiate into other somatic cell types including osteogenic cells (27). Interestingly, it is a topic of great discussion whether fibroblasts are in fact the same cell type as mesenchymal stem cells (MSCs). According to the guidelines of the International Society for Cellular Therapy they are, as both cell types are plastic-adherent, share the presence and absence of the same MSC markers, and can differentiate into cells of the osteogenic, adipogenic, and chondrogenic lineages (27–29). MSCs were originally isolated from the bone marrow but have been subsequently identified in many tissues, including the skin. Their osteogenic properties have raised scientific attention with regards to their application in regenerative medicine (30–32). The similarities they share with the bone-forming MSC progenitors support the use of fibroblasts as an appropriate cell type to study osteogenesis.

FIBROBLAST-BASED MODELS FOR THE STUDY OF BONE DISORDERS

In the recent years, a plethora of reports have emerged, exploring the osteogenic properties of fibroblasts in producing osteoblasts suitable as disease models, as well as for potential bone regenerative applications. These refer mainly to two different ways of cell reprogramming for derivation of osteoblast cells: induced pluripotent stem cell (iPSC)-mediated differentiation and transdifferentiation. The first is based on the dedifferentiation of fibroblasts to an artificial stem cell type (iPSCs) by the induction of pluripotency. It is accomplished by the forced expression of the "Yamanaka factors" which typically include the Oct3/4, Sox2, c-Myc, and Klf4 transcription factors (33, 34). The iPSCs can be then directed toward the osteogenic cell lineage. The excitement revolving around the promising results of this approach is undoubtedly reflected in the numerous ongoing studies (35, 36). In addition to the multipotent plasticity of these cells, their patient specificity for autologous treatment and the lack of associated ethical issues, iPSCs have emerged in the last decade as a source of induced MSCs (iMSCs) (37), which are reported to have superior qualities to those of primary MSCs in cell survival and engraftment (38–40).

Despite these advantages, it is widely acknowledged that there are certain considerations with the use of iPSCs, such as the requirement for specialized technical resources for reprogramming and the consequences of manipulation for the induction of pluripotency, which include teratoma formation in regenerative applications. Minimization of these risks could be accomplished by optimizing the delivery of pluripotency factors by switching to non-integrative approaches, ensuring the absence of residual undifferentiated iPSCs, and monitoring the off-target effects (41). Another point of consideration is the potential disturbance of the cells differentiation potential as a result of reprogramming. Thus, iPSCs may not be suited to study a disorder in which the defect lies in cell differentiation. This is exemplified by the inhibition of iPSC generation and maintenance from fibroblasts of patients with fibrodysplasia ossificans progressiva (FOP), a severe disorder of heterotopic ossification. This was reported to be caused by the gene defect

TABLE 1 | Overview of osteoblast cell derivation approaches.

Fibroblast	Approach	Mouse model	Reference
Gingival, dermal	Retroviral delivery of RUNX-2,Osterix,Oct3/4 and L-Myc in combination with ascorbic acid, β-glycerophosphate, dexamethasone.	NOD/SCID	(6)
Dermal	Retroviral delivery of Oct9 with N-Myc in combination with ascorbic acid, β-glycerophosphate, dexamethasone.	-	(7)
Gingival, dermal	Plasmid delivery of Oct4, Osterix, and L-Myc in combination with ascorbic acid, β-glycerophosphate, dexamethasone.	NOD/SCID	(8)
Gingival, dermal foreskin	Adenovirus delivery of BMP7.	NIH III, C57BL/6	(9–11)
Gingival, dermal foreskin	Ascorbic acid, β-glycerophosphate, dexamethasone.	–	(12)
Dermal	Ascorbic acid, β-glycerophosphate, human platelet lysate.	–	(13, 14)
Gingival	Ascorbic acid, β-glycerophosphate.	–	(15)
Interspinous ligament	Osteoclast cell-like conditioned media.	–	(16)
Dermal	Ascorbic acid, β-glycerophosphate, dexamethasone, ALK5 inhibitor II, vitamin D.	NOG	(17)
Dermal	Ascorbic acid, β-glycerophosphate, dexamethasone, TGF-β.	–	(18)
Dermal	Ascorbic acid, β-glycerophosphate, vitamin D, p-tricalcium phosphate scaffold.	–	(19)
Gingival	5-aza-dC and BMP-2.	BNX	(20)

of the disease in the activin receptor-like kinase 2 (*ALK2*) gene (42). These problems have been resolved in studies, in which iPSCs, and iMSCs from patient fibroblast-derived iPSCs, have been successfully used in FOP disease modeling (43, 44). Perhaps partly owing to these limitations, a low number of studies exist, concerning iPSCs from patients with rare bone disorders. In addition to FOP, these include iPSCs from Marfan syndrome fibroblasts (45), osteogenesis imperfecta bone marrow MSCs (38), craniometaphyseal dysplasia peripheral blood cells (46), thanatophoric dysplasia, and achondroplasia (47).

In an attempt to overcome these issues, research focus has shifted toward differentiation methods that can bypass the cumbersome step of iPSC generation (**Figure 1**). Transdifferentiation is the direct conversion of one differentiated cell type to another without the intermediate generation of iPSCs; however, whether and to which extent the pluripotency state is lacking, remains a point of discussion (48, 49). In addition to avoiding genomic instability and the risk of oncogenesis, an important advantage of transdifferentiation is primarily the lack of extensive cell manipulation, which means that the cells are possibly more likely to maintain their genetic makeup that may play a role in the accurate investigation of the disease mechanism. Below, several approaches of human osteoblast transdifferentiation in the field of bone disorders are summarized.

TRANSGENE-MEDIATED OSTEOGENIC TRANSDIFFERENTIATION

Yamamoto et al. showed that the retroviral transduction of human gingival fibroblasts with the two osteoblast-differentiation regulators RUNX-2 and Osterix, and the two reprogramming factors Oct3/4 and L-Myc, could induce their direct conversion to osteoblast-like cells (6). The differentiated cells showed a high expression of osteoblast-related genes, produced a high amount of calcified ECM, shared a similar global gene expression pattern with primary osteoblasts, and they were able to regenerate bone defect lesions that were surgically created in the femurs of NOD/SCID mice. They differed in the lower CpG methylation at the osteocalcin gene upstream region, compared to primary osteoblasts, but which was higher compared to their progenitor fibroblast cell line. Induction of the osteoblast generation could also be achieved by transient expression of the aforementioned factors. In a similar study, the retroviral-mediated expression of combined Oct9 with N-Myc was identified as the most potent for the osteogenic conversion, which was also based on the expression of osteogenic genes RUNX-2 and osteocalcin, as well as on the production of calcified bone matrix (7). In order to avoid the unwanted effects of retroviral integration in the genome, the same group attempted the expression of Oct4, Osterix, and L-Myc with a plasmid vector in human fibroblasts (8). This led to the induction of an osteoblast-like phenotype based on the expression of osteoblast-specific genes, *in vitro* deposition of minerals, alkaline phosphatase activity, and calcified body formation following implantation in the testis of NOD/SCID mice. Regarding the latter, no teratoma formation was observed in sharp contrast to implanted iPSCs. A pertinent question is the requirement of pluripotency factors in combination with the expression of master switch genes or the common osteogenic media. Even though pluripotency was not detected in the transitioning cells, it can be assumed that they provide some level of stemness, which can prime them for osteogenic conversion by the cues provided from the other factors (6).

Considering that the TGF-β superfamily regulates diverse aspects of the skeletal system (50), the osteo-inductive properties of bone morphogenetic proteins (BMPs) have been explored.

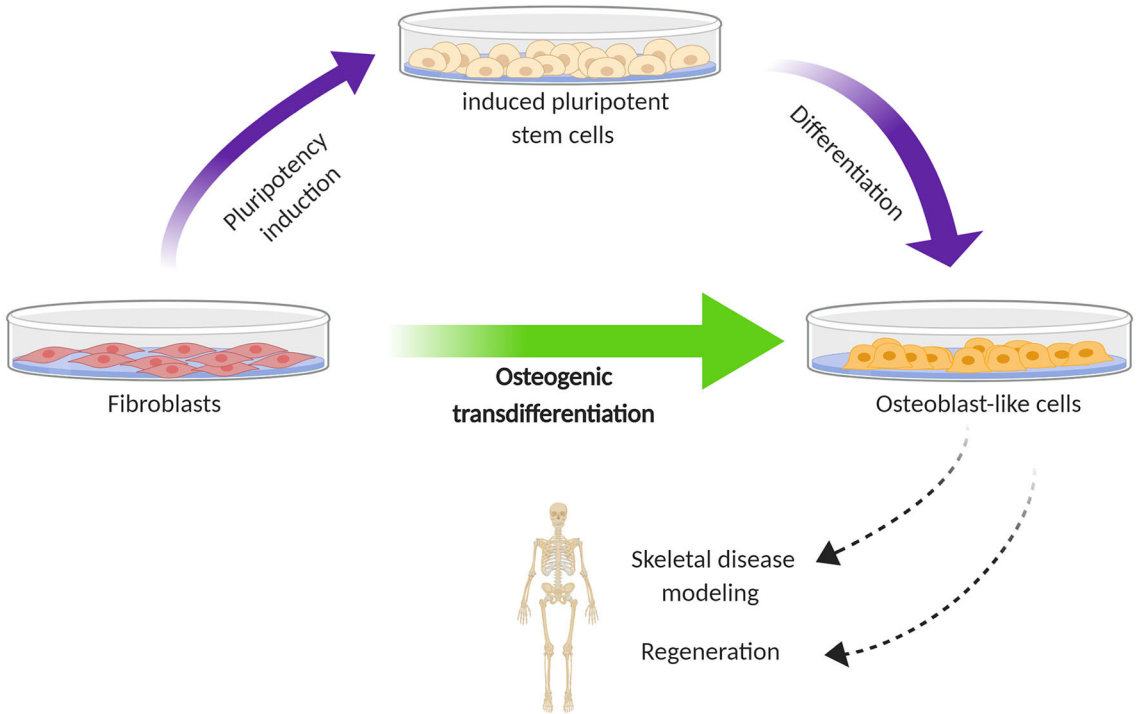


FIGURE 1 | Schematic diagram illustrating the difference in cell reprogramming between iPSC-mediated differentiation (two step) and transdifferentiation (one step) for the generation of osteoblast-like cells from human fibroblasts. The first is based on directing fibroblasts toward induced pluripotent stem cells, which are subsequently subjected to osteogenic differentiation. In the second, this pluripotency stage is bypassed; fibroblasts are directly converted to osteoblast-like cells. The generation of osteoblast-like cells from fibroblasts holds promise for modeling the process of skeletal disorders and exploring regenerative therapies.

Krebsbach et al. reported that *ex vivo* adenovirus BMP-7-transduced fibroblasts have bone-forming properties when transplanted into immunocompromised mice (9). The same group subsequently showed that adenovirus BMP-7-transduced fibroblasts via subcutaneous injection can form ossicles in mice and they can also repair segmental defects in rat femurs. The *in vivo* osteoblast conversion of the transduced fibroblasts in the diffusion chambers took place without contact with the host cells, stressing the osteogenic role of BMP-7 (10). This is in agreement with the suppression of the osteoblast phenotype after addition of the BMP inhibitor noggin in osteogenic media of human fibroblasts seeded in p-tricalcium phosphate scaffolds (19). Chen et al. also showed that the knockdown of the BMP signaling-regulator SMAD4 attenuates the osteogenic differentiation of fibroblasts after adenovirus-mediated BMP-7 expression (11). These studies have not compared BMP-7 with other BMPs, which have also shown to have osteogenic capacity in mouse fibroblasts (51); whether this applies to human fibroblasts remains to be shown.

TRANSGENE FREE-MEDIATED OSTEOGENIC TRANSDIFFERENTIATION

In the described studies, as well as in other studies with untransfected fibroblasts (12), cells were treated with osteogenic media, which included supplementation with ascorbic acid, β-glycerophosphate, and dexamethasone. Dexamethasone is a synthetic glucocorticoid which is frequently used in recipes of osteogenic media to promote the *in vitro* commitment of cells toward the osteogenic cell lineage (52). However, glucocorticoid-induced osteoporosis clearly points to a differential effect on osteogenesis; the boundary distinguishing its ability to promote or suppress bone formation is still undefined (53, 54). In order to provide an alternative for dexamethasone and fetal calf serum, we have turned to growth factors. We have developed an *in vitro* method of osteogenic transdifferentiation based on human platelet lysate (13, 14). Platelet lysate provides numerous growth factors (55), which have been shown to promote osteogenic differentiation of bone marrow-derived MSCs (56, 57) and prevent osteoporosis development in ovariectomized mice (57). In this model, we have observed that dermal fibroblasts from FOP patients show an enhanced potential for osteogenic transdifferentiation in agreement with the heterotopic ossification characterizing this disease (13) A similar model for osteogenesis in FOP also exists with periodontal ligament fibroblasts (15). We also used our model to investigate the effect of the identified genetic variants in *AIFM1* on protein level in patients with spondylometaphyseal dysplasia (14). *AIFM1* encodes the mitochondrial apoptosis-inducing factor 1, which was undetectable in dermal fibroblasts; the osteogenic transdifferentiation of fibroblasts to osteoblast-like cells allowed the confirmation of the pathogenic effect

of the *AIFM1* variant in the differentiated cell type modeling the disease. Differentiation of fibroblasts toward the osteoblast lineage was also demonstrated with treatment of osteoclast-conditioned media (16). It is known that osteoclasts secrete factors affecting osteoblast differentiation (58); the osteoclast factors mediating the osteoblast conversion were not addressed in this study. The identification of key osteogenic factors in the platelet lysate and osteoclast-conditioned media can aid the optimization of transgene-free protocols.

The chemical inhibition of the ALK5 receptor, a TGF-β type I receptor mediating signaling by TGF-β ligands, with the use of the ALK5 inhibitor II, directed the conversion of human dermal fibroblasts to osteoblast-like cells (17). In particular, the combination of ALK5 inhibitor II and vitamin D3 yielded the highest enhancement in osteoblast differentiation. The implantation of the differentiated cells in created bone lesions of immunodeficient NOG mice resulted in bone healing, as evaluated by histological analysis of callus formation and ossification. Interestingly, the stimulation of osteoblastogenesis by ALK5 inhibition is the opposite of what we observed in our study with the different ALK5 inhibitor GW788388 (13). In another study, the addition of TGF-β to osteogenic media was shown to improve the capacity of dermal fibroblasts for osteogenic transdifferentiation, although it did not contribute to mineralization (18). These differences may be attributed to the different growth factor compositions between these models, as well as to the different properties of the chemical inhibitors; for example, GW788388 is known to additionally target ALK4 and ALK7, which are TGF-β type I receptors for activin signaling (59). In a different approach, Cho et al. used BMP2 treatment combined with compound-induced demethylation of the hypermethylated CpG islands of the *RUNX2* and *ALP* genes to drive the direct differentiation of human gingival fibroblasts to functional osteoblasts, as shown by the subcutaneous ectopic bone formation in BNX mice after implantation of the epigenetically modified cells (20). These studies highlight the potential of chemical approaches in osteogenic transdifferentiation as a more controlled, simple, and low-cost alternative to growth factors.

CHONDROGENIC TRANSDIFFERENTIATION

Bone development takes place through two different modes: intramembranous or endochondral ossification. The first is characterized by the differentiation of progenitor mesenchymal cells to osteoblasts, whereas the second is mediated by an intermediate cartilage phase preceding bone tissue development (60). Thus, given that endochondral ossification is an integral part of skeletogenesis, the availability of models to study the chondrocytes certainly has the possibility to deliver significant insights into the dysregulation of this process in certain disorders (61). Similar to osteogenic transdifferentiation, several protocols exist for chondrogenic transdifferentiation of fibroblasts, which are based on growth factor stimulation, forced expression of key transcription factors, scaffold biomaterials, and hypoxic conditions (62).

CONCLUSIONS

Osteogenic transdifferentiation is an attractive route to generate cells of the osteogenic cell lineage. Available examples show that they can be promising in modeling of bone diseases. Several studies exist presenting different experimental options for fibroblast commitment toward the osteogenic cells; many of these do not make use of transgene introduction, which offers an advantage over iPSCs. The latter are derived after fibroblast reprogramming and subsequent differentiation, a process that requires extensive genetic modification, is technically demanding, and may pose malignancy risks in tissue regeneration. On the other hand, the fact that limited studies exist about osteogenic transdifferentiation means that we still have an incomplete understanding of the mechanism and the whole spectrum of potential advantages and shortcomings. With this review, we hope to generate excitement and ideas about the under-investigated osteogenic transdifferentiation as an alternative for the iPSC detour. Harnessing the osteogenic potential of the easily attainable fibroblasts is an attractive prospect for the study of bone pathophysiology and the future development of new technologies for bone regeneration therapy.

AUTHOR CONTRIBUTIONS

DM has carried out the conception and writing of the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was kindly funded by the Horstingstuit foundation.

ACKNOWLEDGMENTS

Figure 1 was created with BioRender.com.

REFERENCES

1. Mäkitie RE, Costantini A, Kämpe A, Alm JJ, Mäkitie O. New insights into monogenic causes of osteoporosis. *Front Endocrinol.* (2019) 10:70. doi: 10.3389/fendo.2019.00070
2. McCarthy EF, Genetic diseases of bones and joints. *Semin Diagn Pathol.* (2011) 28:26–36. doi: 10.1053/j.semdp.2011.01.004
3. Demkow U, Płoski R. *Clinical Applications for Next-Generation Sequencing*. Elsevier Inc., (2015). doi: 10.1016/C2014-0-00597-7
4. Parker RC, The functional characteristics of nine races of fibroblasts. *Science.* (1932) 76:219–20. doi: 10.1126/science.76.1966.219-a
5. Lynch MD, Watt FM. Fibroblast heterogeneity: implications for human disease. *J Clin Invest.* (2018) 128:26–35. doi: 10.1172/JCI 93555

6. Yamamoto K, Kishida T, Sato Y, Nishioka K, Ejima A, Fujiwara H, et al. Direct conversion of human fibroblasts into functional osteoblasts by defined factors. *Proc Natl Acad Sci USA. (*2015*)* 112:6152–7. doi: 10.1073/pnas.1420713112
7. Mizoshiri N, Kishida T, Yamamoto K, Shirai T, Terauchi R, Tsuchida S, et al. Transduction of Oct 6 or Oct 9 gene concomitant with myc family gene induced osteoblast-like phenotypic conversion in normal human fibroblasts. *Biochem Biophys Res Commun.* (2015) 467:1110–6. doi: 10.1016/j.bbrc.2015.10.098
8. Yamamoto K, Sato Y, Honjo K, Ichioka H, Oseko F, Sowa Y, et al. Generation of directly converted human osteoblasts that are free of exogenous gene and xenogenic protein. *J Cell Biochem.* (2016) 117:2538–45. doi: 10.1002/jcb.25546
9. Krebsbach PH, Gu K, Franceschi RT, Rutherford RB. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone *in vivo*. *Hum Gene Ther.* (2000) 11:1201–10. doi: 10.1089/10430340050015248
10. Rutherford RB, Moalli M, Franceschi RT, Wang D, Gu K, Krebsbach PH. Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone *in vivo*. *Tissue Eng.* (2002) 8:441–52. doi: 10.1089/107632702760184709
11. Chen F, Bi D, Cheng C, Ma S, Liu Y, Cheng K. Bone morphogenetic protein 7 enhances the osteogenic differentiation of human dermal-derived CD105+ fibroblast cells through the Smad and MAPK pathways. *Int J Mol Med.* (2019) 43:37–46. doi: 10.3892/ijmm.2018.3938
12. Monterubbianesi R, Bencun M, Pagella P, Woloszyk A, Orsini G, Mitsiadis TA. A comparative *in vitro* study of the osteogenic and adipogenic potential of human dental pulp stem cells, gingival fibroblasts and foreskin fibroblasts. *Sci Rep.* (2019) 9:1761. doi: 10.1038/s41598-018-37981-x
13. Micha D, Voermans E, Eekhoff MEW, van Essen HW, Zandieh-Doulabi B, Netelenbos C, et al. Inhibition of TGFβ signaling decreases osteogenic differentiation of fibrodysplasia ossificans progressiva fibroblasts in a novel *in vitro* model of the disease. *Bone.*, (2016) 84:169–80. doi: 10.1016/j.bone.2016.01.004
14. Miyake N, Wolf NI, Cayami FK, Crawford J, Bley A, Bulas D, et al. X-linked hypomyelination with spondylometaphyseal dysplasia (H-SMD) associated with mutations in AIFM1. *Neurogenetics.* (2017) 18:185–94. doi: 10.1007/s10048-017-0520-x
15. de Vries TJ, Schoenmaker T, Micha D, Hogervorst J, Bouskla S, Forouzanfar T, et al. Periodontal ligament fibroblasts as a cell model to study osteogenesis and osteoclastogenesis in fibrodysplasia ossificans progressiva. *Bone.* (2018) 109:168–177. doi: 10.1016/j.bone.2017.07.007
16. Yu F, Cui Y, Zhou X, Zhang X, Han J. Osteogenic differentiation of human ligament fibroblasts induced by conditioned medium of osteoclast-like cells. *Biosci Trends.* (2011) 5:46–51. doi: 10.5582/bst.2011.v5.2.46
17. Yamamoto K, Kishida T, Nakai K, Sato Y, Kotani SI, Nishizawa Y, et al. Direct phenotypic conversion of human fibroblasts into functional osteoblasts triggered by a blockade of the transforming growth factor-β signal. *Sci Rep.* (2018) 8:8463. doi: 10.1038/s41598-018-26745-2
18. Aloise AC, Pereira MD, Duailibi SE, Gragnani A, Ferreira LM. TGF-β1 on induced osteogenic differentiation of human dermal fibroblast. *Acta Cir Bras.* (2014) 29:1–6. doi: 10.1590/S0102-86502014001300001
19. Hee CK, Nicoll SB. Induction of osteoblast differentiation markers in human dermal fibroblasts: potential application to bone tissue engineering. *Conf Proc IEEE Eng Med Biol Soc.* (2006) 2006:521–4. doi: 10.1109/IEMBS.2006.259308
20. Cho Y, Kim B, Bae H, Kim W, Baek J, Woo K, et al. Direct gingival fibroblast/osteoblast transdifferentiation via epigenetics. *J Dent Res.* (2017) 96:555–61. doi: 10.1177/0022034516686745
21. Bonewald LF. The amazing osteocyte. *J Bone Miner Res.* (2011) 26:229–38. doi: 10.1002/jbmr.320
22. Marie PJ, Kassem M. Osteoblasts in osteoporosis: past, emerging, and future anabolic targets. *Eur J Endocrinol.* (2011)165:1–10. doi: 10.1530/EJE-11-0132
23. Johnson ML. How rare bone diseases have informed our knowledge of complex diseases. *Bonekey Rep.* (2016) 5:839. doi: 10.1038/bonekey.2016.69
24. Tracy LE, Minasian RA, Caterson EJ. Extracellular matrix and dermal fibroblast function in the healing wound. *Adv Wound Care.* (2016) 5:119–36. doi: 10.1089/wound.2014.0561
25. Sorrell JM, Caplan AI. Fibroblasts-a diverse population at the center of it all. *Int Rev Cell Mol Biol.* (2009)276:161–214. doi: 10.1016/S1937-6448(09)76004-6
26. Sorrell JM, Caplan AI. Fibroblast heterogeneity: more than skin deep. *J Cell Sci.* (2004) 117:667–75. doi: 10.1242/jcs.01005
27. Denu RA, Nemcek S, Bloom DD, Goodrich AD, Kim J, Mosher DF, et al. Fibroblasts and mesenchymal stromal/stem cells are phenotypically indistinguishable. *Acta Haematol.* (2016) 136:85–97. doi: 10.1159/000445096
28. Haniffa MA, Collin MP, Buckley CD, Dazzi F. Mesenchymal stem cells: the fibroblasts' new clothes? *Haematologica.* (2009) 94:258–63. doi: 10.3324/haematol.13699
29. Soundararajan M, Kannan S. Fibroblasts and mesenchymal stem cells: two sides of the same coin? *J Cell Physiol.* (2018) 233:9099–109. doi: 10.1002/jcp.26860
30. Infante A, Rodríguez CI. Osteogenesis and aging: lessons from mesenchymal stem cells. *Stem Cell Res Ther.* (2018) 9:244. doi: 10.1186/s13287-018-0995-x
31. Wei X, Yang X, Han ZP, Qu FF, Shao L, Shi YF. Mesenchymal stem cells: a new trend for cell therapy. *Acta Pharmacol Sinica.* (2013) 34:747–54. doi: 10.1038/aps.2013.50
32. Ichim TE, O'Heeron P, Kesari S. Fibroblasts as a practical alternative to mesenchymal stem cells. *J Transl Med.* (2018) 16:212. doi: 10.1186/s12967-018-1536-1
33. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* (2007) 131:861–72. doi: 10.1016/j.cell.2007.11.019
34. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science.* (2007) 318:1917–20. doi: 10.1126/science.1151526
35. Bastami F, Nazeman P, Moslemi H, Rezai Rad M, Sharifi K, Khojasteh A. Induced pluripotent stem cells as a new getaway for bone tissue engineering: a systematic review. *Cell Prolif.* (2017) 50:e12321. doi: 10.1111/cpr.12321
36. Csobonyeiova M, Polak S, Zamborsky R, Danisovic L. iPS cell technologies and their prospect for bone regeneration and disease modeling: a mini review. *J Adv Res.* (2017) 8:321–7. doi: 10.1016/j.jare.2017.02.004
37. Sabapathy V, Kumar S. hiPSC-derived iMSCs: nextGen MSCs as an advanced therapeutically active cell resource for regenerative medicine. *J Cell Mol Med.* (2016) 20:1571–88. doi: 10.1111/jcmm.12839
38. Deyle DR, Khan IF, Ren G, Wang PR, Kho J, Schwarze U, et al. Normal collagen and bone production by gene-targeted human osteogenesis imperfecta iPSCs. *Mol Ther.* (2012) 20:204–13. doi: 10.1038/mt.2011.209
39. Villa-Diaz LG, Brown SE, Liu Y, Ross AM, Lahann J, Parent JM, Krebsbach PH. Derivation of mesenchymal stem cells from human induced pluripotent stem cells cultured on synthetic substrates. *Stem Cells.* (2012) 30:1174–81. doi: 10.1002/stem.1084
40. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Kinoshita S. Exosomes released from human induced pluripotent stem cells-derived MSCs facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis. *J Transl Med.* (2013) 13:49.
41. Doss MX, Sachinidis A. Current challenges of iPSC-based disease modeling and therapeutic implications. *Cells.* (2019) 8:403. doi: 10.3390/cells8050403
42. Hamasaki M, Hashizume Y, Yamada Y, Katayama T, Hohjoh H, Fusaki N, et al. Pathogenic mutation of ALK2 inhibits induced pluripotent stem cell reprogramming and maintenance: mechanisms of reprogramming and strategy for drug identification. *Stem Cells.* (2012) 30:2437–49. doi: 10.1002/stem.1221
43. Cai J, Orlova VV, Cai X, Eekhoff EMW, Zhang K, Pei D, et al. Induced pluripotent stem cells to model human fibrodysplasia ossificans progressiva. *Stem Cell Rep.* (2015) 5:963–70. doi: 10.1016/j.stemcr.2015.10.020
44. Matsumoto Y, Ikeya M, Hino K, Horigome K, Fukuta M, Watanabe M, et al. New protocol to optimize ips cells for genome analysis of fibrodysplasia ossificans progressiva. *Stem Cells.* (2015) 33:1730–42. doi: 10.1002/stem.1981
45. Quarto N, Leonard B, Li S, Marchand M, Anderson E, Behr B, et al. Skeletogenic phenotype of human marfan embryonic stem cells faithfully phenocopied by patient-specific induced-pluripotent stem cells. *Proc Natl Acad Sci USA.* (2012). 109:215–20. doi: 10.1073/pnas.1113442109
46. Chen IP, Fukuda K, Fusaki N, Iida A, Hasegawa M, Lichtler A, et al. Induced pluripotent stem cell reprogramming by integration-free sendai virus vectors from peripheral blood of patients with craniometaphyseal dysplasia. *Cell Reprogram.* (2013) 15:503–13. doi: 10.1089/cell.2013.0037

47. Yamashita A, Morioka M, Kishi H, Kimura T, Yahara Y, Okada M, et al. Statin treatment rescues FGFR3 skeletal dysplasia phenotypes. *Nature*. (2014) 513:507–11. doi: 10.1038/nature13775
48. Cieślar-Pobuda A, Knoflach V, Ringh MV, Stark J, Likus W, Siemianowicz K, et al. Transdifferentiation and reprogramming: overview of the processes, their similarities and differences. *Biochim Biophys Acta Mol Cell Res.* (2017) 1864:1359–69 doi: 10.1016/j.bbamcr.2017.04.017
49. Reid A, and Tursun B. Transdifferentiation: do transition states lie on the path of development? *Curr Opin Syst Biol.* (2018) 11:18–23. doi: 10.1016/j.coisb.2018.07.004
50. Poniatowski LA, Wojdasiewicz P, Gasik R, Szukiewicz D. Transforming growth factor beta family: Insight into the role of growth factors in regulation of fracture healing biology and potential clinical applications. *Mediat Inflam.* (2015). 2015:137823. doi: 10.1155/2015/137823
51. Myllylä RM, Haapasaari KM, Lehenkari P, Tuukkanen J. Bone morphogenetic proteins 4 and 2/7 induce osteogenic differentiation of mouse skin derived fibroblast and dermal papilla cells. *Cell Tissue Res.* (2014) 355:463–70. doi: 10.1007/s00441-013-1745-0
52. Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells *in vitro*. *Stem Cell Res Ther.* (2013) 4:117. doi: 10.1186/scrt328
53. Rimando MG, Wu H, Lee C, Kuo S, Lo Y, Liu Y, et al. Bimodal effects of dexamethasone on osteogenic differentiation of mesenchymal stromal cells. *Cytotherapy.* (2016) 18:S77–8. doi: 10.1016/j.jcyt.2016.03.158
54. Zhou H, Cooper MS, Seibel MJ. Endogenous glucocorticoids and bone. *Bone Res.* (2013) 1:107–19. doi: 10.4248/BR201302001
55. Fekete N, Gadelorge M, Fürst D, Maurer C, Dausend J, Fleury-Cappellesso S, et al. Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active comp. *Cytotherapy*. (2012) 14:540–54. doi: 10.3109/14653249.2012.655420
56. Jonsdottir-Buch SM, Lieder R, Sigurjonsson OE. Platelet lysates produced from expired platelet concentrates support growth and osteogenic differentiation of mesenchymal stem cells. *PLoS ONE.* (2013) 8:e68984. doi: 10.1371/journal.pone.0068984
57. W-Lo C, J-Chiou F, Gelovani JG, M-Cheong L, C-Lee M, H-Liu Y, et al. Transplantation of embryonic fibroblasts treated with platelet-rich plasma induces osteogenesis in SAMP8 mice monitored by molecular imaging. *J Nucl Med.* (2009) 50:765–73. doi: 10.2967/jnumed.108.057372
58. Sims NA, Martin TJ. Coupling signals between the osteoclast and osteoblast: how are messages transmitted between these temporary visitors to the bone surface? *Front Endocrinol.* (2015) 6:41. doi: 10.3389/fendo.2015.00041
59. Petersen M, Thorikay M, Deckers M, Van Dinther M, Grygielko ET, Gellibert F, et al. Oral administration of GW788388, an inhibitor of TGF-β type I and II receptor kinases, decreases renal fibrosis. *Kidney Int.* (2008) 73:705–15. doi: 10.1038/sj.ki.5002717
60. Berendsen AD, Olsen BR. Bone development. *Bone.* (2015) 80:14–8. doi: 10.1016/j.bone.2015.04.035
61. Shore EM. Fibrodysplasia ossificans progressiva (FOP): a disorder of extraskeletal endochondral ossification. *Semin Arthritis Rheum.* (2013) 42:546. doi: 10.1016/j.semarthrit.2012.12.010
62. Driessen BJH, Logie C, Vonk LA. Cellular reprogramming for clinical cartilage repair. *Cell Biol Toxicol.* (2017) 33:329–49. doi: 10.1007/s10565-017-9382-0

Conflict of Interest: 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 Endocrinology

REVIEW
published: 08 July 2020
doi: 10.3389/fendo.2020.00405

The Osteocyte as the New Discovery of Therapeutic Options in Rare Bone Diseases

Janak L. Pathak[1], *Nathalie Bravenboer*[2] *and Jenneke Klein-Nulend*[1,3]*

[1] *Key Laboratory of Oral Medicine, Guangzhou Institute of Oral Disease, Affiliated Stomatology Hospital of Guangzhou Medical University, Guangzhou, China,* [2] *Department of Clinical Chemistry, Amsterdam University Medical Centers, Amsterdam Movement Sciences, Vrije Universiteit Amsterdam, Amsterdam, Netherlands,* [3] *Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam, Amsterdam Movement Sciences, University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam, Netherlands*

Osteocytes are the most abundant (∼95%) cells in bone with the longest half-life (∼25 years) in humans. In the past osteocytes have been regarded as vestigial cells in bone, since they are buried inside the tough bone matrix. However, during the last 30 years it has become clear that osteocytes are as important as bone forming osteoblasts and bone resorbing osteoclasts in maintaining bone homeostasis. The osteocyte cell body and dendritic processes reside in bone in a complex lacuno-canalicular system, which allows the direct networking of osteocytes to their neighboring osteocytes, osteoblasts, osteoclasts, bone marrow, blood vessels, and nerves. Mechanosensing of osteocytes translates the applied mechanical force on bone to cellular signaling and regulation of bone adaptation. The osteocyte lacuno-canalicular system is highly efficient in transferring external mechanical force on bone to the osteocyte cell body and dendritic processes via displacement of fluid in the lacuno-canalicular space. Osteocyte mechanotransduction regulates the formation and function of the osteoblasts and osteoclasts to maintain bone homeostasis. Osteocytes produce a variety of proteins and signaling molecules such as sclerostin, cathepsin K, Wnts, DKK1, DMP1, IGF1, and RANKL/OPG to regulate osteoblast and osteoclast activity. Various genetic abnormality-associated rare bone diseases are related to disrupted osteocyte functions, including sclerosteosis, van Buchem disease, hypophosphatemic rickets, and WNT1 and plastin3 mutation-related disorders. Meticulous studies during the last 15 years on disrupted osteocyte function in rare bone diseases guided for the development of various novel therapeutic agents to treat bone diseases. Studies on genetic, molecular, and cellular mechanisms of sclerosteosis and van Buchem disease revealed a role for sclerostin in bone homeostasis, which led to the development of the sclerostin antibody to treat osteoporosis and other bone degenerative diseases. The mechanism of many other rare bone diseases and the role of the osteocyte in the development of such conditions still needs to be investigated. In this review, we mainly discuss the knowledge obtained during the last 30 years on the role of the osteocyte in rare bone diseases. We speculate

OPEN ACCESS

Edited by:
Wim Van Hul,
University of Antwerp, Belgium

Reviewed by:
Niklas Rye Jørgensen,
Copenhagen University Hospital, Denmark
Reinhold Gottfried Erben,
University of Veterinary Medicine Vienna, Austria

****Correspondence:***
Jenneke Klein-Nulend
j.kleinnulend@acta.nl

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *10 February 2020*
Accepted: *20 May 2020*
Published: *08 July 2020*

Citation:
Pathak JL, Bravenboer N and Klein-Nulend J (2020) The Osteocyte as the New Discovery of Therapeutic Options in Rare Bone Diseases. Front. Endocrinol. 11:405. doi: 10.3389/fendo.2020.00405

about future research directions to develop novel therapeutic drugs targeting osteocyte functions to treat both common and rare bone diseases.

Keywords: osteocyte, rare bone disease, mechanotransduction, bone remodeling, niche, sost/sclerostin, phosphate-homeostasis, RANKL

INTRODUCTION

Bone mainly contains three types of cells, i.e., osteocytes, osteoblasts, and osteoclasts. The osteocytes are the most abundant cells comprising 95% of the total cell population in bone with an average half-life of 25 years (1, 2). The bone-forming osteoblasts and bone-resorbing osteoclasts account for only ∼5% of the total bone cell population, and live for only a few days to weeks. The characteristics and function of osteoblasts and osteoclasts during physiological bone remodeling and bone diseases have been extensively studied (3–6). However, the cellular and molecular mechanisms of osteocyte-mediated effects on skeletal health have not been fully elucidated. Five decades ago the osteocytes were still regarded as inert cells buried alive inside the bone matrix, despite the fact that the healthy human skeleton contains ∼42 billion osteocytes (7). The mechanosensing property of osteocytes has been reported for the first time about three decades ago (8). With the advancement of new technologies in molecular and cellular mechanisms, imaging, transgenic approaches, and RNA sequencing, important functions of osteocytes and their role in bone homeostasis and vital systemic functions have become clear in the last two decades (1). Osteocytes are descendants of osteoblasts. During the bone mineralization process, some osteoblasts bury themselves in the bone matrix. They regulate mineralization, develop connective dendritic processes, and become osteocytes. Although osteocytes are buried deep inside the bone matrix, their dendritic processes are well-connected with neighboring osteocytes, osteoblasts, blood vessels, nerve cells, and bone marrow. The osteocyte cell body resides in a lacunar space inside the bone matrix. From the cell body 50–60 dendritic processes radiate in canaliculi space, forming a complex osteocyte lacuno-canalicular system (9). Mechanical loading of bone triggers interstitial fluid flow in this lacuno-canalicular system. Osteocyte dendritic processes sense the fluid flow, resulting in cellular signaling (10–12). In response to mechanical stimuli, osteocytes release nitric oxide (NO), prostaglandins (PGs), and ATP (within milliseconds), which affects many other cellular signaling pathways including interleukin-6 (IL-6), receptor activator of nuclear factor κB ligand/osteoprotegerin (RANKL/OPG), Wnt/β-catenin, and calcium signaling pathways (10, 11, 13–15). During the last 30 years various mechanisms of osteocyte mechanotransduction have been reported. Calcium oscillation in osteocytes has been shown to be a critical regulator of osteocyte mechanotransduction (16–18). Recently, mechanical loading-induced Ca^{2+} oscillation has been shown to cause the release of extracellular vesicles from osteocytes and to promote bone regeneration (19). Loading-induced Ca^{2+} oscillation in osteocytes triggers the release of downstream signaling molecules, e.g., NO (14, 20–22), prostaglandin E_2 (PGE_2) (23), matrix extracellular phosphoglycoprotein (MEPE), insulin-like growth factor-1 (IGF-1) (24), and β-catenin (25). Similarly, primary cilia on the osteocyte cell body as well as the dendritic processes play a regulatory role in the mechanotransduction process in osteocytes (26). Focal adhesions are macromolecular complexes consisting of multiple actin-associated proteins, such as paxillin, vinculin, connexin-43, integrins, and talin, that serve as physical linkages between a cell's cytoskeleton and the ECM. The mechanism of focal adhesion-mediated osteocyte mechanotransduction has been partly unraveled (27–30).

Osteocytes produce various signaling proteins such as sclerostin, WNT1, WNT3a, Dickkopf-related protein 1 (DKK1), phosphate regulating endopeptidase homolog X-linked (PHEX), RANKL, MEPE, fibroblast growth factor-23 (FGF23), sclerostin, and vascular endothelial growth factor (VEGF) (31–34). These proteins and growth factors not only play a crucial role in bone biology, but also in other organs such as kidney, and in fat metabolism (34, 35). Disruption of the production of these proteins by impaired osteocyte function causes bone diseases, including rare bone diseases (36–40). Osteocyte-specific release of growth factors and signaling molecules is disturbed during long-term unloading, such as occurs in astronauts during space traveling and long-term bed rest (11). Similarly, inflammatory conditions caused by various inflammatory diseases also affect osteocyte function and signaling (41, 42). Various genetic abnormality-associated rare bone diseases are related to disrupted osteocyte functions.

Wnt signaling plays a vital role in skeletal health, mainly via osteogenic differentiation of precursor cells, osteocyte viability, and osteocyte signaling to other bone cells (43, 44). Wnt/β-catenin activation in osteocytes mainly contributes to the anabolic effect in bone (45). Mechanical loading-induced early release of PGE_2 causes rapid activation of Wnt/β-catenin signaling in osteocytes (46, 47). Wnt ligand co-receptor LRP5 is essential for osteocyte mechanotransduction and mechanical loading-induced bone formation (43, 48–50). This suggests a crucial role of osteocytic Wnt signaling in the process of mechanotransduction. The consequence of disturbed Wnt signaling in osteocytes is demonstrated by a mutation in the WNT1 gene, which causes autosomal-recessive osteogenesis imperfecta, a childhood rare bone disease (51). The osteocyte is the main source of sclerostin, a negative regulator of Wnt/β-catenin signaling. Mechanical loading reduces, while proinflammatory cytokines enhance sclerostin production in osteocytes (31, 41). Sclerostin deficiency in various rare genetic bone diseases, such as sclerosteosis and van Buchem disease, causes osteopetrosis, a high bone mass phenotype (36).

Parathyroid hormone (PTH) signaling contributes via PTH-related protein (PTHrP)-derived peptides, to the mechanical loading-induced osteocyte-mediated adaptation of bone tissue

composition (52, 53). Inherited hypoparathyroidism is a rare disease that reduces bone turnover causing higher bone mineral density (BMD) and brittle bone (54). However, the osteocyte mechanotransduction-mediated bone adaptation in inherited hypoparathyroidism is still unknown. Similarly, mechanical loading upregulates insulin growth factor-1 (IGF1) expression in osteocytes, and IGF1 signaling plays an important role in the osteogenic response to mechanical loading (24, 55, 56). Moreover, IGF1 regulates PTH/PTHrP signaling in osteocytes, and bone regeneration (57–61). Osteocytic IGF1 signaling in rare bone diseases still needs to be investigated. Osteocytes produce RANKL and OPG to regulate osteoclastogenesis and osteoclast activity (6, 62). The RANKL/OPG ratio in osteocytes is upregulated by proinflammatory cytokines (31, 41, 63), but reduced by mechanical loading (64). Mechanical loading of osteocytes downregulates the expression of most proinflammatory cytokines, except IL-6. Interestingly, mechanical loading upregulates IL-6 expression in parallel with PGE_2 production in bone cells (63, 65). However, the exact role of mechanical loading-induced osteocytic IL-6 signaling in bone biology and rare bone diseases is poorly understood. Osteocytes not only regulate osteoblast and osteoclast formation and activity, but also phosphate homeostasis and the function of vital organs in an endocrine fashion (62, 66). Osteocytes respond to PTH by inducing osteolysis that releases calcium in the bloodstream to maintain the systemic mineral homeostasis (67). During lactation, osteocytic sclerostin modulates the production of the osteoclast markers tartrate-resistant acid phosphatase (TRAP), cathepsin K, and carbonic anhydrase-2 in osteocytes to regulate the release of calcium from bone (68). Mutation of the cathepsin K encoding gene causes a rare autosomal recessive osteochondrodysplasia (69). Although cathepsin K is mainly required for osteoclastic bone resorption, osteocytes also release cathepsin K and regulate mechanotransduction (70). Osteocytes release FGF23, dentin matrix acidic phosphoprotein 1 (DMP1), PHEX, and MEPE, and act as endocrine cells to regulate phosphate metabolism (1, 71–73). Osteocytes release sclerostin to control bone mineralization via the modulation of DMP1, PHEX, MEPE, and FGF-23 expression (74, 75). The osteocyte is a critical player in chronic kidney disease-associated adverse effects on bone and heart (76). Osteocyte-derived DMP1 reduces FGF23 expression and enhances bone mineralization (35). Chronic kidney disease reduces DMP1 expression in osteocytes, while DMP1 supplementation prevents osteocyte apoptosis, lowers FGF23 expression, increases serum phosphate, and prevents the development of left ventricular hypertrophy in a chronic kidney disease mice model (35, 76). PHEX indirectly regulates FGF23, and *PHEX* gene mutation causes hypophosphatemic rickets, a rare hereditary bone disease (39). The MEPE-PHEX interaction regulates bone turnover, mineralization, and bone-renal vascularization (77). MEPE is highly expressed in human osteocytes embedded within mineralized bone (78). $MEPE^{-/-}$ mice develop increased bone mass, hyperphosphatemia and creatinine-clearance, and transgenic overexpression of MEPE C-terminal acidic serine aspartate-rich MEPE-associated (ASARM)-motif corrects these abnormalities (79). C-terminal ASARM-motif plays a major role in regulation of bone mass and renal function in aging mice showing the association of MEPE in age-dependent osteoporosis. This unveils the endocrine function of osteocytes affecting the function of distant organs such a kidney and heart. Thus, osteocytes play a vital role in bone homeostasis, and several osteocyte-specific proteins are involved in the pathogenesis of rare bone diseases. In this review, we mainly focus on the role of disturbed development and activity of osteocytes in rare bone diseases. We will discuss the existing insights on the role of osteocytes in the pathophysiology of rare metabolic bone disorders as well as the consequences of these rare metabolic bone disorders for the development and function of osteocytes.

DISTURBED OSTEOCYTE FUNCTION CAN CAUSE METABOLIC BONE DISEASES

Many factors, including aging, osteoporosis, inflammatory diseases, and systemic diseases, disrupt osteocyte functions (2, 41, 76, 80). Aging causes 15–30% reduction in lacunar density or osteocyte numbers (81). Smaller and more round osteocyte lacunae are common in aged mice compared to young mice (82). The age-related decrease in lacunar density is accompanied by osteocyte death, hypermineralization, and micropetrosis (83). Aging also reduces the number of osteocyte dendrites and canaliculi by ∼30% (80, 84). The remarkable decrease in osteocyte lacunar density, canaliculi, and dendrites number will reduce the entire osteocyte network connectivity that affects osteocyte function and bone homeostasis. Since the osteocyte lacuno-canalicular system plays a crucial role in mechanotransduction, abnormalities in this system might directly affect osteocyte mechanotransduction-mediated bone adaptation and remodeling (85). Estrogen, PTH, bisphosphonates, and muscle-derived irisin increase osteocyte survival (86–88). Postmenopausal estrogen deficiency, imbalance in PTH signaling, long-term glucocorticoid treatment, and oxidative stress caused by disuse may cause osteocyte death resulting in imbalanced bone remodeling and decreased bone mass (89). Systemic inflammatory conditions, such as periodontitis, rheumatoid arthritis, chronic kidney disease, and cancer, affect osteocyte function mainly via elevated levels of proinflammatory cytokines.

Advanced glycation end products (AGEs) are inflammatory mediators in diabetes. AGEs induce osteocyte apoptosis and upregulate osteocytic expression of IL-6 and VEGF (90, 91). Periodontitis-mediated inflammation causes sclerostin production and NF-κβ activation in alveolar osteocytes (92). Diabetic rats with periodontitis show a higher expression of sclerostin, RANKL, tumor necrosis factor-α (TNFα), and IL-1β in osteocytes, which affects osteoblast and osteoclast function (93–95). Brucella abortus infection is a common cause of osteomyelitis, which not only inhibits connexin-43 expression in osteocytes, but also induces osteocyte apoptosis and upregulates expression of inflammatory mediators RANKL, TNFα, and IL-6 in osteocytes (96). Multiple myeloma, a cancer that directly affects bone, induces osteocyte apoptosis and osteocyte-derived sclerostin and RANKL expression (97). Osteocytic sclerostin

and FGF23 expression are highly upregulated in chronic kidney disease (98). In rheumatoid arthritis, a systemic inflammatory disease, elevated levels of inflammatory cytokines enhance IL-1β, TNFα, sclerostin (*SOST*), and DKK1 gene expression in osteocytes (31).

RARE BONE DISEASES AND OSTEOCYTE FUNCTION

Genetic defects cause various rare bone diseases such as Paget disease, fibrous dysplasia, pycnodysostosis, sclerosteosis, osteogenesis imperfecta, X-linked hypophosphatemia, and hypophosphatasia. Osteocyte functions are disturbed in many genetic defect-mediated rare bone diseases (99, 100). Possible mechanisms of disrupted osteocyte functions in rare bone diseases are depicted in **Figure 1**. An impaired activity/function of osteoblasts, osteoclasts, and/or osteocytes could lead to alterations in the mechanical environment of osteocytes, variations in ECM structure, and de-regulation of mechanotransduction-related pathways, resulting in disturbed mechanotransduction possibly via primary cilium, calcium channels, physical deformation of bone matrix, canalicular fluid flow, shear stress, adhesion molecules, and/or cytoskeleton.

SCLEROSTEOSIS AND VAN BUCHEM DISEASE

Sclerosteosis and van Buchem disease are autosomal recessive skeletal dysplasia causing deficiency of sclerostin protein and progressive skeletal growth (36). Sclerosteosis is primarily reported in the descendants of Dutch settlers from the seventeenth century in South Africa (101). Van Buchem disease is mainly found in a Dutch population in The Netherlands (102, 103). Skeletal manifestations of sclerosteosis and van Buchem disease are similar, including increased thickening of skull, jaw bones, long bones, and ribs. Gigantism, and hand abnormalities in sclerosteosis are distinguishing features between sclerosteosis and gigantism (104). *SOST*, the gene responsible for sclerosteosis and van Buchem disease, is localized on chromosome 17q12-q21, and encodes sclerostin protein. A point mutation in the *SOST* gene causes sclerosteosis, and a 52 kb deletion of the downstream gene of *SOST* causes van Buchem disease (36, 37). A study on the genetics and pathophysiology of sclerosteosis and van Buchem disease led to the discovery of sclerostin and its function that contributed to the development of an anti-sclerostin drug to treat osteoporosis (105). Mature osteoblasts produce sclerostin to some extent, but osteocytes are the primary source of sclerostin (106). Activation of Wnt/β-catenin signaling enhances osteogenic differentiation and bone formation (107). Sclerostin, a potent Wnt inhibitor, controls osteogenic differentiation of precursor cells and bone formation (108). On the other hand, Wnt inhibition causes overexpression of RANKL and deregulation of OPG resulting in osteoclastogenesis (38). Studies on rare bone diseases, sclerosteosis, and van Buchem disease, have unraveled the role of sclerostin in bone homeostasis (99). In the case of sclerostin deficiency, osteocytes become like a "snake without fang" and are unable to control new bone deposition by osteoblasts (36, 37). Sclerostin deficiency results in excessive bone formation (109), as observed in sclerosteosis and van Buchem disease. Since both sclerosteosis and van Buchem disease are genetic diseases caused by osteocytic sclerostin deficiency, the osteocyte could be the possible target cell to treat these diseases.

HYPOPHOSPHATEMIC RICKETS

Hypophosphatemic rickets is a hereditary disease with a prevalence of 1/20,000. *PHEX* gene mutations have been reported to cause hypophosphatemia and a hypomineralized bone phenotype (39, 40). Hypophosphatemic rickets is characterized by a generalized bone mineralization defect resulting in a decreased total volumetric bone mineral density (vBMD) at the radius and tibia, and lower cortical vBMD and cortical thickness at the radius compared to healthy adults (110). However, the exact mechanism of *PHEX* gene mutation-mediated FGF23 upregulation, hypophosphatemia, and development of rickets is still unclear. Both PHEX and FGF23 are mainly produced by osteocytes (111). One autosomal recessive hypophosphatemic rickets family carried a mutation affecting the dentin matrix protein (*DMP1*) start codon (112). DMP1 is essential for osteocyte maturation, while *DMP1* mutation leads to altered skeletal mineralization and disturbed phosphate homeostasis associated with increased FGF23 production via an effect on the function of osteocytes (112). A combination of oral phosphorous supplementation and active vitamin D analogs is the conventional therapy to counteract the consequences of excessive FGF23 in hypophosphatemic rickets (113). Anti-FGF23 antibody or gene therapy targeting *DMP1*, *FGF23*, or *PHEX*, could be a future direction to treat hypophosphatemic rickets. This has been demonstrated already in children with X-linked hypophosphatemia, where treatment with anti-FGF23 antibody Burosumab improved linear growth and physical function, and reduced the pain and the severity of rickets (114).

WNT1 AND PLS3 MUTATION

WNT1 is a key ligand of the canonical WNT signaling pathway, which is the most important signaling pathway in bone (115). The WNT family contains a total of 19 WNT proteins, including WNT1, which are essential for fetal bone development and maintenance of postnatal bone health (38). The plastin protein family belongs to the actin bundling proteins and is ubiquitously expressed in solid tissue, including neurons in the brain, osteoblasts and osteocytes in bone, hematopoietic cells, and many cancer cell types (116). Plastin3 (PLS3) expression in mesenchymal stem cells and osteoblasts increases during osteogenic differentiation (117, 118). Missense mutation c.652T>G (p.C218G) in *WNT1*, and an X-linked form resulting from a splice mutation c.73-24T>A in PLS3 are associated with osteoporosis in children (115, 119). The role of WNT1 and PLS3 in the function of osteocytes is not yet fully understood. *WNT1* mutation affects WNT/β-catenin signaling that might affect osteocyte function, and causes an imbalance in bone

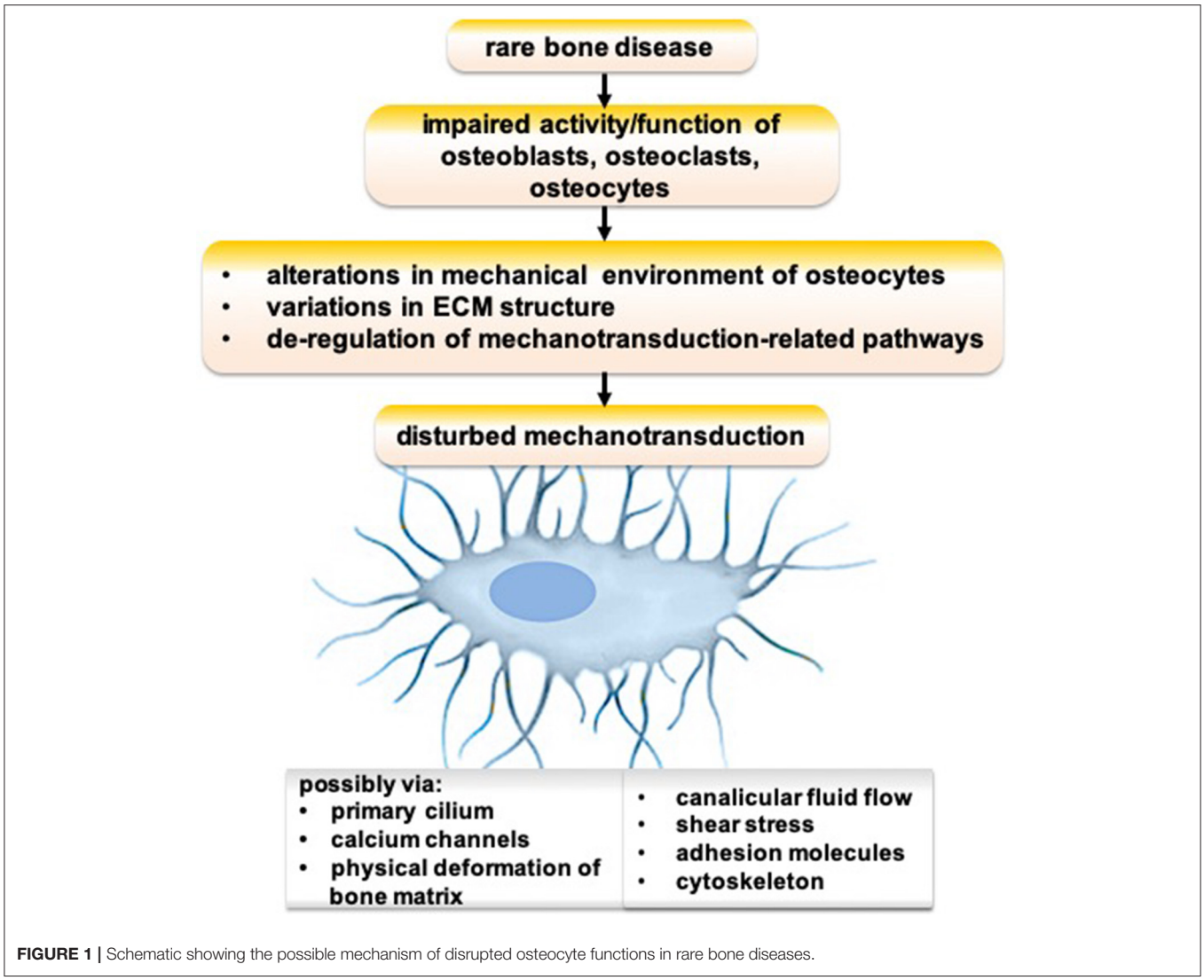


FIGURE 1 | Schematic showing the possible mechanism of disrupted osteocyte functions in rare bone diseases.

homeostasis resulting in osteoporosis (51). PLS3 has been suggested to play a role in osteocyte dendrite function and mechanotransduction (120). High FGF23 expression has been reported in osteocytes of a patient with a *WNT1* mutation compared to a *PLS3* mutation (121). The expression pattern of DMP1, sclerostin, and phospo-β-catenin is similar in patients with a *WNT1* and *PLS3* mutation (121). This suggests that WNT1 and PLS3-mediated osteoporosis might have a similar mechanism of disease progression. Osteocyte-derived WNT1 is a key regulator of osteoblast function and bone homeostasis (122). Deletion of *Wnt1* in osteocytes results in low bone mass and increased fracture risk, similar as *WNT1* mutation-related osteoporosis (122). Interestingly, *Wnt1* overexpression in osteocytes stimulates bone formation by increasing the osteoblast number and activity partly via activation of mTORC1 signaling (122). Anti-sclerostin antibody robustly increases bone mass and reduces the fracture rate in *Wnt1* global knockout mice (122). These findings suggest that *WNT1* mutation-related osteoporosis is caused in part by a loss of WNT1 signaling in osteocytes, which decreases mTORC1-dependent osteoblast formation and bone regeneration. The sclerostin antibody has been suggested to be an effective treatment option for *WNT1* mutation-related osteoporosis (122). However, osteocytic mechanotransduction in patients with a *WNT1* mutation is not yet fully understood. Microgravity, or unloading, decreases WNT3a, WNT5a, DKK1, cyclinD1, LEF-1, and CX43, but increases *WNT1* and *SOST* expression in osteocytes (11, 123). Microgravity dramatically reduces the number of F-actin filaments in osteocytes (123). This suggests a role for WNT1 in the formation of the osteocyte cytoskeleton and in osteocyte mechanosensitivity. *PLS3* mutation or deficiency causes low bone mass, possibly via hyperactivity of osteoclasts. PLS3-deficient mice show no effect in trabecular bone, but cortical bone mass is highly reduced (124). Normal osteocyte morphology is observed in PLS3-deficient mice (125). Bone marrow stem cells from PLS3-deficient mice show compromised osteogenic differentiation with reduced expression of osteocalcin, Wnt16, and Sfrp4 mRNA (125). This indicates a role of PLS3 in bone regeneration via osteoblast

differentiation and function (125). A lack of PLS3 has been shown to decrease the expression of NFkB repressing factor, thereby augmenting Nfatc1 transcription and osteoclastogenesis, indicating osteoclast-mediated bone loss in PLS3-deficient mice (124). The actin cytoskeleton and focal adhesions play an important role in osteocyte mechanotransduction. Since the plastin protein family belongs to the actin bundling protein, plastin might have a direct or focal adhesion-mediated indirect effect on osteocyte mechanotransduction. However, the role of PLS3 in osteocyte functions, such as mechanotransduction, osteocyte-to-osteoblast communication, and osteocyte-to-osteoclast signaling, and its cellular and molecular influence on bone remodeling has not been investigated yet.

OSTEOGENESIS IMPERFECTA

Osteogenesis imperfecta is mainly an autosomal dominant disease of connective tissue that lowers bone mass and causes fracture. Very few cases of recessive and X-chromosome-linked forms of osteogenesis imperfecta have been reported so far. Osteogenesis imperfecta is one of the most common bone fragility disorders with an incidence of about 1/15–20,000 (126). It is a brittle bone disease directly related to abnormalities of type I collagen primary posttranslational modification, folding, structure, strength, and quantity (127). Mutations in the *COL1A1* or *COL1A2* gene, encoding the α1(I) or α2(I) chain of type I collagen, are associated with ∼85% of osteogenesis imperfecta cases (128). Mutation-mediated alteration in processing, structure, and secretion of type I collagen, as well as ER stress causes a subclinical to lethal skeletal phenotype. Loss-of-function mutations in WNT1 lead to moderately severe and progressive forms of osteogenesis imperfecta (119, 129). Since osteocytes are embedded in the bone ECM, ECM-to-osteocyte interaction plays a vital role in bone homeostasis. The effect of deregulated collagen matrix-to-osteocyte interaction in osteogenesis imperfecta could influence the severity of bone fragility. However, the role of osteocytes in osteogenesis imperfecta disease progression has rarely been investigated yet. Future studies focusing on the role of the collagen matrix-to-osteocyte interaction in osteocytes function, including mechanotransduction, and osteoblast-to-osteoclast communication could guide in the development of new therapeutic targets to treat osteogenesis imperfecta.

PYCNODYSOSTOSIS

Pycnodysostosis (OMIM 265800) is a rare autosomal recessive osteochondrodysplasia with a prevalence rate of 1–1.7/million and without gender specificity (130). Pycnodysostosis is characterized by a short stature with increased bone mineral density and an increased bone fragility phenotype (105, 131). Cortical and trabecular osteosclerosis with increased cortical width and high bone mineral density is observed in patients with pycnodysostosis (11, 12). Gelb et al. reported mutation of the gene encoding cathepsin K in chromosome 1q21 in patients with pycnodysostosis (69). Cathepsin K degrades bone matrix proteins, including collagen type I, and is therefore essential for osteoclastic bone resorption (132). A study on the genetics and pathophysiology of pycnodysostosis revealed the role of cathepsin K in osteoclast activity that led to the development of cathepsin K inhibitors to treat osteoporosis by inhibiting osteoclastic bone resorption (105). Unfortunately cathepsin K inhibitors did not lead to new osteoporosis medication because of serious side effects (stroke). In pycnodysostosis the number of osteoclasts is not affected, but bone resorption is highly reduced (133). Osteoclastic bone resorption is essential for bone homeostasis, as old and cracked bone is removed as well as the fibrous extracellular matrix that provides the signal to osteoblasts to deposit new bone and increase bone strength. Cathepsin K is also produced by osteoblasts and osteocytes (70, 134). Osteocytic cathepsin K is responsible for lactation-induced bone loss (135). Mechanical loading increases cathepsin K expression in cortical bone of wild type mice (70). Globally knocking out of cathepsin K enhances mechanotransduction signals resulting in cortical bone formation (70). Cathepsin K regulates bone remodeling not only by enhancing osteoclast activity, but also by inhibiting osteogenic differentiation via modulation of Wnt signaling (70). Cathepsin K deficiency in osteoclasts increases sphingosine kinase 1 (Sphk1) that catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P) (136, 137). S1P promotes osteoblast differentiation, bone regeneration (136), and osteocytic mechanotransduction (138). New research approaches reducing the mechanosensitivity of osteocytes by inhibiting S1P could be important to develop therapeutics for the treatment of cathepsin K deficiency-mediated high bone mass phenotype.

Cathepsin K regulates bone remodeling and cortical bone formation by degrading periostin (139). Periostin is mainly expressed in the periosteum and in osteocytes, and enhances bone formation via activation of Wnt signaling (70). Bonnet et al. nicely depicted the role of osteoblastic and osteocytic periostin in cathepsin K-mediated bone modeling and remodeling (70). Osteocyte-mediated periostin could be a possible target in pycnodystostosis.

ANALYSIS OF OSTEOCYTE FUNCTION

Multiple approaches have been developed to analyze osteocyte morphology (80). Confocal laser scanning electron microscopy (CLSM) (140), scanning electron microscopy (SEM) (141), ultra-high voltage electron microscopy, tomography on silver stained bone sections (117, 142), and SEM of acid-etching technique of non-decalcified bone samples (143) have been developed to visualize osteocyte density, morphology, and osteocyte lacuno-canalicular network in bone biopsies from patients. Van Hove and colleagues nicely show differences in osteocyte morphology in patients with osteoarthritis, osteopenia, and osteopetrosis using CLSM (144). Schneider and colleagues developed serial focused ion beam/SEM imaging for quantitative 3D-assessment of the osteocyte lacuno-canalicular network (145). Micropetrotic lacunae, as seen in old age, in cortical and trabecular bone can be visualized by transmission electron microscopy (TEM)

and SEM (81). High power backscattered SEM images of a bone tissue section visualizes the mineralized micropetrotic lacunae (146). Osteocyte-specific expression of proteins such as sclerostin, IL-1β, TNFα, DKK1, DMP1, and FGF23 is altered in different disease conditions. Immunohistochemistry using specific antibodies easily visualizes the expression pattern in bone sections (33, 121, 147). Serum sclerostin is a key marker of osteocyte function in different disease conditions (148, 149). Serum sclerostin levels are upregulated in osteoporosis and downregulated in high bone mass conditions (150). Enzyme-linked immune assays and automated chemiluminescent assays have been developed and validated for high precision analysis of serum sclerostin (151, 152). Spinal cord injury causes patient immobilization and bed rest that mimics unloading conditions. Serum of patients with spinal cord injury contains increased periostin and decreased sclerostin levels (153). Since sclerostin and periostin are mainly secreted by osteocytes, these proteins could possibly be used as serum markers to analyze osteocyte function in different diseases.

Osteocyte mechanotransduction alters in different disease conditions, such as aging, osteoporosis, and inflammatory diseases (82, 154–157). Various *in vitro* and *ex vivo* methods have been developed to analyze osteocyte functions (158). However, most of these methods are invasive and difficult to perform routinely in clinical setting. Non-invasive bone loading methods are available to analyze osteocyte functions in murine models (59, 159, 160). Future research is recommended to develop non-invasive approaches to analyze osteocyte mechanotransduction *in vivo*.

Recently, extracellular vesicles and exosomes are regarded as the key cargo-carrying organelles affecting the local and systemic cellular activities. Exosomes are released from living cells and carry miRNAs, circular RNAs, mRNAs, and various proteins from one cell to other cells. Osteocyte-derived exosomes detected in the circulation are enriched with osteocyte-specific miRNAs (161). A possible role of extracellular vesicles and exosomes in bone biology has been presented nicely in a recent review from Tao and Guo (162). Mechanically loaded osteocytes release exosomes with bone regenerating potential, via Ca^{2+} oscillation (19). Proteomic analysis of exosomes from cortical bone osteocytes provide a clear picture of osteocyte function in different disease conditions, including rare bone diseases (32). The osteocyte transcriptome is extensively deregulated in a mouse model of osteogenesis imperfecta (163). Transcriptome and proteomic analysis in osteocytic exosomes could unravel the role of exosomes in the pathophysiology of rare bone diseases. Recent advancements in RNA sequencing, functional analysis tools, and bioinformatic tools reveal a role of non-coding RNAs such as miRNAs, circular RNAs, piRNAs, and lncRNAs in various cellular signaling and biological activities including development and diseases (164–168). Various mRNAs and their translated proteins play a role in osteocyte function (36, 56). Only few studies address the role of non-coding RNAs in osteocyte function (161, 169, 170). Disruption of the Cx43/miR21 pathway results in osteocyte apoptosis and increases osteocyte-mediated osteoclastogenesis in old-age subjects (170). miR-29b-3p regulates osteogenic differentiation of precursor cells via modulating IGF1 secretion in mechanically loaded osteocytes (169). The role of circular RNAs, piRNAs, lncRNAs, and other miRNAs on osteocyte functions in physiological and disease conditions is poorly understood. The differential expression pattern of non-coding RNAs in osteocytes during rare bone diseases has not been investigated yet. Altered expression pattern of non-coding RNAs in osteocytes during rare bone diseases could play role in disease development and pathophysiology. We believe that this research direction could guide the development of new targets and techniques to analyze the function of osteocytes in patients.

THERAPIES TO IMPROVE OSTEOCYTE FUNCTION

Intermittent PTH therapy enhances bone regeneration and bone mineral density (171). PTH signaling affects the function of osteoblasts, osteoclasts, and osteocytes. Intermittent PTH treatment enhances the commitment of precursor cells to an osteogenic fate (172). PTH signaling in osteocytes regulates sclerostin expression and controls osteocyte-mediated osteoblastogenesis (58, 87, 173, 174). PTH treatment (teriparatide, PTH1-34) in osteogenesis imperfecta increases bone mineral density and vertebral strength (175, 176). PTH inhibits Notch signaling in osteoblasts and osteocytes, which might exert the anabolic effect on bone (177).

Studies on sclerostin deficiency-related high bone mass phenotype illustrate the role of sclerostin in bone biology guiding the development of anti-sclerostin bone anabolic agents. Anti-sclerostin monoclonal antibody has the potency to treat diseases with low bone mass phenotype, including osteoporosis (178, 179). There is increasing evidence suggesting a role of sclerostin in myeloma bone diseases and breast cancer bone metastasis-mediated complications (149, 180). In the bone niche, sclerostin is mainly produced by mature osteoblasts and osteocytes (181). Interestingly, multiple myeloma cells and breast cancer cells also produce sclerostin that might have a catabolic effect on bone (180, 181). Furthermore, cancer metastasis-induced inflammation upregulates osteocytic sclerostin that inhibits osteoblast function (181). Therefore, sclerostin monoclonal antibody could be beneficial to reduce myeloma and breast cancer-mediated complications in bone (182–184). Sclerostin antibody romosozumab clears a phase III trial with satisfactory outcomes and already got approval for osteoporosis treatment (185). This sclerostin antibody has shown promising potential to treat osteogenesis imperfecta (127, 186, 187). Therefore, romosozumab might be beneficial to treat rare bone disease patients with low bone mass phenotypes, such as osteogenesis imperfecta, Wnt1 mutation, and PLS3 mutation.

DKK1 is another potent Wnt inhibitor, that is also mainly produced by osteocytes in bone. Similar to sclerostin, DKK1 is also produced by breast, prostate, and multiple myeloma cancer cells (188–190). Increased levels of DKK1 in various cancers cause osteolytic bone disease and inhibit osteoblast function (188, 189). DKK1 is an osteocyte-specific target to treat osteoporosis and other low bone mass diseases (191). In systemic

inflammation, the neutralization of DKK1 reduces sclerostin expression and protects systemic bone loss (192). Monoclonal antibodies against DKK1 showed DKK1 inhibitory potential *in vitro* and increased bone mass *in vivo* (192). Moreover, a bispecific antibody targeting both sclerostin and DKK1 shows higher efficiency on bone formation and fracture repair (193). Phase I and phase II clinical trials have been performed to test the efficacy of anti-DKK1 antibodies on myeloma and myeloma-induced skeletal events (194, 195).

Studies on the role of osteocytic RANKL in bone homeostasis have led to the development of an anti-RANKL monoclonal antibody to treat common metabolic bone diseases, including osteoporosis (196, 197). During the last 10 years, the use of denosumab proved to be satisfactory with rare adverse effects (198). An imbalance in RANK-RANKL-OPG signaling is also observed in many rare bone diseases such as Juvenile Paget disease, fibrous dysplasia, Hajdu Cheney syndrome, and Langerhans cell histiocytosis (199). Therefore, denosumab has also been used off-label in rare metabolic bone diseases, including Paget's disease, osteogenesis imperfecta, and aneurysmal bone cysts (200). Bisphosphonate treatment prevents bone loss and fractures caused by rare bone disease-mediated osteogenesis imperfecta (201–203). Physical therapy/rehabilitation regimes in children with osteogenesis imperfecta improved mobility and bone mineral density, and thereby prevented fractures (175). Most treatment approaches for rare bone diseases directly act on osteoblast or osteoclast activity, and are symptomatic treatments.

The meticulous research on the molecular mechanism of osteocytic sclerostin on bone remodeling led to the development of anti-sclerostin antibodies to treat osteoporosis and other skeletal disorders demanding an increase in bone mass. Anti-sclerostin antibody primarily targets bone-lining cells, rather than the osteocytes imbedded in bone matrix (204). Anti-sclerostin antibody activates selected canonical Wnt target genes in a mature osteoblast subpopulation and increases bone formation (204). Sclerostin monoclonal antibody romosozumab treatment significantly increases bone mineral density in postmenopausal women with low bone mineral density and reduces fracture risk in postmenopausal women with osteoporosis (205). However, adverse side effects of a loss of sclerostin are osteoarthritis (206), TNF-dependent inflammatory joint destruction (207), negative effect on B cells (208), and risk of cardiac failure (205), which should be carefully evaluated before romosozumab treatment is considered. Although research on the cellular and molecular mechanisms of sclerosteosis and van Buchem disease guided the development of anti-sclerostin antibody to treat osteoporosis, an osteocyte function-targeted therapy for sclerosteosis and van Buchem disease has not yet been developed. Genetic disorders disrupt the expression of osteocytic proteins that play a role in the pathophysiology of various rare bone diseases (**Figure 2**). Since osteocyte functions play a crucial role in bone homeostasis, and since these functions are disrupted in many rare bone diseases, a better understanding of the molecular mechanisms of disrupted osteocyte functions in

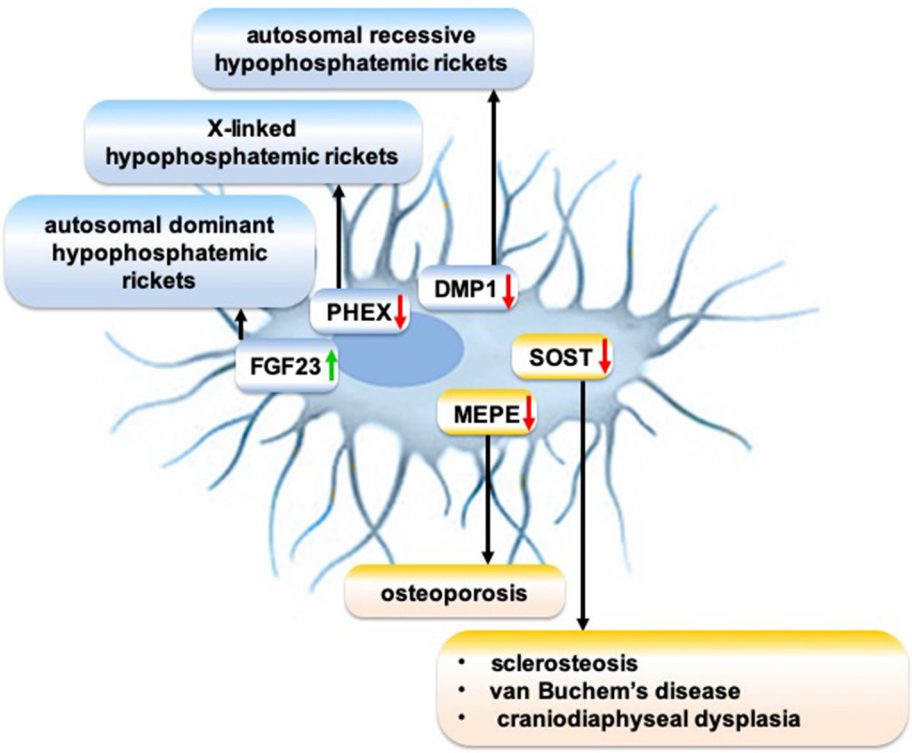


FIGURE 2 | Schematic showing the role of disrupted expression of osteocytic proteins on the pathophysiology of rare bone diseases. Green arrow: Gain-of-function mutation; red arrow: Loss-of-function mutation.

rare bone diseases may guide to discover novel targets to treat these rare bone diseases.

CONCLUSIONS

Genetic and pathophysiological research on three rare bone diseases, i.e., sclerosteosis, pycnodysostosis, and van Buchem disease, provided new effective interventions to treat osteoporosis. The current available therapeutic approaches for rare bone diseases are symptomatic and mainly target osteoblast and osteoclast formation and activity. Since osteocytes play a vital role in bone homeostasis, and because their function is disrupted in many rare bone diseases, it would be wise to focus on unraveling the osteocyte-specific targets to treat rare bone diseases. The role of coding RNAs (mRNAs) in osteocyte function during pathophysiological conditions has been widely investigated. Non-coding RNAs (piRNAs, circRNAs, lncRNAs, shRNAs, etc.) represent 97% of the total RNA in the cell, and recent technological advances have unveiled a crucial role of non-coding RNAs in various biological processes including bone homeostasis. Therefore, meticulous research focusing on the role of non-coding RNAs in osteocyte functions under physiological conditions and in various bone diseases including rare bone diseases could be the future research direction. The results of this research could provide clues for the discovery of novel osteocyte-specific targets to treat rare bone diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by a Project of Education of Guangdong Province, China (2017KQNCX162) and High-Level University Construction Talents of Guangzhou Medical University (B185006003014 and B19502003017).

ACKNOWLEDGMENTS

The authors thank Wei Cao for help with the illustrations.

REFERENCES

1. Bonewald LF. The amazing osteocyte. *J Bone Miner Res.* (2011) 26:229–38. doi: 10.1002/jbmr.320
2. Bonewald LF. The role of the osteocyte in bone and nonbone disease. *Endocrinol Metab Clin North Am.* (2017) 46:1–18. doi: 10.1016/j.ecl.2016.09.003
3. Chen H, Senda T, Kubo KY. The osteocyte plays multiple roles in bone remodeling and mineral homeostasis. *Med Mol Morphol.* (2015) 48:61–8. doi: 10.1007/s00795-015-0099-y
4. Xiong J, O'Brien CA. Osteocyte RANKL: new insights into the control of bone remodeling. *J Bone Miner Res.* (2012) 27:499–505. doi: 10.1002/jbmr.1547
5. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med.* (2011) 17:1235–41. doi: 10.1038/nm.2448
6. Xiong J, Piemontese M, Onal M, Campbell J, Goellner JJ, Dusevich V, et al. Osteocytes, not osteoblasts or lining cells, are the main source of the RANKL required for osteoclast formation in remodeling bone. *PLoS ONE.* (2015) 10:e0138189. doi: 10.1371/journal.pone.0138189
7. Buenzli PR, Sims NA. Quantifying the osteocyte network in the human skeleton. *Bone.* (2015) 75:144–50. doi: 10.1016/j.bone.2015.02.016
8. Pead MJ, Suswillo R, Skerry TM, Vedi S, Lanyon LE. Increased 3H-uridine levels in osteocytes following a single short period of dynamic bone loading *in vivo*. *Calcif Tissue Int.* (1988) 43:92–6. doi: 10.1007/BF02555153
9. Thi MM, Suadicani SO, Schaffler MB, Weinbaum S, Spray DC. Mechanosensory responses of osteocytes to physiological forces occur along processes and not cell body and require alphaVbeta3 integrin. *Proc Natl Acad Sci USA.* (2013) 110:21012–7. doi: 10.1073/pnas.1321210110
10. Bacabac RG, Smit TH, Van Loon JJWA, Zandieh Doulabi B, Helder MN, Klein-Nulend J. Bone cell responses to high-frequency vibration stress: does the nucleus oscillate within the cytoplasm? *FASEB J.* (2006) 20:858–64. doi: 10.1096/fj.05-4966.com
11. Burger EH, Klein-Nulend J. Microgravity and bone cell mechanosensitivity. *Bone.* (1998) 22(Suppl. 5):127–30S. doi: 10.1016/S8756-3282(98)00010-6
12. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, et al. Sensitivity of osteocytes to biomechanical stress *in vitro*. *FASEB J.* (1995) 9:441–5. doi: 10.1096/fasebj.9.5.7896017
13. Klein-Nulend J, Bacabac RG, Bakker AD. Mechanical loading and how it affects bone cells: the role of the osteocyte cytoskeleton in maintaining our skeleton. *Eur Cells Mater.* (2012) 24:278–91. doi: 10.22203/eCM.v024a20
14. Vatsa A, Smit TH, Klein-Nulend J. Extracellular NO signalling from a mechanically stimulated osteocyte. *J Biomech.* (2007) 40:S89–95. doi: 10.1016/j.jbiomech.2007.02.015
15. Klein-Nulend J, Bakker AD, Bacabac RG, Vatsa A, Weinbaum S. Mechanosensation and transduction in osteocytes. *Bone.* (2013) 54:182–90. doi: 10.1016/j.bone.2012.10.013
16. Hung CT, Allen FD, Pollack SR, Brighton CT. Intracellular Ca2+ stores and extracellular Ca2+ are required in the real-time Ca2+ response of bone cells experiencing fluid flow. *J Biomech.* (1996) 29:1411–7. doi: 10.1016/0021-9290(96)84536-2
17. Lewis KJ, Frikha-Benayed D, Louie J, Stephen S, Spray DC, Thi MM, et al. Osteocyte calcium signals encode strain magnitude and loading frequency *in vivo*. *Proc Natl Acad Sci USA.* (2017) 114:11775–80. doi: 10.1073/pnas.1707863114
18. Santos A, Bakker AD, Klein-Nulend J. The role of osteocytes in bone mechanotransduction. *Osteoporos Int.* (2009) 20:1027–31. doi: 10.1007/s00198-009-0858-5
19. Morrell AE, Brown GN, Robinson ST, Sattler RL, Baik AD, Zhen G, et al. Mechanically induced Ca(2+) oscillations in osteocytes release extracellular vesicles and enhance bone formation. *Bone Res.* (2018) 6:6. doi: 10.1038/s41413-018-0007-x
20. Zaman G, Pitsillides AA, Rawlinson SC, Suswillo RF, Mosley JR, Cheng MZ, et al. Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. *J Bone Miner Res.* (1999) 14:1123–31. doi: 10.1359/jbmr.1999.14.7.1123
21. Vatsa A, Mizuno D, Smit TH, Schmidt CF, MacKintosh FC, Klein-Nulend J. Bio imaging of intracellular NO production in single bone cells after mechanical stimulation. *J Bone Miner Res.* (2006) 21:1722–8. doi: 10.1359/jbmr.060720
22. Klein-Nulend J, van Oers RF, Bakker AD, Bacabac RG. Nitric oxide signaling in mechanical adaptation of bone. *Osteoporos Int.* (2014) 25:1427–37. doi: 10.1007/s00198-013-2590-4
23. Ajubi NE, Klein-Nulend J, Alblas MJ, Burger EH, Nijweide PJ. Signal transduction pathways involved in fluid flow-induced PGE2 production by cultured osteocytes. *Am J Physiol.* (1999) 276:E171–8. doi: 10.1152/ajpendo.1999.276.1.E171

24. Lean JM, Jagger CJ, Chambers TJ, Chow JW. Increased insulin-like growth factor I mRNA expression in rat osteocytes in response to mechanical stimulation. *Am J Physiol.* (1995) 268(Pt 1):E318–27. doi: 10.1152/ajpendo.1995.268.2.E318
25. Kamel MA, Picconi JL, Lara-Castillo N, Johnson ML. Activation of beta-catenin signaling in MLO-Y4 osteocytic cells versus 2T3 osteoblastic cells by fluid flow shear stress and PGE2: implications for the study of mechanosensation in bone. *Bone.* (2010) 47:872–81. doi: 10.1016/j.bone.2010.08.007
26. Xiao Z, Dallas M, Qiu N, Nicolella D, Cao L, Johnson M, et al. Conditional deletion of Pkd1 in osteocytes disrupts skeletal mechanosensing in mice. *FASEB J.* (2011) 25:2418–32. doi: 10.1096/fj.10-180299
27. Santos A, Bakker AD, Zandieh-Doulabi B, de Blieck-Hogervorst JMA, Klein-Nulend J. Early activation of the beta-catenin pathway in osteocytes is mediated by nitric oxide, phosphatidyl inositol-3 kinase/Akt, and focal adhesion kinase. *Biochem Bioph Res Commun.* (2010) 391:364–9. doi: 10.1016/j.bbrc.2009.11.064
28. Geoghegan IP, Hoey DA, McNamara LM. Integrins in osteocyte biology and mechanotransduction. *Curr Osteoporos Rep.* (2019) 17:195–206. doi: 10.1007/s11914-019-00520-2
29. Geoghegan IP, Hoey DA, McNamara LM. Estrogen deficiency impairs integrin alphavbeta3-mediated mechanosensation by osteocytes and alters osteoclastogenic paracrine signalling. *Sci Rep.* (2019) 9:4654. doi: 10.1038/s41598-019-41095-3
30. Vatsa A, Semeins CM, Smit TH, Klein-Nulend J. Paxillin localisation in osteocytes–is it determined by the direction of loading? *Biochem Biophys Res Commun.* (2008) 377:1019–24. doi: 10.1016/j.bbrc.2007.12.174
31. Pathak JL, Bakker AD, Luyten FP, Verschueren P, Lems WF, Klein-Nulend J, et al. Systemic inflammation affects human osteocyte-specific protein and cytokine expression. *Calcif Tissue Int.* (2016) 98:596–608. doi: 10.1007/s00223-016-0116-8
32. Zhang C, Xu S, Zhang S, Liu M, Du H, Sun R, et al. Ageing characteristics of bone indicated by transcriptomic and exosomal proteomic analysis of cortical bone cells. *J Orthop Surg Res.* (2019) 14:129. doi: 10.1186/s13018-019-1163-4
33. Shah KM, Stern MM, Stern AR, Pathak JL, Bravenboer N, Bakker AD. Osteocyte isolation and culture methods. *Bonekey Rep.* (2016) 5:838. doi: 10.1038/bonekey.2016.65
34. Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell and more. *Endocr Rev.* (2013) 34:658–90. doi: 10.1210/er.2012-1026
35. Dussold C, Gerber C, White S, Wang X, Qi L, Francis C, et al. DMP1 prevents osteocyte alterations, FGF23 elevation and left ventricular hypertrophy in mice with chronic kidney disease. *Bone Res.* (2019) 7:12. doi: 10.1038/s41413-019-0051-1
36. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, et al. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet.* (2001) 10:537–43. doi: 10.1093/hmg/10.5.537
37. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, et al. Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *J Med Genet.* (2002) 39:91–7. doi: 10.1136/jmg.39.2.91
38. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med.* (2013) 19:179–92. doi: 10.1038/nm.3074
39. Goldsweig BK, Carpenter TO. Hypophosphatemic rickets: lessons from disrupted FGF23 control of phosphorus homeostasis. *Curr Osteoporos Rep.* (2015) 13:88–97. doi: 10.1007/s11914-015-0259-y
40. Pavone V, Testa G, Gioitta Iachino S, Evola FR, Avondo S, Sessa G. Hypophosphatemic rickets: etiology, clinical features and treatment. *Eur J Orthop Surg Traumatol.* (2015) 25:221–6. doi: 10.1007/s00590-014-1496-y
41. Zhou M, Li S, Pathak JL. Pro-inflammatory cytokines and osteocytes. *Curr Osteoporos Rep.* (2019) 17:97–104. doi: 10.1007/s11914-019-00507-z
42. Bakker AD, Klein-Nulend J, Tanck E, Heyligers IC, Albers GH, Lips P, et al. Different responsiveness to mechanical stress of bone cells from osteoporotic versus osteoarthritic donors. *Osteoporos Int.* (2006) 17:827–33. doi: 10.1007/s00198-006-0072-7
43. Cui Y, Niziolek PJ, MacDonald BT, Zylstra CR, Alenina N, Robinson DR, et al. Lrp5 functions in bone to regulate bone mass. *Nat Med.* (2011) 17:684–91. doi: 10.1038/nm.2388
44. Bullock WA, Pavalko FM, Robling AG. Osteocytes and mechanical loading: the Wnt connection. *Orthod Craniofac Res.* (2019) 22(Suppl. 1):175–9. doi: 10.1111/ocr.12282
45. Tu X, Delgado-Calle J, Condon KW, Maycas M, Zhang H, Carlesso N, et al. Osteocytes mediate the anabolic actions of canonical Wnt/beta-catenin signaling in bone. *Proc Natl Acad Sci USA.* (2015) 112:E478–86. doi: 10.1073/pnas.1409857112
46. Lara-Castillo N, Kim-Weroha NA, Kamel MA, Javaheri B, Ellies DL, Krumlauf RE, et al. *In vivo* mechanical loading rapidly activates beta-catenin signaling in osteocytes through a prostaglandin mediated mechanism. *Bone.* (2015) 76:58–66. doi: 10.1016/j.bone.2015.03.019
47. Klein-Nulend J, Burger EH, Semeins CM, Raisz LG, Pilbeam CC. Pulsating fluid flow stimulates prostaglandin release and inducible prostaglandin G/H synthase mRNA expression in primary mouse bone cells. *J Bone Miner Res.* (1997) 12:45–51. doi: 10.1359/jbmr.1997.12.1.45
48. Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, et al. The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. *J Biol Chem.* (2006) 281:23698–711. doi: 10.1074/jbc.M601000200
49. Saxon LK, Jackson BF, Sugiyama T, Lanyon LE, Price JS. Analysis of multiple bone responses to graded strains above functional levels, and to disuse, in mice *in vivo* show that the human Lrp5 G171V High Bone Mass mutation increases the osteogenic response to loading but that lack of Lrp5 activity reduces it. *Bone.* (2011) 49:184–93. doi: 10.1016/j.bone.2011.03.683
50. Zhao L, Shim JW, Dodge TR, Robling AG, Yokota H. Inactivation of Lrp5 in osteocytes reduces young's modulus and responsiveness to the mechanical loading. *Bone.* (2013) 54:35–43. doi: 10.1016/j.bone.2013.01.033
51. Kampe AJ, Makitie RE, Makitie O. New genetic forms of childhood-onset primary osteoporosis. *Horm Res Paediatr.* (2015) 84:361–9. doi: 10.1159/000439566
52. Gardinier JD, Al-Omaishi S, Morris MD, Kohn DH. PTH signaling mediates perilacunar remodeling during exercise. *Matrix Biol.* (2016) 52–54:162–75. doi: 10.1016/j.matbio.2016.02.010
53. Camirand A, Goltzman D, Gupta A, Kaouass M, Panda D, Karaplis A. The role of Parathyroid Hormone-Related Protein (PTHrP) in osteoblast response to microgravity: mechanistic implications for osteoporosis development. *PLoS ONE.* (2016) 11:e0160034. doi: 10.1371/journal.pone.0160034
54. Clarke BL. Bone disease in hypoparathyroidism. *Arq Bras Endocrinol Metabol.* (2014) 58:545–52. doi: 10.1590/0004-2730000003399
55. Reijnders CM, Bravenboer N, Tromp AM, Blankenstein MA, Lips P. Effect of mechanical loading on insulin-like growth factor-I gene expression in rat tibia. *J Endocrinol.* (2007) 192:131–40. doi: 10.1677/joe.1.06880
56. Lau KHW, Baylink DJ, Zhou XD, Rodriguez D, Bonewald LF, Li ZH, et al. Osteocyte-derived insulin-like growth factor I is essential for determining bone mechanosensitivity. *Am J Physiol Endocr Metab.* (2013) 305:E271–81. doi: 10.1152/ajpendo.00092.2013
57. Saini V, Marengi DA, Barry KJ, Fulzele KS, Heiden E, Liu X, et al. Parathyroid hormone (PTH)/PTH-related peptide type 1 receptor (PPR) signaling in osteocytes regulates anabolic and catabolic skeletal responses to PTH. *J Biol Chem.* (2013) 288:20122–34. doi: 10.1074/jbc.M112.441360
58. Rhee Y, Allen MR, Condon K, Lezcano V, Ronda AC, Galli C, et al. PTH receptor signaling in osteocytes governs periosteal bone formation and intracortical remodeling. *J Bone Miner Res.* (2011) 26:1035–46. doi: 10.1002/jbmr.304
59. Gross TS, Srinivasan S, Liu CC, Clemens TL, Bain SD. Noninvasive loading of the murine tibia: an *in vivo* model for the study of mechanotransduction. *J Bone Miner Res.* (2002) 17:493–501. doi: 10.1359/jbmr.2002.17.3.493
60. Kesavan C, Wergedal JE, Lau KH, Mohan S. Conditional disruption of IGF-I gene in type 1alpha collagen-expressing cells shows an essential role of IGF-I in skeletal anabolic response to loading. *Am J Physiol Endocrinol Metab.* (2011) 301:E1191–7. doi: 10.1152/ajpendo.00440.2011
61. Sakata T, Wang Y, Halloran BP, Elalieh HZ, Cao J, Bikle DD. Skeletal unloading induces resistance to insulin-like growth factor-I (IGF-I) by

inhibiting activation of the IGF-I signaling pathways. *J Bone Miner Res.* (2004) 19:436–46. doi: 10.1359/JBMR.0301241
62. Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J. Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone.* (2007) 41:745–51. doi: 10.1016/j.bone.2007.07.019
63. Pathak JL, Bravenboer N, Luyten FP, Verschueren P, Lems WF, Klein-Nulend J, et al. Mechanical loading reduces inflammation-induced human osteocyte-to-osteoclast communication. *Calcif Tissue Int.* (2015) 97:169–78. doi: 10.1007/s00223-015-9999-z
64. You L, Temiyasathit S, Lee P, Kim CH, Tummala P, Yao W, et al. Osteocytes as mechanosensors in the inhibition of bone resorption due to mechanical loading. *Bone.* (2008) 42:172–9. doi: 10.1016/j.bone.2007.09.047
65. Sanchez C, Gabay O, Salvat C, Henrotin YE, Berenbaum F. Mechanical loading highly increases IL-6 production and decreases OPG expression by osteoblasts. *Osteoarthritis Cartilage.* (2009) 17:473–81. doi: 10.1016/j.joca.2008.09.007
66. Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J. Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. *Biochem Biophys Res Commun.* (2006) 348:1082–8. doi: 10.1016/j.bbrc.2006.07.146
67. Tsourdi E, Jahn K, Rauner M, Busse B, Bonewald LF. Physiological and pathological osteocytic osteolysis. *J Musculoskelet Neuronal Interact.* (2018) 18:292–303.
68. Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF, et al. Sclerostin regulates release of bone mineral by osteocytes by induction of carbonic anhydrase 2. *J Bone Miner Res.* (2013) 28:2436–48. doi: 10.1002/jbmr.2003
69. Gelb BD, Edelson JG, Desnick RJ. Linkage of pycnodysostosis to chromosome 1q21 by homozygosity mapping. *Nat Genet.* (1995) 10:235–7. doi: 10.1038/ng0695-235
70. Bonnet N, Brun J, Rousseau JC, Duong LT, Ferrari SL. Cathepsin K controls cortical bone formation by degrading periostin. *J Bone Miner Res.* (2017) 32:1432–41. doi: 10.1002/jbmr.3136
71. Fujii O, Tatsumi S, Ogata M, Arakaki T, Sakaguchi H, Nomura K, et al. Effect of osteocyte-ablation on inorganic phosphate metabolism: analysis of bone-kidney-gut axis. *Front Endocrinol.* (2017) 8:359. doi: 10.3389/fendo.2017.00359
72. Tresguerres FGF, Torres J, Lopez-Quiles J, Hernandez G, Vega JA, Tresguerres IF. The osteocyte: a multifunctional cell within the bone. *Ann Anat.* (2020) 227:151422. doi: 10.1016/j.aanat.2019.151422
73. Quarles LD. FGF23, PHEX, and MEPE regulation of phosphate homeostasis and skeletal mineralization. *Am J Physiol Endocrinol Metab.* (2003) 285:E1–9. doi: 10.1152/ajpendo.00016.2003
74. Sapir-Koren R, Livshits G. Osteocyte control of bone remodeling: is sclerostin a key molecular coordinator of the balanced bone resorption-formation cycles? *Osteoporos Int.* (2014) 25:2685–700. doi: 10.1007/s00198-014-2808-0
75. Ryan ZC, Ketha H, McNulty MS, McGee-Lawrence M, Craig TA, Grande JP, et al. Sclerostin alters serum vitamin D metabolite and fibroblast growth factor 23 concentrations and the urinary excretion of calcium. *Proc Natl Acad Sci USA.* (2013) 110:6199–204. doi: 10.1073/pnas.1221255110
76. Martin A. Bone and heart health in chronic kidney disease: role of dentin matrix protein 1. *Curr Opin Nephrol Hypertens.* (2019) 28:297–303. doi: 10.1097/MNH.0000000000000512
77. David V, Martin A, Hedge AM, Rowe PSN. Matrix Extracellular Phosphoglycoprotein (MEPE) is a new bone renal hormone and vascularization modulator. *Endocrinology.* (2009) 150:4012–23. doi: 10.1210/en.2009-0216
78. Nampei A, Hashimoto J, Hayashida K, Tsuboi H, Shi K, Tsuji I, et al. Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. *J Bone Miner Metab.* (2004) 22:176–84. doi: 10.1007/s00774-003-0468-9
79. Zelenchuk LV, Hedge AM, Rowe PS. Age dependent regulation of bone-mass and renal function by the MEPE ASARM-motif. *Bone.* (2015) 79:131–42. doi: 10.1016/j.bone.2015.05.030
80. Tiede-Lewis LM, Dallas SL. Changes in the osteocyte lacunocanalicular network with aging. *Bone.* (2019) 122:101–13. doi: 10.1016/j.bone.2019.01.025
81. Busse B, Djonic D, Milovanovic P, Hahn M, Puschel K, Ritchie RO, et al. Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveals failure and delay of remodeling in aged human bone. *Aging Cell.* (2010) 9:1065–75. doi: 10.1111/j.1474-9726.2010.00633.x
82. Hemmatian H, Bakker AD, Klein-Nulend J, van Lenthe GH. Aging, osteocytes, and mechanotransduction. *Curr Osteoporos Rep.* (2017) 15:401–11. doi: 10.1007/s11914-017-0402-z
83. Rolvien T, Schmidt FN, Milovanovic P, Jahn K, Riedel C, Butscheidt S, et al. Early bone tissue aging in human auditory ossicles is accompanied by excessive hypermineralization, osteocyte death and micropetrosis. *Sci Rep.* (2018) 8:1920. doi: 10.1038/s41598-018-19803-2
84. Tiede-Lewis LM, Xie Y, Hulbert MA, Campos R, Dallas MR, Dusevich V, et al. Degeneration of the osteocyte network in the C57BL/6 mouse model of aging. *Aging.* (2017) 9:2190–208. doi: 10.18632/aging.101308
85. Burger EH, Klein-Nulend J. Mechanotransduction in bone–role of the lacuno-canalicular network. *FASEB J.* (1999) 13:S101–12. doi: 10.1096/fasebj.13.9001.s101
86. Kim H, Wrann CD, Jedrychowski M, Vidoni S, Kitase Y, Nagano K, et al. Irisin mediates effects on bone and fat via alphav integrin receptors. *Cell.* (2018) 175:1756–68 e17. doi: 10.1016/j.cell.2018.10.025
87. Bellido T, Plotkin LI. Novel actions of bisphosphonates in bone: preservation of osteoblast and osteocyte viability. *Bone.* (2011) 49:50–5. doi: 10.1016/j.bone.2010.08.008
88. Jilka RL, Noble B, Weinstein RS. Osteocyte apoptosis. *Bone.* (2013) 54:264–71. doi: 10.1016/j.bone.2012.11.038
89. Jilka RL, O'Brien CA. The role of osteocytes in age-related bone loss. *Curr Osteoporos Rep.* (2016) 14:16–25. doi: 10.1007/s11914-016-0297-0
90. Chen H, Liu W, Wu X, Gou M, Shen J, Wang H. Advanced glycation end products induced IL-6 and VEGF-A production and apoptosis in osteocyte-like MLO-Y4 cells by activating RAGE and ERK1/2, P38 and STAT3 signalling pathways. *Int Immunopharmacol.* (2017) 52:143–9. doi: 10.1016/j.intimp.2017.09.004
91. Tanaka K, Yamaguchi T, Kanazawa I, Sugimoto T. Effects of high glucose and advanced glycation end products on the expressions of sclerostin and RANKL as well as apoptosis in osteocyte-like MLO-Y4-A2 cells. *Biochem Biophys Res Commun.* (2015) 461:193–9. doi: 10.1016/j.bbrc.2015.02.091
92. Pacios S, Xiao W, Mattos M, Lim J, Tarapore RS, Alsadun S, et al. Osteoblast lineage cells play an essential role in periodontal bone loss through activation of nuclear factor-kappa B. *Sci Rep.* (2015) 5:16694. doi: 10.1038/srep16694
93. Graves DT, Alshabab A, Albiero ML, Mattos M, Correa JD, Chen SS, et al. Osteocytes play an important role in experimental periodontitis in healthy and diabetic mice through expression of RANKL. *J Clin Periodontol.* (2018) 45:285–92. doi: 10.1111/jcpe.12851
94. Kim JH, Kim AR, Choi YH, Jang S, Woo GH, Cha JH, et al. Tumor necrosis factor-alpha antagonist diminishes osteocytic RANKL and sclerostin expression in diabetes rats with periodontitis. *PLoS ONE.* (2017) 12:e0189702. doi: 10.1371/journal.pone.0189702
95. Kim JH, Lee DE, Woo GH, Cha JH, Bak EJ, Yoo YJ. Osteocytic sclerostin expression in alveolar bone in rats with diabetes mellitus and ligature-induced periodontitis. *J Periodontol.* (2015) 86:1005–11. doi: 10.1902/jop.2015.150083
96. Pesce Viglietti AI, Arriola Benitez PC, Gentilini MV, Velasquez LN, Fossati CA, Giambartolomei GH, et al. *Brucella abortus* invasion of osteocytes modulates connexin 43 and integrin expression and induces osteoclastogenesis via receptor activator of NF-kappaB ligand and tumor necrosis factor alpha secretion. *Infect Immun.* (2016) 84:11–20. doi: 10.1128/IAI.01049-15
97. Delgado-Calle J, Bellido T, Roodman GD. Role of osteocytes in multiple myeloma bone disease. *Curr Opin Support Palliat Care.* (2014) 8:407–13. doi: 10.1097/SPC.0000000000000090
98. Morena M, Jaussent I, Dupuy AM, Bargnoux AS, Kuster N, Chenine L, et al. Osteoprotegerin and sclerostin in chronic kidney disease prior to dialysis: potential partners in vascular calcifications. *Nephrol Dial Transplant.* (2015) 30:1345–56. doi: 10.1093/ndt/gfv081
99. van Bezooijen RL, Bronckers AL, Gortzak RA, Hogendoorn PC, van der Wee-Pals L, Balemans W, et al. Sclerostin in mineralized matrices and van Buchem disease. *J Dent Res.* (2009) 88:569–74. doi: 10.1177/0022034509338340

100. Sebastian A, Loots GG. Genetics of Sost/SOST in sclerosteosis and van Buchem disease animal models. *Metabolism.* (2018) 80:38–47. doi: 10.1016/j.metabol.2017.10.005
101. Hamersma H, Gardner J, Beighton P. The natural history of sclerosteosis. *Clin Genet.* (2003) 63:192–7. doi: 10.1034/j.1399-0004.2003.00036.x
102. Van Buchem FS, Hadders HN, Ubbens R. An uncommon familial systemic disease of the skeleton: hyperostosis corticalis generalisata familiaris. *Acta radiol.* (1955) 44:109–20. doi: 10.3109/00016925509170789
103. Van Hul W, Balemans W, Van Hul E, Dikkers FG, Obee H, Stokroos RJ, et al. Van Buchem disease (hyperostosis corticalis generalisata) maps to chromosome 17q12-q21. *Am J Hum Genet.* (1998) 62:391–9. doi: 10.1086/301721
104. Beighton P, Barnard A, Hamersma H, van der Wouden A. The syndromic status of sclerosteosis and van Buchem disease. *Clin Genet.* (1984) 25:175–81. doi: 10.1111/j.1399-0004.1984.tb00481.x
105. Appelman-Dijkstra NM, Papapoulos SE. From disease to treatment: from rare skeletal disorders to treatments for osteoporosis. *Endocrine.* (2016) 52:414–26. doi: 10.1007/s12020-016-0888-7
106. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW, et al. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J.* (2005) 19:1842–4. doi: 10.1096/fj.05-4221fje
107. Case N, Rubin J. beta-catenin-a supporting role in the skeleton. *J Cell Biochem.* (2010) 110:545–53. doi: 10.1002/jcb.22574
108. van Bezooijen RL, ten Dijke P, Papapoulos SE, Lowik CW. SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytok Growth Factor Rev.* (2005) 16:319–27. doi: 10.1016/j.cytogfr.2005.02.005
109. Delgado-Calle J, Sato AY, Bellido T. Role and mechanism of action of sclerostin in bone. *Bone.* (2017) 96:29–37. doi: 10.1016/j.bone.2016.10.007
110. Shanbhogue VV, Hansen S, Folkestad L, Brixen K, Beck-Nielsen SS. Bone geometry, volumetric density, microarchitecture, and estimated bone strength assessed by HR-pQCT in adult patients with hypophosphatemic rickets. *J Bone Miner Res.* (2015) 30:176–83. doi: 10.1002/jbmr.2310
111. Lambert AS, Linglart A. Hypocalcaemic and hypophosphatemic rickets. *Best Pract Res Clin Endocrinol Metab.* (2018) 32:455–76. doi: 10.1016/j.beem.2018.05.009
112. Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, et al. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet.* (2006) 38:1310–5. doi: 10.1038/ng1905
113. Linglart A, Biosse-Duplan M, Briot K, Chaussain C, Esterle L, Guillaume-Czitrom S, et al. Therapeutic management of hypophosphatemic rickets from infancy to adulthood. *Endocr Connect.* (2014) 3:R13–30. doi: 10.1530/EC-13-0103
114. Carpenter TO, Whyte MP, Imel EA, Boot AM, Hogler W, Linglart A, et al. Burosumab therapy in children with x-linked hypophosphatemia. *N Engl J Med.* (2018) 378:1987–98. doi: 10.1056/NEJMoa1714641
115. Laine CM, Joeng KS, Campeau PM, Kiviranta R, Tarkkonen K, Grover M, et al. WNT1 mutations in early-onset osteoporosis and osteogenesis imperfecta. *N Engl J Med.* (2013) 368:1809–16. doi: 10.1056/NEJMoa1215458
116. Shinomiya H. Plastin family of actin-bundling proteins: its functions in leukocytes, neurons, intestines, and cancer. *Int J Cell Biol.* (2012) 2012:213492. doi: 10.1155/2012/213492
117. Kamioka H, Sugawara Y, Honjo T, Yamashiro T, Takano-Yamamoto T. Terminal differentiation of osteoblasts to osteocytes is accompanied by dramatic changes in the distribution of actin-binding proteins. *J Bone Miner Res.* (2004) 19:471–8. doi: 10.1359/JBMR.040128
118. Kim JM, Kim J, Kim YH, Kim KT, Ryu SH, Lee TG, et al. Comparative secretome analysis of human bone marrow-derived mesenchymal stem cells during osteogenesis. *J Cell Physiol.* (2013) 228:216–24. doi: 10.1002/jcp.24123
119. Laine CM, Wessman M, Toiviainen-Salo S, Kaunisto MA, Mayranpaa MK, Laine T, et al. A novel splice mutation in PLS3 causes X-linked early onset low-turnover osteoporosis. *J Bone Miner Res.* (2015) 30:510–8. doi: 10.1002/jbmr.2355
120. Boudin E, Fijalkowski I, Hendrickx G, Van Hul W. Genetic control of bone mass. *Mol Cell Endocrinol.* (2016) 432:3–13. doi: 10.1016/j.mce.2015.12.021
121. Wesseling-Perry K, Makitie RE, Valimaki VV, Laine T, Laine CM, Valimaki MJ, et al. Osteocyte protein expression is altered in low-turnover osteoporosis caused by mutations in WNT1 and PLS3. *J Clin Endocr Metab.* (2017) 102:2340–8. doi: 10.1210/jc.2017-00099
122. Joeng KS, Lee YC, Lim J, Chen Y, Jiang MM, Munivez E, et al. Osteocyte-specific WNT1 regulates osteoblast function during bone homeostasis. *J Clin Invest.* (2017) 127:2678–88. doi: 10.1172/JCI92617
123. Yang X, Sun, LW, Liang M, Wang X, Fan Y-B. The response of wnt/ß-catenin signaling pathway in osteocytes under simulated microgravity. *Micrograv Sci Technol.* (2015) 27:473–83. doi: 10.1007/s12217-015-9439-8
124. Neugebauer J, Heilig J, Hosseinibarkooie S, Ross BC, Mendoza-Ferreira N, Nolte F, et al. Plastin 3 influences bone homeostasis through regulation of osteoclast activity. *Human Mol Genet.* (2018) 27:4249–62. doi: 10.1093/hmg/ddy318
125. Yorgan TA, Sari H, Rolvien T, Windhorst S, Failla AV, Kornak U, et al. Mice lacking plastin-3 display a specific defect of cortical bone acquisition. *Bone.* (2020) 130:115062. doi: 10.1016/j.bone.2019.115062
126. Martin E, Shapiro JR. Osteogenesis imperfecta:epidemiology and pathophysiology. *Curr Osteoporos Rep.* (2007) 5:91–7. doi: 10.1007/s11914-007-0023-z
127. Tauer JT, Robinson ME, Rauch F. Osteogenesis imperfecta: new perspectives from clinical and translational research. *JBMR Plus.* (2019) 3:e10174. doi: 10.1002/jbm4.10174
128. Morello R. Osteogenesis imperfecta and therapeutics. *Matrix Biol.* (2018) 71–72:294–312. doi: 10.1016/j.matbio.2018.03.010
129. Pyott SM, Tran TT, Leistritz DF, Pepin MG, Mendelsohn NJ, Temme RT, et al. WNT1 mutations in families affected by moderately severe and progressive recessive osteogenesis imperfecta. *Am J Hum Genet.* (2013) 92:590–7. doi: 10.1016/j.ajhg.2013.02.009
130. Arman A, Bereket A, Coker A, Kiper PO, Guran T, Ozkan B, et al. Cathepsin K analysis in a pycnodysostosis cohort: demographic, genotypic and phenotypic features. *Orphanet J Rare Dis.* (2014) 9:60. doi: 10.1186/1750-1172-9-60
131. Maroteaux P, Lamy M. [2 cases of a condensing osseous disease: pynodysostosis]. *Arch Fr Pediatr.* (1962) 19:267–74.
132. Vaaraniemi J, Halleen JM, Kaarlonen K, Ylipahkala H, Alatalo SL, Andersson G, et al. Intracellular machinery for matrix degradation in bone-resorbing osteoclasts. *J Bone Miner Res.* (2004) 19:1432–40. doi: 10.1359/JBMR.040603
133. Motyckova G, Fisher DE. Pycnodysostosis: role and regulation of cathepsin K in osteoclast function and human disease. *Curr Mol Med.* (2002) 2:407–21. doi: 10.2174/1566524023362401
134. Mandelin J, Hukkanen M, Li TF, Korhonen M, Liljestrom M, Sillat T, et al. Human osteoblasts produce cathepsin K. *Bone.* (2006) 38:769–77. doi: 10.1016/j.bone.2005.10.017
135. Lotinun S, Ishihara Y, Nagano K, Kiviranta R, Carpentier VT, Neff L, et al. Cathepsin K-deficient osteocytes prevent lactation-induced bone loss and parathyroid hormone suppression. *J Clin Invest.* (2019) 129:3058–71. doi: 10.1172/JCI122936
136. Lotinun S, Kiviranta R, Matsubara T, Alzate JA, Neff L, Luth A, et al. Osteoclast-specific cathepsin K deletion stimulates S1P-dependent bone formation. *J Clin Invest.* (2013) 123:666–81. doi: 10.1172/JCI64840
137. Ryu J, Kim HJ, Chang EJ, Huang H, Banno Y, Kim HH. Sphingosine 1-phosphate as a regulator of osteoclast differentiation and osteoclast-osteoblast coupling. *EMBO J.* (2006) 25:5840–51. doi: 10.1038/sj.emboj.7601430
138. Zhang JN, Zhao Y, Liu C, Han ES, Yu X, Lidington D, et al. The role of the sphingosine-1-phosphate signaling pathway in osteocyte mechanotransduction. *Bone.* (2015) 79:71–8. doi: 10.1016/j.bone.2015.05.017
139. Bonnet N, Garnero P, Ferrari S. Periostin action in bone. *Mol Cell Endocrinol.* (2016) 432:75–82. doi: 10.1016/j.mce.2015.12.014
140. Blouin S, Roschger A, Varga F, Misof B, Spitzer S, Roschger P, et al. Confocal laser scanning microscopy-a powerful tool in bone research. *Wien Med Wochenschr.* (2018) 168:314–21. doi: 10.1007/s10354-018-0639-x
141. Boyde A. Scanning electron microscopy of bone. *Methods Mol Biol.* (2019) 1914:571–616. doi: 10.1007/978-1-4939-8997-3_31
142. Kamioka H, Murshid SA, Ishihara Y, Kajimura N, Hasegawa T, Ando R, et al. A method for observing silver-stained osteocytes *in situ* in 3-microm sections using ultra-high voltage electron microscopy tomography. *Microsc Microanal.* (2009) 15:377–83. doi: 10.1017/S1431927609990420

143. Lampi T, Dekker H, Ten Bruggenkate CM, Schulten E, Mikkonen JJW, Koistinen A, et al. Acid-etching technique of non-decalcified bone samples for visualizing osteocyte-lacuno-canalicular network using scanning electron microscope. *Ultrastruct Pathol.* (2018) 42:74–9. doi: 10.1080/01913123.2017.1384418
144. van Hove RP, Nolte PA, Vatsa A, Semeins CM, Salmon PL, Smit TH, et al. Osteocyte morphology in human tibiae of different bone pathologies with different bone mineral density - is there a role for mechanosensing? *Bone.* (2009) 45:321–9. doi: 10.1016/j.bone.2009.04.238
145. Schneider P, Meier M, Wepf R, Muller R. Serial FIB/SEM imaging for quantitative 3D assessment of the osteocyte lacuno-canalicular network. *Bone.* (2011) 49:304–11. doi: 10.1016/j.bone.2011.04.005
146. Milovanovic P, Zimmermann EA, Vom Scheidt A, Hoffmann B, Sarau G, Yorgan T, et al. The formation of calcified nanospherites during micropetrosis represents a unique mineralization mechanism in aged human bone. *Small.* (2017) 13. doi: 10.1002/smll.201602215
147. Santos FR, Moyses RM, Montenegro FL, Jorgetti V, Noronha IL. IL-1beta, TNF-alpha, TGF-beta, and bFGF expression in bone biopsies before and after parathyroidectomy. *Kidney Int.* (2003) 63:899–907. doi: 10.1046/j.1523-1755.2003.00835.x
148. Chatzopoulos GS, Mansky KC, Lunos S, Costalonga M, Wolff LF. Sclerostin and WNT-5a gingival protein levels in chronic periodontitis and health. *J Periodontal Res.* (2019) 54:555-65. doi: 10.1111/jre.12659
149. Costa AG, Cremers S, Bilezikian JP. Sclerostin measurement in human disease: validity and current limitations. *Bone.* (2017) 96:24–8. doi: 10.1016/j.bone.2016.10.012
150. Durosier C, van Lierop A, Ferrari S, Chevalley T, Papapoulos S, Rizzoli R. Association of circulating sclerostin with bone mineral mass, microstructure, and turnover biochemical markers in healthy elderly men and women. *J Clin Endocrinol Metab.* (2013) 98:3873–83. doi: 10.1210/jc.2013-2113
151. McNulty M, Singh RJ, Li X, Bergstralh EJ, Kumar R. Determination of serum and plasma sclerostin concentrations by enzyme-linked immunoassays. *J Clin Endocrinol Metab.* (2011) 96:E1159–62. doi: 10.1210/jc.2011-0254
152. Drake MT, Fenske JS, Blocki FA, Zierold C, Appelman-Dijkstra N, Papapoulos S, et al. Validation of a novel, rapid, high precision sclerostin assay not confounded by sclerostin fragments. *Bone.* (2018) 111:36–43. doi: 10.1016/j.bone.2018.03.013
153. Maimoun L, Ben Bouallegue F, Gelis A, Aouinti S, Mura T, Philibert P, et al. Periostin and sclerostin levels in individuals with spinal cord injury and their relationship with bone mass, bone turnover, fracture and osteoporosis status. *Bone.* (2019) 127:612–9. doi: 10.1016/j.bone.2019.07.019
154. Hemmatian H, Jalali R, Semeins CM, Hogervorst JMA, van Lenthe GH, Klein-Nulend J, et al. Mechanical loading differentially affects osteocytes in fibulae from lactating mice compared to osteocytes in virgin mice: possible role for lacuna size. *Calcif Tissue Int.* (2018) 103:675–85. doi: 10.1007/s00223-018-0463-8
155. Bakker AD, Kulkarni RN, Klein-Nulend J, Lems WF. IL-6 alters osteocyte signaling toward osteoblasts but not osteoclasts. *J Dent Res.* (2014) 93:394–9. doi: 10.1177/0022034514522485
156. Bakker AD, Silva VC, Krishnan R, Bacabac RG, Blaauboer ME, Lin YC, et al. Tumor necrosis factor alpha and interleukin-1beta modulate calcium and nitric oxide signaling in mechanically stimulated osteocytes. *Arthritis Rheum.* (2009) 60:3336–45. doi: 10.1002/art.24920
157. Yan Y, Wang L, Ge L, Pathak JL. Osteocyte-mediated translation of mechanical stimuli to cellular signaling and its role in bone and non-bone-related clinical complications. *Curr Osteoporos Rep*. (2020) 18:67–80. doi: 10.1007/s11914-020-00564-9
158. Hinton PV, Rackard SM, Kennedy OD. *In vivo* osteocyte mechanotransduction: recent developments and future directions. *Curr Osteoporos Rep.* (2018) 16:746–53. doi: 10.1007/s11914-018-0485-1
159. Seref-Ferlengez Z, Basta-Pljakic J, Kennedy OD, Philemon CJ, Schaffler MB. Structural and mechanical repair of diffuse damage in cortical bone *in vivo*. *J Bone Miner Res.* (2014) 29:2537–44. doi: 10.1002/jbmr.2309
160. Seref-Ferlengez Z, Maung S, Schaffler MB, Spray DC, Suadicani SO, Thi MM. P2X7R-Panx1 complex impairs bone mechanosignaling under high glucose levels associated with type-1 diabetes. *Plos ONE.* (2016) 11:e0155107. doi: 10.1371/journal.pone.0155107
161. Sato M, Suzuki T, Kawano M, Tamura M. Circulating osteocyte-derived exosomes contain miRNAs which are enriched in exosomes from MLO-Y4 cells. *Biomed Rep.* (2017) 6:223–31. doi: 10.3892/br.2016.824
162. Tao SC, Guo SC. Extracellular vesicles in bone: "dogrobbers" in the "eternal battle field". *Cell Commun Signal.* (2019) 17:6. doi: 10.1186/s12964-019-0319-5
163. Zimmerman SM, Dimori M, Heard-Lipsmeyer ME, Morello R. The osteocyte transcriptome is extensively dysregulated in mouse models of osteogenesis imperfecta. *JBMR Plus.* (2019) 3:e10171. doi: 10.1002/jbm4.10171
164. Kristensen LS, Andersen MS, Stagsted LVW, Ebbesen KK, Hansen TB, Kjems J. The biogenesis, biology and characterization of circular RNAs. *Nat Rev Genet.* (2019) 20:675–91. doi: 10.1038/s41576-019-0158-7
165. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics.* (2018) 34:i884–90. doi: 10.1093/bioinformatics/bty560
166. Gao Y, Wang J, Zhao F. CIRI: an efficient and unbiased algorithm for *de novo* circular RNA identification. *Genome Biol.* (2015) 16:4. doi: 10.1186/s13059-014-0571-3
167. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* (2014) 15:550. doi: 10.1186/s13059-014-0550-8
168. Qian DY, Yan GB, Bai B, Chen Y, Zhang SJ, Yao YC, et al. Differential circRNA expression profiles during the BMP2-induced osteogenic differentiation of MC3T3-E1 cells. *Biomed Pharmacother.* (2017) 90:492–9. doi: 10.1016/j.biopha.2017.03.051
169. Zeng Q, Wang Y, Gao J, Yan Z, Li Z, Zou X, et al. miR-29b-3p regulated osteoblast differentiation via regulating IGF-1 secretion of mechanically stimulated osteocytes. *Cell Mol Biol Lett.* (2019) 24:11. doi: 10.1186/s11658-019-0136-2
170. Davis HM, Pacheco-Costa R, Atkinson EG, Brun LR, Gortazar AR, Harris J, et al. Disruption of the Cx43/miR21 pathway leads to osteocyte apoptosis and increased osteoclastogenesis with aging. *Aging Cell.* (2017) 16:551–63. doi: 10.1111/acel.12586
171. Thomas T. Intermittent parathyroid hormone therapy to increase bone formation. *Joint Bone Spine.* (2006) 73:262–9. doi: 10.1016/j.jbspin.2005.11.007
172. Wang YH, Liu Y, Rowe DW. Effects of transient PTH on early proliferation, apoptosis, and subsequent differentiation of osteoblast in primary osteoblast cultures. *Am J Physiol Endocrinol Metab.* (2007) 292:E594–603. doi: 10.1152/ajpendo.00216.2006
173. Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O'Brien CA, et al. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology.* (2005) 146:4577–83. doi: 10.1210/en.2005-0239
174. Wein MN. Parathyroid hormone signaling in osteocytes. *JBMR Plus.* (2018) 2:22–30. doi: 10.1002/jbm4.10021
175. Hoyer-Kuhn H, Semler O, Stark C, Struebing N, Goebel O, Schoenau E. A specialized rehabilitation approach improves mobility in children with osteogenesis imperfecta. *J Musculoskelet Neuronal Interact.* (2014) 14:445–53.
176. Gatti D, Rossini M, Viapiana O, Povino MR, Liuzza S, Fracassi E, et al. Teriparatide treatment in adult patients with osteogenesis imperfecta type I. *Calcif Tissue Int.* (2013) 93:448–52. doi: 10.1007/s00223-013-9770-2
177. Zanotti S, Canalis E. Parathyroid hormone inhibits Notch signaling in osteoblasts and osteocytes. *Bone.* (2017) 103:159–67. doi: 10.1016/j.bone.2017.06.027
178. McClung MR. Sclerostin antibodies in osteoporosis: latest evidence and therapeutic potential. *Ther Adv Musculoskelet Dis.* (2017) 9:263–70. doi: 10.1177/1759720X17726744
179. Ke HZ, Richards WG, Li XD, Ominsky MS. Sclerostin and Dickkopf-1 as therapeutic targets in bone diseases. *Endocr Rev.* (2012) 33:747–83. doi: 10.1210/er.2011-1060
180. Colucci S, Brunetti G, Oranger A, Mori G, Sardone F, Specchia G, et al. Myeloma cells suppress osteoblasts through sclerostin secretion. *Blood Cancer J.* (2011) 1:e27. doi: 10.1038/bcj.2011.22
181. Mendoza-Villanueva D, Zeef L, Shore P. Metastatic breast cancer cells inhibit osteoblast differentiation through the Runx2/CBFbeta-dependent

expression of the Wnt antagonist, sclerostin. *Breast Cancer Res.* (2011) 13:R106. doi: 10.1186/bcr3048
182. Kleber M, Ntanasis-Stathopoulos I, Dimopoulos MA, Terpos E. Monoclonal antibodies against RANKL and sclerostin for myeloma-related bone disease: can they change the standard of care? *Expert Rev Hematol.* (2019) 12:651–63. doi: 10.1080/17474086.2019.1640115
183. Toscani D, Bolzoni M, Ferretti M, Palumbo C, Giuliani N. Role of osteocytes in myeloma bone disease: anti-sclerostin antibody as new therapeutic strategy. *Front Immunol.* (2018) 9:2467. doi: 10.3389/fimmu.2018.02467
184. Hesse E, Schroder S, Brandt D, Pamperin J, Saito H, Taipaleenmaki H. Sclerostin inhibition alleviates breast cancer-induced bone metastases and muscle weakness. *JCI Insight.* (2019) 5:e125543. doi: 10.1172/jci.insight.125543
185. Markham A. Romosozumab: first global approval. *Drugs.* (2019) 79:471–6. doi: 10.1007/s40265-019-01072-6
186. Surowiec RK, Battle LF, Ward FS, Schlecht SH, Khoury BM, Robbins C, et al. A xenograft model to evaluate the bone forming effects of sclerostin antibody in human bone derived from pediatric osteogenesis imperfecta patients. *Bone.* (2020) 130:115118. doi: 10.1016/j.bone.2019.115118
187. Scheiber AL, Barton DK, Khoury BM, Marini JC, Swiderski DL, Caird MS, et al. Sclerostin antibody-induced changes in bone mass are site specific in developing crania. *J Bone Miner Res.* (2019) 34:2301–10. doi: 10.1002/jbmr.3858
188. Voorzanger-Rousselot N, Goehrig D, Journe F, Doriath V, Body JJ, Clezardin P, et al. Increased Dickkopf-1 expression in breast cancer bone metastases. *Br J Cancer.* (2007) 97:964–70. doi: 10.1038/sj.bjc.6603959
189. Rachner TD, Gobel A, Benad-Mehner P, Hofbauer LC, Rauner M. Dickkopf-1 as a mediator and novel target in malignant bone disease. *Cancer Lett.* (2014) 346:172–7. doi: 10.1016/j.canlet.2014.01.010
190. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. *N Engl J Med.* (2003) 349:2483–94. doi: 10.1056/NEJMoa030847
191. Glantschnig H, Hampton RA, Lu P, Zhao JZ, Vitelli S, Huang L, et al. Generation and selection of novel fully human monoclonal antibodies that neutralize Dickkopf-1 (DKK1) inhibitory function *in vitro* and increase bone mass *in vivo*. *J Biol Chem.* (2010) 285:40135–47. doi: 10.1074/jbc.M110.166892
192. Heiland GR, Zwerina K, Baum W, Kireva T, Distler JH, Grisanti M, et al. Neutralisation of Dkk-1 protects from systemic bone loss during inflammation and reduces sclerostin expression. *Ann Rheum Dis.* (2010) 69:2152–9. doi: 10.1136/ard.2010.132852
193. Florio M, Gunasekaran K, Stolina M, Li X, Liu L, Tipton B, et al. A bispecific antibody targeting sclerostin and DKK-1 promotes bone mass accrual and fracture repair. *Nat Commun.* (2016) 7:11505. doi: 10.1038/ncomms11505
194. Iyer SP, Beck JT, Stewart AK, Shah J, Kelly KR, Isaacs R, et al. A Phase IB multicentre dose-determination study of BHQ880 in combination with anti-myeloma therapy and zoledronic acid in patients with relapsed or refractory multiple myeloma and prior skeletal-related events. *Br J Haematol.* (2014) 167:366–75. doi: 10.1111/bjh.13056
195. Sousa S, Clezardin P. Bone-targeted therapies in cancer-induced bone disease. *Calcif Tissue Int.* (2018) 102:227–50. doi: 10.1007/s00223-017-0353-5
196. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med.* (2011) 17:1231–4. doi: 10.1038/nm.2452
197. Wijenayaka AR, Kogawa M, Lim HP, Bonewald LF, Findlay DM, Atkins GJ. Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway. *PLoS ONE.* (2011) 6:e25900. doi: 10.1371/journal.pone.0025900
198. Zaheer S, LeBoff M, Lewiecki EM. Denosumab for the treatment of osteoporosis. *Expert Opin Drug Metab Toxicol.* (2015) 11:461–70. doi: 10.1517/17425255.2015.1000860
199. Walsh MC, Choi Y. Biology of the RANKL-RANK-OPG system in immunity, bone, and beyond. *Front Immunol.* (2014) 5:511. doi: 10.3389/fimmu.2014.00511
200. Polyzos SA, Makras P, Tournis S, Anastasilakis AD. Off-label uses of denosumab in metabolic bone diseases. *Bone.* (2019) 129:115048. doi: 10.1016/j.bone.2019.115048
201. Rauch F, Travers R, Plotkin H, Glorieux FH. The effects of intravenous pamidronate on the bone tissue of children and adolescents with osteogenesis imperfecta. *J Clin Invest.* (2002) 110:1293–9. doi: 10.1172/JCI0215952
202. Alcausin MB, Briody J, Pacey V, Ault J, McQuade M, Bridge C, et al. Intravenous pamidronate treatment in children with moderate-to-severe osteogenesis imperfecta started under three years of age. *Horm Res Paediatr.* (2013) 79:333–40. doi: 10.1159/000351374
203. Bishop N, Adami S, Ahmed SF, Anton J, Arundel P, Burren CP, et al. Risedronate in children with osteogenesis imperfecta: a randomised, double-blind, placebo-controlled trial. *Lancet.* (2013) 382:1424–32. doi: 10.1016/S0140-6736(13)61091-0
204. Nioi P, Taylor S, Hu R, Pacheco E, He YDD, Hamadeh H, et al. Transcriptional profiling of laser capture microdissected subpopulations of the osteoblast lineage provides insight into the early response to sclerostin antibody in rats. *J Bone Miner Res.* (2015) 30:1457–67. doi: 10.1002/jbmr.2482
205. Saag KG, Petersen J, Brandi ML, Karaplis AC, Lorentzon M, Thomas T, et al. Romosozumab or alendronate for fracture prevention in women with osteoporosis. *N Engl J Med.* (2017) 377:1417–27. doi: 10.1056/NEJMoa1708322
206. Bouaziz W, Funck-Brentano T, Lin H, Marty C, Ea HK, Hay E, et al. Loss of sclerostin promotes osteoarthritis in mice via beta-catenin-dependent and -independent Wnt pathways. *Arthritis Res Ther.* (2015) 17:24. doi: 10.1186/s13075-015-0540-6
207. Wehmeyer C, Frank S, Beckmann D, Bottcher M, Cromme C, Konig U, et al. Sclerostin inhibition promotes TNF-dependent inflammatory joint destruction. *Sci Transl Med.* (2016) 8:330ra35. doi: 10.1126/scitranslmed.aac4351
208. Cain CJ, Rueda R, McLelland B, Collette NM, Loots GG, Manilay JO. Absence of sclerostin adversely affects B-cell survival. *J Bone Miner Res.* (2012) 27:1451–61. doi: 10.1002/jbmr.1608

Conflict of Interest: 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 Endocrinology

ORIGINAL RESEARCH
published: 14 July 2020
doi: 10.3389/fendo.2020.00501

Activin-A Induces Fewer, but Larger Osteoclasts From Monocytes in Both Healthy Controls and Fibrodysplasia Ossificans Progressiva Patients

Ton Schoenmaker[1], Esmée Botman[2], Merve Sariyildiz[1], Dimitra Micha[3], Coen Netelenbos[2], Nathalie Bravenboer[4], Angele Kelder[5], E. Marelise W. Eekhoff[2] and Teun J. De Vries[1]*

[1] Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Vrije Universiteit, Amsterdam, Netherlands, [2] Department of Internal Medicine Section Endocrinology, Amsterdam Movement Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [3] Department of Clinical Genetics, Amsterdam Movement Sciences, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [4] Department of Clinical Chemistry, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [5] Department of Hematology, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands

OPEN ACCESS

Edited by:
Giacomina Brunetti, University of Bari Aldo Moro, Italy

Reviewed by:
Emanuela Galliera, University of Milan, Italy
Eleonora Palagano, National Research Council (CNR), Italy

***Correspondence:**
Ton Schoenmaker
t.schoenmaker@acta.nl

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *17 December 2019*
Accepted: *23 June 2020*
Published: *14 July 2020*

Citation:
Schoenmaker T, Botman E, Sariyildiz M, Micha D, Netelenbos C, Bravenboer N, Kelder A, Eekhoff EMW and De Vries TJ (2020) Activin-A Induces Fewer, but Larger Osteoclasts From Monocytes in Both Healthy Controls and Fibrodysplasia Ossificans Progressiva Patients. Front. Endocrinol. 11:501. doi: 10.3389/fendo.2020.00501

Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disease characterized by heterotopic ossification (HO) that occurs in muscle tissue, tendons, and ligaments. The disease is caused by mutations in the Activin receptor type I (ACVR1) gene resulting in enhanced responsiveness to Activin-A. Binding of this molecule to the mutated receptor induces HO. Bone metabolism normally requires the coupled action of osteoblasts and osteoclasts, which seems to be disturbed during HO. We hypothesize that Activin-A may also counteract the formation of osteoclasts in FOP patients. In this study we investigated the effect of Activin-A on osteoclast differentiation of CD14+ monocytes from FOP patients and healthy controls. The lymphocytic and monocytic cell populations were determined by FACS analysis. Expression of the mutated R206H receptor was assessed and confirmed by allele specific PCR. The effect of Activin-A on osteoclastogenesis was assessed by counting the number and size of multinucleated cells. Osteoclast activity was determined by culturing the cells on Osteo Assay plates. The influence of Activin-A on expression of various osteoclast related genes was studied with QPCR. Blood from FOP patients contained similar percentages of classical, intermediate, or non-classical monocytes as healthy controls. Addition of Activin-A to the osteoclastogenesis cultures resulted in fewer osteoclasts in both control and FOP cultures. The osteoclasts formed in the presence of Activin-A were, however, much larger and more active compared to the cultures without Activin-A. This effect was tempered when the Activin-A inhibitor follistatin was added to the Activin-A containing cultures. Expression of osteoclast specific genes Cathepsin K and TRAcP was upregulated, gene expression of osteoclastogenesis related genes M-CSF and DC-STAMP was downregulated by Activin-A. Since Activin-A is a promising target for inhibiting the formation of HO in FOP, it is important to know its effects on both osteoblasts and osteoclasts. Our study shows that Activin-A induces fewer, but larger and more active osteoclasts independent of the presence of the mutated ACVR1 receptor. When considering FOP as an Activin-A driven disease that acts locally,

our findings suggest that Activin-A could cause a more pronounced local resorption by larger osteoclasts. Thus, when targeting Activin-A in patients with neutralizing antibodies, HO formation could potentially be inhibited, and osteoclastic activity could be slightly reduced, but then performed dispersedly by more and smaller osteoclasts.

Keywords: fibrodysplasia ossificans progressiva, Activin-A, *ACVR1*, CD14+ monocyte, osteoclast

INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) is a autosomal dominant severe genetic disease characterized by heterotopic bone formation where muscles, tendons, and ligaments are being converted into bone (1–3). Heterotopic ossification (HO) in FOP might appear after a flare-up, during inflammation, following injury or spontaneously. The new extra-skeletal bone is formed by endochondral ossification (1), the metabolism and composition of this heterotopic bone formed in FOP patients appears to be comparable to skeletal bone in healthy subjects. It ultimately connects to the existing skeleton, hereby gradually causing irreversible movement impairments throughout the body (4).

FOP is caused by mutations in the gene encoding the Activin receptor type 1/activin kinase 2 (ACVR1/ALK2), a bone morphogenetic protein (BMP) type 1 receptor (2, 5). Upon binding of different ligands of the TGF-β superfamily, ACVR1 receptor dimers normally form a complex with a BMP type 2-receptor dimer stimulating the SMAD1/5/8 pathway and thereby osteogenesis. The most frequent mutation causing FOP is the single nucleotide c.617G>A mutation resulting in the replacement of the amino acid arginine by histidine (R206H). This replacement stimulates osteogenic differentiation of osteoblast-like cells by causing both decreased binding of the ACVR1-inhibitor FK binding protein 12 (FKBP12) (6, 7) and increased responsiveness to BMP4 (8–10). Recently Activin-A, a TGF- β superfamily ligand that normally inhibits BMP signaling through ACVR1 (11) has been shown to induce osteogenic differentiation via the mutated receptor. In FOP-patient derived induced pluripotent stem cells (12) as well as in a mouse model of FOP (13) Activin-A was specifically shown to signal through the canonical BMP-pSMAD1/5/8 pathway, thus stimulating osteogenesis. FOP mice that received an inhibitory antibody against Activin A were protected for HO formation, holding promise for therapies addressing Activin A activity (13, 14). Recently the first results from the LUMINA-1 trial using the Activin-A antibody Garetosmab indeed showed a reduction in the formation of new lesions in patients. This study also showed a small decrease in bone lesion volume, suggesting that osteoclasts could be activated in this process (https://newsroom.regeneron.com/index.php/news-releases/news-release-details/regeneron-announces-encouraging-garetosmab-phase-2-results).

Over the past years, research on FOP has focused on the osteogenic properties of osteoblast-like cells harboring the R206H mutation. Endochondral ossification as well as normal bone remodeling however, requires the coupled action of both the bone forming osteoblasts as well as the bone resorbing osteoclasts (15, 16). Therefore, it is pivotal to investigate the potential role of the ACVR1 R206H mutation on osteoclast formation as well as the influence of Activin-A on this process.

Osteoclasts are multinucleated cells that arise through fusion of CD14 positive (CD14+) monocytic cells (17). This fusion is mediated by Macrophage Colony Stimulating Factor (M-CSF) and Receptor Activator of Nuclear factor Kappa-B Ligand (RANK-L). These molecules are normally produced by, amongst others, osteoblast like cells when bone resorption is required (15). Our group previously described the use of human periodontal ligament fibroblasts (PLF) from healthy controls and FOP patients as osteoblast-like cells. When these PLF were cocultured with non-mutated CD14+ osteoclast precursors, no significant difference in PLF induced osteoclastogenesis between the control and FOP PLF was observed (18). In addition, adding Activin-A to these cocultures inhibited osteoclast formation regardless of the mutation in the PLF cells (19). This suggests a direct effect of Activin-A on the CD14+ osteoclast precursors. All these experiments however, were performed with CD14+ not bearing the mutation of ACVR1. Monocytes from FOP patients might respond differently to Activin-A.

Elucidating the effect of Activin-A on human osteoclastogenesis in cells bearing the ACVR1-R206H mutation is especially relevant since the first clinical trial in FOP using Activin-A blocking antibodies is currently in phase II.

In this study we investigated osteoclast formation from CD14+ cells from healthy controls and FOP patients, and the potential effect of Activin-A on this osteoclastogenesis. Since the CD14+ cells express ACVR-1 (19), we hypothesize that Activin-A interacts more strongly with FOP-patient derived CD14+ cells resulting in a stronger inhibition of osteoclast formation in FOP patients compared with the healthy controls.

MATERIALS AND METHODS

Flow Cytometry

Blood was drawn from six sex and age matched controls and patients (2 males, 4 females, age range 20–68 years, maximal age difference between control and FOP 2 years). Five of the FOP patients harbor the R206H mutation, one patient harbors a variant mutation (Q207E). This Q207E mutation is adjacent to the classical R206H mutation and also located in the GS domain. Patients with this mutation show comparable phenotypes with the R206H patients (20, 21). Written informed consent was obtained from all donors as required by the Medical Ethics Review Committee of the Amsterdam UMC, Vrije Universiteit Amsterdam (research protocol 2012.467). The blood cell composition was analyzed by four color Flow

Cytometry using the FACSCanto Flow Cytometry system (BD Biosciences, Franklin Lakes, NJ, USA). First the erythrocytes were lysed using Pharmlyse (BD Biosciences, Franklin Lakes, NJ, USA) and washed once with phosphate-buffered saline (PBS) containing 0.1% human serum albumin. The cells were incubated for 15 min at room temperature with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinyl chlorophyllin (PerCP), Phycoerythrin-Cyanine 7 (PC7), allophycocyanin (APC) allophycocyanin-cyanine (APC-H7), or Krome Orange (KO) conjugated monoclonal antibodies and washed once with PBS containing 0.1% human serum albumin. The pan-leucocyte marker CD45 (KO-labeled) was used to discriminate between white blood cells and unlysed red blood cells or debris. Lymphoid markers (CD4 APC-H7, CD8 FITC, CD19 APC) were used to discriminate between T-cells (CD4, CD8), B-cells (CD19). CD8 (FITC-labeled), and CD4 (APC-H7-labeled) were included to identify the cytotoxic/suppressor T-cells and T-helper/inducer cells, respectively. CD14 (PercP-labeled) and CD16 (PE labeled) were used to discriminate between classical monocytes (CD14++CD16−), intermediate monocytes (CD14++CD16+) and non-classical monocytes (CD14+CD16+), all of which have the capacity to differentiate into osteoclasts (22). The different monoclonal antibody clones used are listed in **Supplementary Table 1**. Data acquisition was performed on the FACSCanto system and analysis was performed using Infincyt software (Cytognos).

CD14+ Cell Isolation

For the initial TRAcP staining and QPCR experiments $CD14^+$ monocytes were isolated from the blood from 6 FOP patients and sex- and age- matched controls (20–40 ml blood per donor), for all other experiments human buffy coats (Sanquin, The Netherlands) or blood from healthy donors was used. The CD14+ cells were isolated as described before (23).

Briefly, peripheral blood mononuclear cells were isolated using Ficoll-Paque density gradient centrifugation. Subsequently cells were incubated with CD14-antibody tagged microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and sorted using a manual MACS magnetic cell sorter (Miltenyi Biotec) according to the manufacturer's instructions (24). The purity of the cells was determined with flow cytometry (FACSverse™, BD Biosciences, Piscataway, USA). For the analysis, cells were incubated with FITC labeled anti-human CD14 (Miltenyi Biotec) or its equivalent isotype control IgG2a (Miltenyi Biotec) for 30 min in the dark on ice. After incubation, cells were washed to remove unbound antibodies, recovered in FACS buffer and analyzed (30 s or 100,000 viable events) on a BD Bioscience FACSverse flow cytometer. Purity was confirmed to be at least 80%.

Osteoclastogenesis

Purified CD14+ cells were suspended in culture medium consisting of αMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (HyClone, Logan, UT), and 1% antibiotics: 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Sigma, St. Louis, MO, USA). Cells were cultured in a 96 well-plate at a density of 1×10^5 cells/well, for the first 3 days with 25 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D systems, Oxon, UK), without or with 50 ng/ml Activin-A (Sigma). After 3 days the medium composition was changed to 10 ng/ml M-CSF and 2 ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL) (R&D Systems), without or with 50 ng/ml Activin-A. In the blocking experiments cells were also cultured with 50 ng/ml Activin-A and 500 ng/ml follistatin (R&D systems) according to advice from Wang (25). All cultures were maintained at 37°C in a humidified atmosphere under 5% CO_2 for 21 days. Culture media were replaced every 3–4 days.

In the experiments with CD14+ cells from control and FOP, blood from 6 controls and 6 FOP patients were used. For the TRAcP staining all experimental conditions were plated in triplicate. For the counting of the osteoclasts five designated fields per well were counted and the number of TRAcP positive multinuclear cells (>3 nuclei) were counted. For the follistatin experiments blood from 3 healthy donors was used and each experimental condition was plated in quadruplicate. Counting of osteoclasts was performed as described above. For the size of the osteoclasts four low magnification (10X) pictures per well were taken at designated fields with a digital camera (Leica, Wetzlar, Germany). Subsequently the size was measured using Image-Pro Plus (MediaCybernetics, Rockville, USA). Osteoclast size is depicted in um^2 per osteoclast or as the percentage of surface area that is occupied by the osteoclasts.

TRACP Staining

Tartrate Resistant Acid Phosphatase (TRACP) staining was performed with the Leukocyte Acid Phosphatase Staining Kit (Sigma, St. Louis, MO, USA) as previously described (18, 19). Nuclei were stained with diamidino-2 phenylindole dihydrochloride (DAPI). For the counting of the osteoclasts five designated fields per well were selected and the number of TRAcP positive multinuclear cells (>3 nuclei) were counted.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (Q-PCR)

Cells were cultured for 7, 14, and 21 days in 96 well-plates. For each experimental condition 3 wells were pooled.

RNA was isolated using the RNeasy mini kit (QIAGEN, Hilden, Germany) following manufacturer's instructions. The reverse transcriptase reaction was performed with the first strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturers protocol, using both the Oligo(dT)18 and the D(N)6 primers.

Allele specific PCR primers were used to distinguish between c.617G>A and non-mutated ACVR-1. Primers were described by Kaplan et al. (26). To detect the FOP allele 300 nM of the *ACVR1 c.617A* primers were used in a standard two step QPCR program with an annealing temperature of 63°C. To detect the control allele 150 nM of the *ACVR1 c.617G* primers were used in a standard two step QPCR program with an annealing temperature of 63°C. For the other genes Q-PCR primers were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA) (**Table 1**). To avoid amplification of genomic DNA, each amplicon spanned at least one intron. Q-PCR was

TABLE 1 | Primer sequences used for quantitative PCR.

Gene	Sequence 5′-3	Amplicon length (bp)	Ensemble gene ID
PBGD	TgCAgTTTgAAATCATTgCTATgTC	84	ENSG00000113721
	AACAggCTTTTCTCTCCAATCTTAgA		
ACVR1 c.617G	TggTACAAAgAACAgTggCTAg	101	ENSG00000115170
	CCATACCTgCCTTTCCCgA		
ACVR1 c.617A	TggTACAAAgAACAgTggCTTA	101	
	CCATACCTgCCTTTCCCgA		
ACVR1	CAgCTgCCCACTAAAggAAAAT	68	
	AATAATgAggCCAACCTCCAAgT		
CSF1	CCgAggAggTgTCggAgTAC	100	ENSG00000184371
	AATTTggCACgAggTCTCCAT		
	CTCggAgCTCTgATgTgTTgAA		
DCSTAMP	ATTTTCTCAgTgAgCAAgCAgTTTC	101	ENSG0000016493
	AgAATCATggATAATATCTTgAgTTCCTT		
ID-1	ACgTgCTgCTCTACgACATgA	56	ENSG00000125968
	TgggCACCAgCTCCTTgA		
TRAcP	CACAATCTgCAgTACCTgCAAgAT	128	ENSG00000102575
	CCCATAgTggAAgCgCAgATA		
CTSK	CCATATgTgggACAggAAgAgAgTT	149	ENSG00000143387
	TgCATCAATggCCACAgAgA		
NFATc1	AgCAgAgCACggACAgCTATC	143	ENSG00000131196
	ggTCAgTTTTCgCTTCCATCTC		
AlphaV Integrin	TACAgCAggTCCCCAAgTCACT	100	ENSG00000138448
	AATTCAgATTCATCCCgCAgAT		

PBGD, porphobilinogen deaminase; ACVR1c.617G, Activin A receptor type I, control allele; ACVR1c.617A, Activin A receptor type I, FOP allele; ACVR1, Activin A receptor type I; CFS1, colony-stimulating factor1 [coding for macrophage-colony stimulating factor (M-CSF)]; DC-STAMP, dendritic cell-specific transmembrane protein; ID-1, Inhibitor of DNA binding 1; protein; TRAcP, tartrate resistant acid phosphatese; CTSK, Cathepsin-K; NFATc1, nuclear factor of activated T-cells 1; Alpha V Integrin, Integrin subunit Alpha V. For each gene, the first oligonucleotide sequence represents the forward primer, the second sequence the reverse primer.

performed on the LC480 light cycler (Roche, Basel, Switzerland). Three nanogram cDNA was used in a total volume of 20 µl containing Light Cycler SybrGreen1 Master mix (Roche) and 1 µM of each primer. A standard two step QPCR program with an annealing temperature of 60°C was performed. Sequence information for all primers are listed in **Table 1**. Expression of housekeeping gene porphobilinogen deaminase (*PBGD*) was not affected by the experimental conditions. Samples were normalized for the expression of *PBGD* by calculating the ΔCt ($Ct_{,geneofinterest} - Ct_{,PBGD}$) and expression of the different genes was expressed as $2^{-(\Delta Ct)}$. All qPCRs had equal efficiencies.

Osteoclast Activity on Osteo Assay Surface

CD14+ cells were isolated from blood from 4 healthy donors as described in the CD14+ cell isolation section. Osteoclastogenesis was performed on 96 well-Osteo Assay surface plates (Corning Costar, Lowell, MA, USA) as described in the osteoclastogenesis section either without or with Actvin-A and with both Activin-A and Follistatin. Each experimental condition was plated in quadruplicate. Cells were cultured for 14 and 21 days. To visualize the lysis of the calcium phosphate coating, wells were incubated for 5 min in 10% bleach, washed with H_2O and air dried. For the quantification of the total resorbed area, 4 pictures were taken per well at 10 x magnification. Resorbed area was measured using Image-Pro Plus (MediaCybernetics, Rockville, USA) and depicted as percentage of resorption per well.

Statistical Analysis

Differences between cultures without and with Activin-A were tested using the Wilcoxon matched-pairs signed rank test. For differences between Control and FOP cultures the Mann-Whitney test was used. In the follistatin experiments the Friedmans test with the Dunn's multiple comparison test was used. Differences were considered to be significant when the *p*-value was lower than 0.05.

RESULTS

No Difference in Lymphocyte and Monocyte Subsets Between Controls and FOP Patients

Although CD14+ cells were isolated for subsequent osteoclastogenesis experiments, blood composition was first determined, since cellular composition of peripheral blood may prime osteoclast precursors [Reviewed in (27)]. No difference was found between the percentage B-lymphocytes and

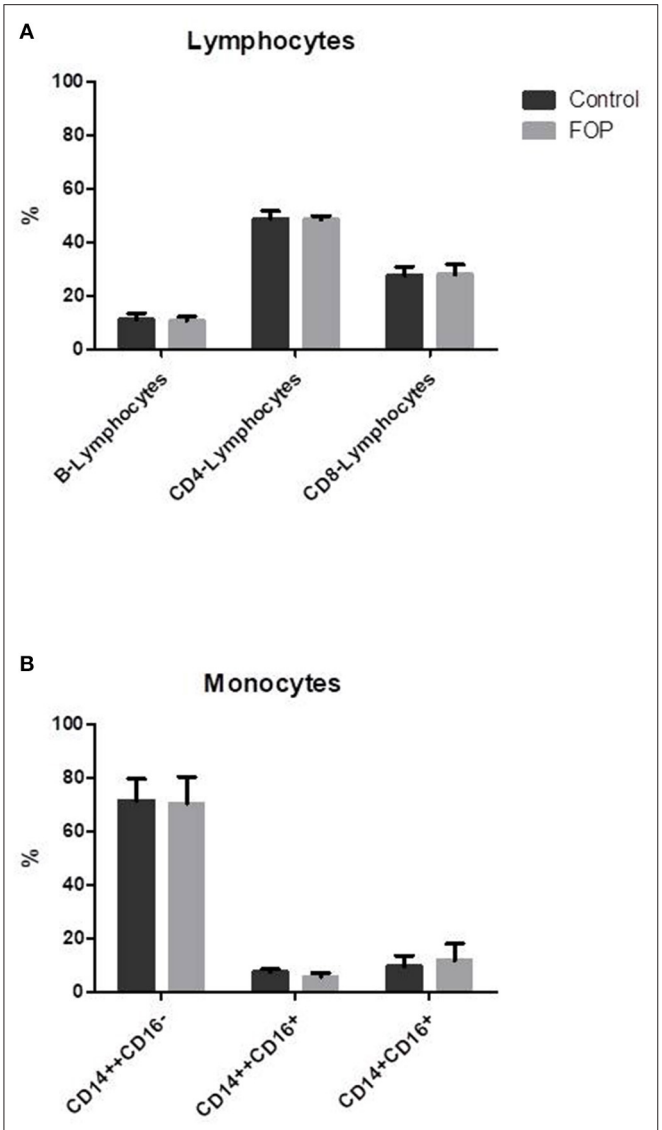


FIGURE 1 | Blood cell composition does not differ between control and FOP blood. Control or FOP blood cell populations were analyzed with FACS analysis. There are no significant differences in the percentages of B-lymphocytes, CD4-lymphocytes, or CD8-lymphocytes between control and FOP blood **(A)**. The percentage of classical CD14++CD16−, intermediate CD14++CD16+ and non-classical CD14+CD16+ was also similar between control and FOP blood **(B)**. $n = 6$ for both control and FOP (unpaired *t*-test).

CD4 or CD8 positive T-lymphocytes between the control and FOP blood samples (**Figure 1A**). Also, there were no differences in the total CD14 positive monocytic cell populations. Since osteoclasts differentiate from CD14 positive cell populations, and different CD14 cell populations show distinct osteoclastogenesis dynamics (23), we next investigated the CD14 cell composition in the blood of the controls and FOP patients. There were no differences between classical monocytes (CD14++CD16−), intermediate monocytes (CD14++CD16+) and non-classical monocytes (CD14+CD16+) between controls and FOP (**Figure 1B**, Gating strategy is shown in **Supplementary Figure 1**).

ACVR-1 Is Expressed in CD14+ Monocytes; The c.617G>A FOP Allele Is Only Expressed in Monocytes From FOP Patients

To show that the CD14+ cells undergoing osteoclast differentiation indeed express the *ACVR1* gene and that only FOP monocytes from the R206H patients express the c.617G>A FOP allele we performed allele specific QPCR as described by Kaplan et al. (26), where the 3′ last nucleotide of the forward primer is complementary to either the control (c.617G) or the FOP (c.617A) allele. The second last 3′ nucleotide is a deliberate mismatch. The reverse primer is the same for both PCR reactions.

After 7 days of culture the wild type c.617G allele is expressed in both the control and the FOP cells at similar levels (**Figure 2A**). The FOP c.617A allele however, is only expressed by the FOP cells (**Figure 2B**). Expression of ACVR1 increases from day 7–14 and stays the same to day 21 (data not shown). The expression of both the control and the FOP allele was not influenced by Activin-A (**Figures 2A,B**).

Activin-A Inhibits Osteoclast Formation but Increases Osteoclast Size in Both Control and FOP Cultures

Having shown that both control and FOP-derived osteoclast precursors express ACVR1, and that only FOP-derived precursors express the c.617G>A variant, we next investigated whether this mutation alters the effect of Activin-A on osteoclast formation. Cells were cultured with M-CSF and RANK-L without and with Activin-A. After 21 days the cells were fixed and stained for TRAcP and the nuclei were stained with DAPI. Similar numbers of osteoclasts formed in control and FOP cultures (**Figures 3A,B**). Although the osteoclasts were significantly larger in the presence of Activin-A, the total number of osteoclasts was significantly inhibited by this molecule in both control and FOP cultures (**Figures 3A–C**).

Activin-A Alters Osteoclast Related Gene Expression in Both Control and FOP Cultures

In order to elucidate the molecular mechanisms by which Activin-A inhibits osteoclast formation in these cultures we analyzed gene expression with qPCR at several time points on osteoclast related genes. Two of the key role players in osteoclast formation, M-CSF, and DC-STAMP, were significantly downregulated in the presence of Activin-A after 7 days of culture (**Figures 4A,B**). In contrast, ID-1, one of the target molecules of SMAD1/5/8 signaling, was significantly upregulated in both cultures after 7 days in the presence of Activin-A (**Figure 4E**). Osteoclast-specific genes TRAcP and Cathepsin-K were both upregulated in the presence of Activin-A after 21 days (**Figures 4C,D**).

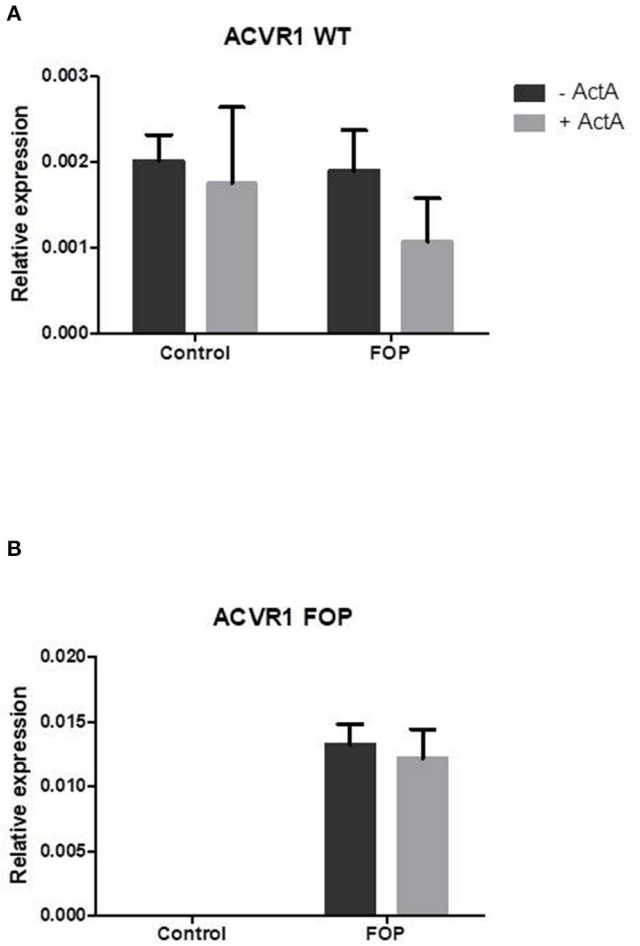


FIGURE 2 | Expression of the FOP c.617G>A allele is only present in the FOP osteoclast precursors. Control or FOP CD14+ cells were cultured with M-CSF and RANK-L, without and with Activin-A 50 ng/ml. RNA was isolated after 7 days and QPCR was performed. **(A)** The control c.617G allele is equally expressed in both control and FOP cells. **(B)** The FOP c.617A allele is only expressed in FOP cells harboring the R206H mutation (the cells from the patient with the Q207E mutation did not show any expression of the c.617A allele, data not shown). Activin-A does not alter the expression of either allele. Expression was normalized based on expression of the housekeeping gene PBGD. $n = 6$ for both control and FOP.

Inhibition With Follistatin Reverses the Activin-A Effect

To further prove that Activin-A results in the formation of less but larger osteoclasts, we performed inhibition experiments with follistatin, a natural inhibitor of Activin-A that can reverse Activin-A effects (25). Initial titration experiments showed that Activin-A's effect on osteoclast formation was reduced at a concentration of 500 ng/ml follistatin (data not shown). Since nor osteoclast formation, nor osteoclast-related gene expression nor the effect of Activin-A is different between control and FOP osteoclast cultures, these experiments were performed on CD14+ cells isolated from buffy coats from healthy donors.

After 21 days of culturing with follistatin the inhibitory effect of Activin-A on osteoclast formation and inductive effect on size was reduced (**Figures 5A,C,D**). The total percentage of area covered by the osteoclasts however, did not differ between the different culture conditions (**Figure 5E**). Early gene expression data also show that the increased expression of NFATc1, AlphaV Integrin, TRAcP, and Cathepsin K by Activin-A is reversed by follistatin (**Supplementary Figure 2**).

Activin-A Increases Overall Resorptive Activity

We next investigated the effect of Activin-A on the resorptive activity of the osteoclasts. Osteoclastogenesis was performed with CD14+ cells from buffy coats from healthy donors on Osteo Assay plates (Corning Costar) in the absence or presence of Activin-A or with both Activin-A and Follistatin. The lysis of the inorganic calcium phosphate coating was used as a measure of the resorptive activity of the osteoclasts. In line with the finding that the osteoclasts are larger in the presence of Activin-A, also the total percentage of resorbed area was increased in this culture condition (**Figures 5B,F**).

DISCUSSION

Heterotopic bone from FOP patients displays similar histological bone parameters such as osteoblast and osteoclast activity as normal skeletal bone. This assumes that remodeling of heterotopic bone is comparable to that of normal bone, but this has not been extensively investigated. One of the recent discoveries is the activating effect of Activin-A specifically on the mutated ACVR1 receptor that induces HO in muscle, tendons, and ligaments (12, 13). The effect of Activin-A on osteoclast formation from monocytes from FOP patients has not been previously investigated. In this study we show for the first time that Activin-A induces fewer but larger osteoclasts from both control and FOP derived human monocytes. Studies using murine cells have reported contradictory findings on the effect of Activin-A on osteoclasts, probably depending on the source of osteoclast precursors used. Some groups show that Activin-A enhances osteoclast formation and activity when using murine whole bone marrow cultures or the mouse macrophage cell line RAW246.7 (28–30). In contrast, Fowler et al. (31) showed that Activin-A suppressed osteoclastogenesis when using stromal cell depleted murine bone marrow macrophages.

We show that control as well as FOP monocytes expressed ACVR-1 at a similar level and only the FOP monocytes expressed the mutated variant of this gene, as expected. This was tested for the 5 out of 6 patients, bearing the classical R206H mutation. Expression of the rarer mutant Q207E, which is one amino acid further on the ACVR-1 protein, was not assessed. At the biochemical level, binding of osteogenic signaling inhibitor FKBP12 is less efficient in both R206H and Q207E. We therefore have reasons to assume that the addition of Activin-A may result in similar effects in both mutations.

Given the expression of the mutated version of ACVR1 in FOP monocytes, which seemed higher even than the unaffected allele,

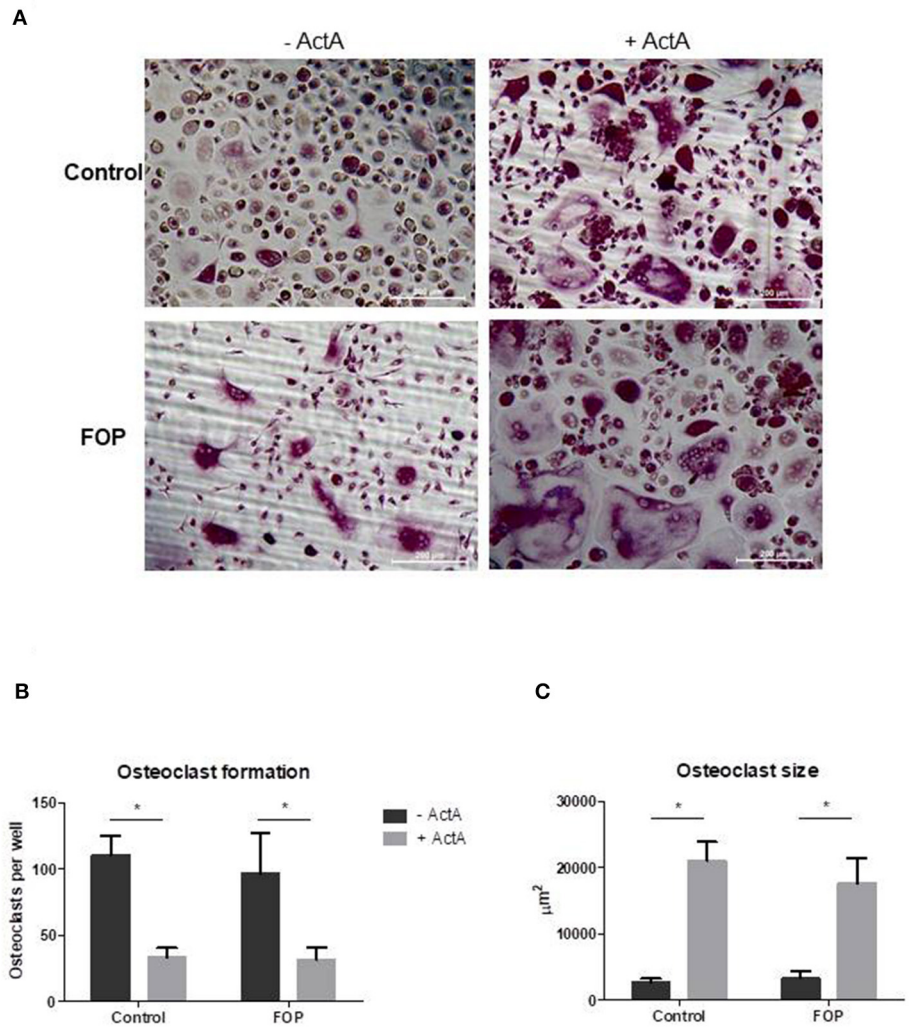


FIGURE 3 | Activin-A inhibits osteoclast formation in both control and FOP cultures. Control or FOP CD14+ cells were cultured with M-CSF and RANK-L, without and with Activin-A 50 ng/ml. Cells were stained for TRAcP and nuclei were stained with DAPI after 21 days of culture. **(A)** Micrograph of the stained cultures of the four different conditions after 21 days. **(B)** Equal numbers of osteoclasts were formed in the control and FOP cultures. Activin-A significantly inhibited osteoclast formation in both cultures. **(C)** The average size of the osteoclasts was significantly increased when Activin-A was added to the cultures. $n = 6$ for both control and FOP (Wilcoxon matched-pairs signed rank test, $^*p \leq 0.05$).

we hypothesized that Activin-A would have a more profound effect on the mutated CD14 cells compared to the control cells. However, we did not observe any difference in osteoclastogenesis between the control and FOP cultures. The addition of Activin-A had an inhibitory effect on the number of osteoclasts. However, these fewer osteoclasts were on average 5–8-fold larger in both cultures, suggesting this effect is not mediated via the mutated receptor. Binding of Activin-A to ACVR1 normally inhibits BMP signaling via this receptor (13, 32). In contrast to the generally accepted role of BMPs in osteogenesis, several studies have shown that BMP signaling is also important for osteoclast formation. Inhibition of this signaling, either via deletion of BMPR2 (33) or by inhibiting SMAD 4 or SMAD 1/5 (34, 35) reduces DC-STAMP expression and inhibits osteoclastogenesis. Similarly, the inhibitory effect of Activin-A on BMP signaling could be the cause of the inhibited osteoclastogenesis seen in this study. QPCR analysis however, showed a higher expression of ID-1, a downstream target molecule of SMAD1/5/8 phosphorylation (25), in the presence of Activin-A especially in the FOP cells, suggesting that enhanced SMAD1/5/8 signaling may occur in FOP patients derived monocytes. The fact that this does not seem to influence osteoclastogenesis might be related to the timing of effects. During the early stages of osteoclastogenesis signaling via the non-canonical BMP pathway seems to be more important, whereas during the later stages the canonical signaling pathway seems to play a more important role (33). The lower expression of M-CSF and DC-STAMP by Activin-A could explain the inhibition of osteoclastogenesis. Next to an

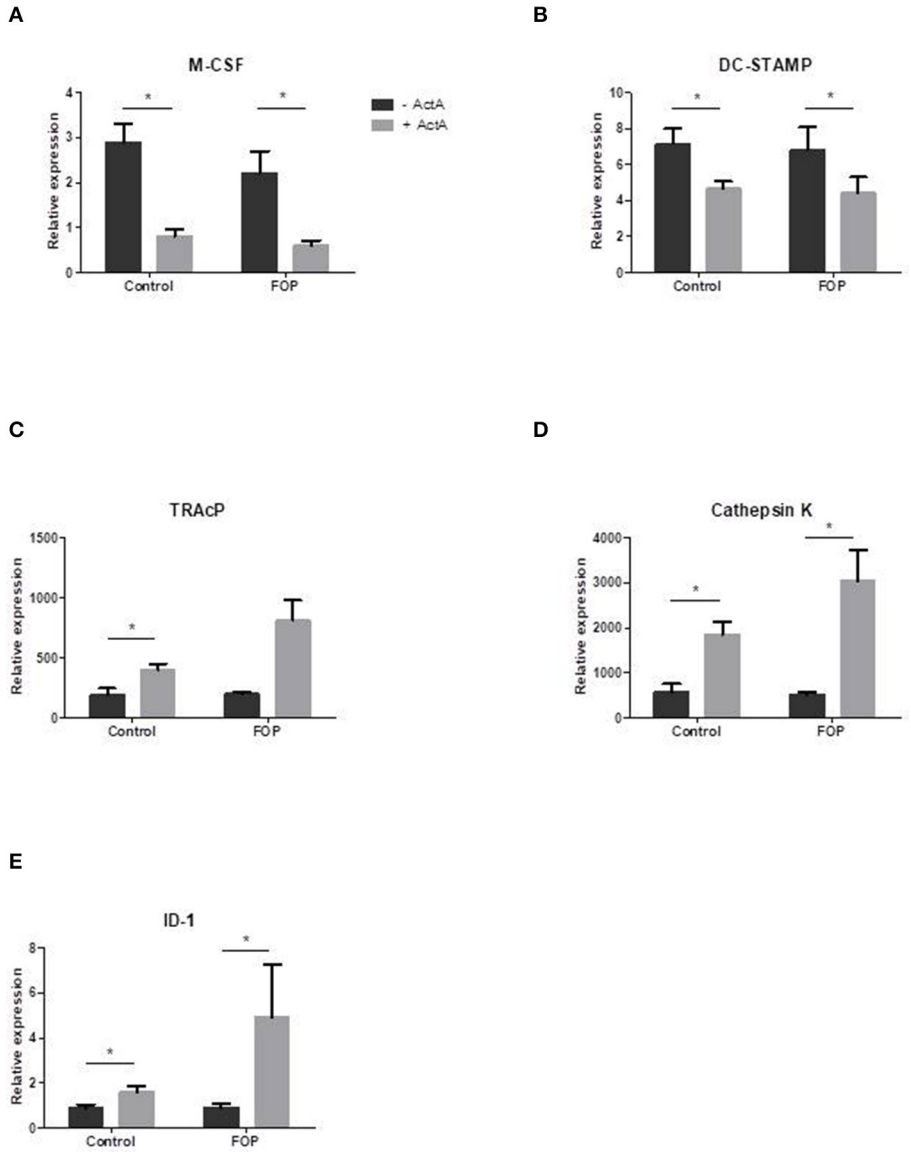


FIGURE 4 | Osteoclast related gene expression is altered in the presence of Activin-A. Control or FOP CD14+ cells were cultured with M-CSF and RANK-L, without and with Activin-A 50 ng/ml. RNA was isolated after 7 and 21 days and QPCR was performed. Gene expression of tested genes was similar in control and FOP cultures. **(A)** Expression of M-CSF was downregulated after 7 days of culturing in the presence of Activin-A. **(B)** Expression of DC-STAMP was downregulated after 7 days of culturing in the presence of Activin-A. **(C)** Expression of TRAcP was upregulated after 21 days of culturing in the presence of Activin-A. **(D)** Expression of Cathepsin K was upregulated after 21 days of culturing in the presence of Activin-A. **(E)** Expression of ID-1 was upregulated after 7 days of culturing in the presence of Activin-A. $n = 6$ for both control and FOP (Wilcoxon matched-pairs signed rank test, $^*p \leq 0.05$).

inhibitory effect on osteoclast formation we also observed that the presence of Activin-A induced larger osteoclasts. Osteoclast precursors with lower expression of the fusion receptor DC-STAMP have been shown to give rise to higher TRAcP expression and bigger osteoclasts compared to precursors with higher DC-STAMP expression (36). This could be the explanation for our observed difference in osteoclast size, since we also see a decreased DC-STAMP and increased TRAcP expression in the presence of Activin-A. Omi et al. recently reported that ACVR1 plays a role in osteoclast formation via BMP7 induced canonical SMAD signaling pathways (37). They also showed that signaling via BMPR1A seems to be more important for the fusion of osteoclast precursors. Possibly the observed increase in osteoclast size in our experiments is due to signaling of Activin-A via a BMPR1A receptor complex, probably bypassing ACVR-1. The correlation between osteoclast size and activity is not entirely clear. In some cases giant osteoclasts seem to be an indication of less active osteoclasts. This is especially apparent

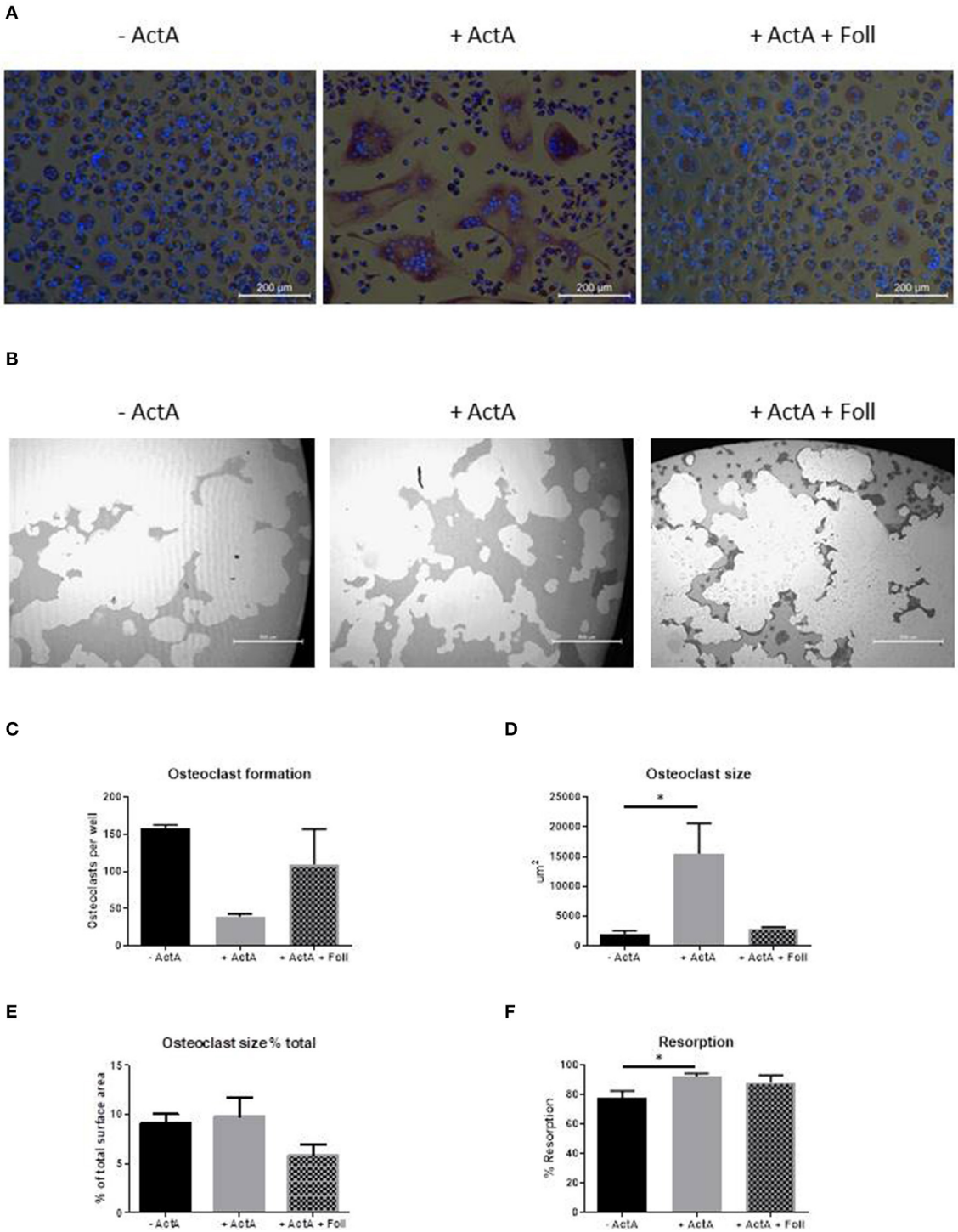


FIGURE 5 | Follistatin reduces the Activin-A effect. CD14+ cells from three healthy donors were cultured with M-CSF and RANK-L, without and with Activin-A (50 ng/ml). Experiments were plated in quadruplicate. To block the Activin-A effect a third experimental condition was added where follistatin (500 ng/ml) was present in the cultures. After 21 days cells were stained for TRAcP and nuclei were stained with DAPI. **(A)** Micrographs of the stained cultures of the three different conditions. **(B)** Micrographs of the lysed calcium phosphate surface from the osteo assay plates of the three different culture conditions. **(C)** Follistatin reduces the inhibitory effect of Activin-A on the number of formed osteoclasts. **(D)** Follistatin reduces the increasing effect of Activin-A on the size of the formed osteoclasts. **(E)** The percentage of the total area occupied by the formed osteoclasts does not differ between the three culture conditions. **(F)** The percentage of resorbed area in the osteo assay plates is higher when Activin-A is present in the cultures, implicating a higher activity per osteoclast. This effect is nullified when Follistatin is added. $n = 3$ (Friedmann test with Dunn's multiple comparisons, $^*p \leq 0.05$).

when osteoclast activity is inhibited by bisphosphonates. In other pathological conditions such as Paget Disease, the giant osteoclasts are also highly active (38, 39). A positive correlation between size and activity has been described by Piper et al., who correlated activity to the number of nuclei per osteoclast (40). We could later confirm this in another way, by correlating osteoclast area to actin ring surface (41). We showed that osteoclast size is proportional to the number of actin rings per osteoclast and that the percentage of actin ring area per osteoclast is relatively constant, being ∼20%. Together, these two studies imply that larger osteoclasts are more active in resorption, albeit that per osteoclast a constant area is used for resorption. Our results, less, but larger and more active osteoclast when cultured with Activin-A that cover a similar area as the more but smaller osteoclasts without Activin-A, also suggest that indeed the larger osteoclasts are more active, but that this activity is performed more localized since the the area covered by osteoclasts is the same in the two conditions. In the context of Activin-A, it is likely the local *in vivo* circumstances that determine overall osteoclast activity. Upadhyay et al. (42) recently described a partial resorption of HO after treatment with Activin-A antibodies in their FOP mouse model. This implies that osteoclast function is inhibited by local access of Activin-A and that the osteoclast function can be restored after Activin-A antibody treatment. Our results, with the obvious limitation of the cell biological approach using CD14+ cells and differentiation factors M-CSF and RANKL in the presence of Activin A, suggest an increase of resorption. Whether inhibition of Activin-A *in vivo* may have different effects, cannot be ruled out. In conjunction with the heterotopic bone formation potential of Activin A in FOP, our results rather show that Activin-A may contribute to an increased bone metabolism altogether.

Our study is the first to investigate the effect of Activin-A on human FOP-patient derived osteoclasts. We demonstrated that Activin-A induces fewer but larger osteoclasts irrespective of the presence of the mutated ACVR1 receptor, but further studies on FOP-patients derived cells are necessary for understanding the full width of the mode of action of Activin-A. This is even more important in the light of promising ongoing clinical trials in FOP that specifically target mutant ACVR-1 in general or Activin-A in particular (43). It remains intriguing that Activin A only causes heterotopic bone formation, and only in FOP, leaving the normal skeleton seemingly untouched. Likewise, it is conceivable that osteoclasts that remodel heterotopic bone, could respond differently to anti-Activin A in the microenvironment of heterotopic bone. We propose that when inhibiting osteogenesis by anti-Activin A in a heterotopic bone context, osteoclast activity could be reduced but more dispersed since it is performed by more but smaller osteoclasts.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Review Committee of the Amsterdam UMC, Vrije Universiteit Amsterdam. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TS designed and conducted the study, performed the data analysis and wrote the manuscript. EB, DM, CN, and NB contributed to the design of the study and writing the manuscript. MS performed the follistatin experiments. AK performed the FACS analysis. EE and TD supervised the study, contributed to the study design, and writing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Over the years TS and TD have received research grants from the International Fibrodysplasia Ossificans Progressiva Organization (IFOPA) and from Friends of Dutch FOP Foundation, which enabled some of the analyses described in this paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00501/full#supplementary-material

Supplementary Figure 1 | Gating strategies for the FACS analysis. Gating strategy used to discriminate between between classical monocytes (CD14++CD16−), intermediate monocytes (CD14++CD16+) and non-classical monocytes (CD14+CD16+).

Supplementary Figure 2 | Follistatin reduces the Activin-A effect on osteoclast related gene expression. CD14+ cells from pne healthy donor were cultured with M-CSF and RANK-L, without and with Activin-A (50 ng/ml). Experiments were plated in quadruplicate. To block the Activin-A effect a third experimental condition was added where follistatin (500 ng/ml) was present in the cultures. RNA was isolated after 7 days and QPCR was performed. The inductive effect of Activin-A on the gene expression of **(A)** the early transcription factor NFATc1, **(B)** the on osteoclasts abundantly expressed AlphaV integrin, **(C,D)** the osteoclast specific markers TRAcP and Cathepsin K is reduces by follistatin. $N = 1$, mRNA was isolated from one of confirmative experiments shown in **Figure 5**, $n = 4$ wells per condition (Friedmann test with Dunn's multiple comparisons).

Supplementary Table 1 | Monoclonal antibody clones used in FACS analysis.

REFERENCES

1. Kaplan FS, Le Merrer M, Glaser DL, Pignolo RJ, Goldsby RE, Kitterman JA, et al. Fibrodysplasia ossificans progressiva. best practice and research. *Clin Rheumatol.* (2008) 22:191–205. doi: 10.1016/j.berh.2007.11.007
2. Pignolo RJ, Shore EM, Kaplan FS. Fibrodysplasia ossificans progressiva: clinical and genetic aspects. *Orphanet J Rare Dis.* (2011) 6:80. doi: 10.1186/1750-1172-6-80
3. Bravenboer N, Micha D, Triffit JT, Bullock AN, Ravazollo R, Bocciardi R, et al. Clinical utility gene card for: fibrodysplasia ossificans

progressiva. *Eur J Hum Genet*. (2015) 23:1431. doi: 10.1038/ejhg.2014.274
4. Huning I, Gillessen-Kaesbach G. Fibrodysplasia ossificans progressiva: clinical course, genetic mutations and genotype-phenotype correlation. *Mol Syndromol*. (2014) 5:201–11. doi: 10.1159/000365770
5. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nat Genet.* (2006) 38:525–7. doi: 10.1038/ng1783
6. Billings PC, Fiori JL, Bentwood JL, O'Connell MP, Jiao X, Nussbaum B, et al. Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP). *J Bone Miner Res.* (2008) 23:305–13. doi: 10.1359/jbmr.071030
7. Song GA, Kim HJ, Woo KM, Baek JH, Kim GS, Choi JY, et al. Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. *J Biol Chem.* (2010) 285:22542–53. doi: 10.1074/jbc.M109.094557
8. de la Pena LS, Billings PC, Fiori JL, Ahn J, Kaplan FS, Shore EM. Fibrodysplasia Ossificans Progressiva (FOP), a disorder of ectopic osteogenesis, misregulates cell surface expression and trafficking of BMPRIA. *J Bone Miner Res.* (2005) 20:1168–76. doi: 10.1359/JBMR.050305
9. Fiori JL, Billings PC, de la Pena LS, Kaplan FS, Shore EM. Dysregulation of the BMP-p38 MAPK signaling pathway in cells from patients with fibrodysplasia ossificans progressiva (FOP). *J Bone Miner Res.* (2006) 21:902–9. doi: 10.1359/jbmr.060215
10. Kaplan FS, Fiori J, DE LA Peña LS, Ahn J, Billings PC, and Shore EM. Dysregulation of the BMP-4 signaling pathway in fibrodysplasia ossificans progressiva. *Ann N Y Acad Sci.* (2006) 1068:54–65. doi: 10.1196/annals.1346.008
11. Olsen OE, Wader KF, Hella H, Mylin AK, Turesson I, Nesthus I, et al. Activin A Inhibits BMP-signaling by Binding ACVR2A and ACVR2B. *Cell Commun Signal.* (2015) 13:27. doi: 10.1186/s12964-015-0104-z
12. Hino K, Ikeya M, Horigome K, Matsumoto Y, Ebise H, Nishio M, et al. Neofunction of ACVR1 in fibrodysplasia ossificans progressiva. *Proc Natl Acad Sci USA.* (2015) 112:15438–43. doi: 10.1073/pnas.1510540112
13. Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, et al. ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. *Sci Transl Med.* (2015) 7:303ra137. doi: 10.1126/scitranslmed.aac4358
14. Hsiao EC, Di Rocco M, Cali A, Zasloff M, Al Mukaddam M, Pignolo RJ, et al. Special considerations for clinical trials in fibrodysplasia ossificans progressiva (FOP). *Br J Clin Pharmacol*. (2019) 85:1199–207. doi: 10.1111/bcp.13777
15. Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simoes MJ, Cerri PS. Biology of bone tissue: structure, function, and factors that influence bone cells. *Biomed Res Int*. (2015) 2015:421746. doi: 10.1155/2015/421746
16. Matsuo K, Irie N. Osteoclast-osteoblast communication. *Arch Biochem Biophys*. (2008) 473:201–9. doi: 10.1016/j.abb.2008.03.027
17. Sorensen MG, Henriksen K, Schaller S, Henriksen DB, Nielsen FC, Dziegiel MH, et al. Characterization of osteoclasts derived from CD14+ monocytes isolated from peripheral blood. *J Bone Miner Metabol.* (2007) 25:36–45. doi: 10.1007/s00774-006-0725-9
18. de Vries TJ, Schoenmaker T, Micha D, Hogervorst J, Bouskla S, Forouzanfar T, et al. Periodontal ligament fibroblasts as a cell model to study osteogenesis and osteoclastogenesis in fibrodysplasia ossificans progressiva. *Bone*. (2018) 109:168–77. doi: 10.1016/j.bone.2017.07.007
19. Schoenmaker T, Wouters F, Micha D, Forouzanfar T, Netelenbos C, Eekhoff EMW, et al. The effect of Activin-A on periodontal ligament fibroblasts-mediated osteoclast formation in healthy donors and in patients with fibrodysplasia ossificans progressiva. *J Cell Physiol.* (2018) 234:10238–47. doi: 10.1002/jcp.27693
20. Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, et al. Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. *Human Mutation.* (2009) 30:379–90. doi: 10.1002/humu.20868
21. Haupt J, Deichsel A, Stange K, Ast C, Bocciardi R, Ravazzolo R, et al. ACVR1 p.Q207E causes classic fibrodysplasia ossificans progressiva and is functionally distinct from the engineered constitutively active ACVR1 p.Q207D variant. *Human Mol Genet.* (2014) 23:5364–77. doi: 10.1093/hmg/ddu255
22. Sprangers S, Schoenmaker T, Cao Y, Everts V, de Vries TJ. Different blood-borne human osteoclast precursors respond in distinct ways to IL-17A. *J Cell Physiol.* (2016) 231:1249–60. doi: 10.1002/jcp.25220
23. ten Harkel B, Schoenmaker T, Picavet DI, Davison NL, de Vries TJ, Everts V. The foreign body giant cell cannot resorb bone, but dissolves hydroxyapatite like osteoclasts. *PLoS ONE.* (2015) 10:e0139564. doi: 10.1371/journal.pone.0139564
24. Davison NL, ten Harkel B, Schoenmaker T, Luo X, Yuan H, Everts VF. Barrere-de Groot, and de Bruijn JD, Osteoclast resorption of beta-tricalcium phosphate controlled by surface architecture. *Biomaterials.* (2014) 35:7441–51. doi: 10.1016/j.biomaterials.2014.05.048
25. Wang H, Shore EM, Pignolo RJ, Kaplan FS. Activin A amplifies dysregulated BMP signaling and induces chondro-osseous differentiation of primary connective tissue progenitor cells in patients with fibrodysplasia ossificans progressiva (FOP). *Bone*. (2017) 109:218–24. doi: 10.1016/j.bone.2017.11.014
26. Kaplan J, Kaplan FS, Shore EM. Restoration of normal BMP signaling levels and osteogenic differentiation in FOP mesenchymal progenitor cells by mutant allele-specific targeting. *Gene Therapy.* (2012) 19:786–90. doi: 10.1038/gt.2011.152
27. de Vries TJ, El Bakkali I, Kamradt T, Schett GI, Jansen DC, D'Amelio P. What are the peripheral blood determinants for increased osteoclast formation in the various inflammatory diseases associated with bone loss? *Front Immunol.* (2019) 10:505. doi: 10.3389/fimmu.2019.00505
28. Gaddy-Kurten D, Coker JK, Abe E, Jilka RL, Manolagas SC. Inhibin suppresses and activin stimulates osteoblastogenesis and osteoclastogenesis in murine bone marrow cultures. *Endocrinology*. (2002) 143:74–83. doi: 10.1210/endo.143.1.8580
29. Pearsall RS, Canalis E, Cornwall-Brady M, Underwood KW, Haigis B, Ucran J, et al. A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. *Proc Natl Acad Sci USA*. (2008) 105:7082–7. doi: 10.1073/pnas.0711263105
30. Kajita T, Ariyoshi W, Okinaga T, Mitsugi S, Tominaga K, Nishihara T, et al. Mechanisms involved in enhancement of osteoclast formation by activin-A. *J Cell Biochem.* (2018) 119:6974–85. doi: 10.1002/jcb.26906
31. Fowler TW, Kamalakar A, Akel NS, Kurten RC, Suva LJ, Gaddy D. Activin A inhibits RANKL-mediated osteoclast formation, movement and function in murine bone marrow macrophage cultures. *J Cell Sci.* (2015) 128:683–94. doi: 10.1242/jcs.157834
32. Lowery JW, Intini G, Gamer L, Lotinun S, Salazar VS, Ote S, et al. Loss of BMPR2 leads to high bone mass due to increased osteoblast activity. *J Cell Sci.* (2015) 128:1308–15. doi: 10.1242/jcs.156737
33. Broege A, Pham L, Jensen ED, Emery A, Huang TH, Stemig M, et al. Bone morphogenetic proteins signal via SMAD and mitogen-activated protein (MAP) kinase pathways at distinct times during osteoclastogenesis. *J Biol Chem.* (2013) 288:37230–40. doi: 10.1074/jbc.M113.496950
34. Tasca A, Stemig M, Broege A, Huang B, Davydova J, Zwijsen A, et al. Smad1/5 and Smad4 expression are important for osteoclast differentiation. *J Cell Biochem.* (2015) 116:1350–60. doi: 10.1002/jcb.25092
35. Tasca A, Astleford K, Blixt NC, Jensen ED, Gopalakrishnan R, Mansky KC. SMAD1/5 signaling in osteoclasts regulates bone formation via coupling factors. *PLoS ONE.* (2018) 13:e0203404. doi: 10.1371/journal.pone.0203404
36. Mensah KA, Ritchlin CT, Schwarz EM. RANKL induces heterogeneous DC-STAMP(lo) and DC-STAMP(hi) osteoclast precursors of which the DC-STAMP(lo) precursors are the master fusogens. *J Cell Physiol.* (2010) 223:76–83. doi: 10.1002/jcp.22012
37. Omi M, Kaartinen V, Mishina Y. Activin A receptor type 1-mediated BMP signaling regulates RANKL-induced osteoclastogenesis via canonical SMAD-signaling pathway. *J Biol Chem.* (2019) 294:17818–36. doi: 10.1074/jbc.RA119.009521
38. Weinstein RS, Roberson PK, Manolagas SC. Giant osteoclast formation and long-term oral bisphosphonate therapy. *N Engl J Med.* (2009) 360:53–62. doi: 10.1056/NEJMoa0802633

39. Jobke B, Milovanovic P, Amling M, Busse B. Bisphosphonate-osteoclasts: changes in osteoclast morphology and function induced by antiresorptive nitrogen-containing bisphosphonate treatment in osteoporosis patients. *Bone*. (2014) 59:37–43. doi: 10.1016/j.bone.2013.10.024
40. Piper K, Boyde A, Jones SJ. The relationship between the number of nuclei of an osteoclast and its resorptive capability *in vitro*. *Anat Embryol*. (1992) 186:291–9. doi: 10.1007/BF00185977
41. Azari A, Schoenmaker T, de Souza Faloni AP, Everts V, de Vries TJ. Jaw and long bone marrow derived osteoclasts differ in shape and their response to bone and dentin. *Biochem Biophys Res Commun.* (2011) 409:205–10. doi: 10.1016/j.bbrc.2011.04.120
42. Upadhyay J, Xie L, Huang L, Das N, Stewart RC, Lyon MC, et al. The expansion of heterotopic bone in fibrodysplasia ossificans progressiva is Activin A-dependent. *J Bone Miner Res.* 32:2489–99. (2017). doi: 10.1002/jbmr.3235
43. Wentworth KL, Masharani U, Hsiao EC. Therapeutic advances for blocking heterotopic ossification in fibrodysplasia ossificans progressiva. *Br J Clin Pharmacol.* (2018) 85:1180–7. doi: 10.1111/bcp.13823

Conflict of Interest: 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 Endocrinology

REVIEW
published: 31 July 2020
doi: 10.3389/fendo.2020.00489

Zebrafish: A Resourceful Vertebrate Model to Investigate Skeletal Disorders

Francesca Tonelli[1†], Jan Willem Bek[2†], Roberta Besio[1†], Adelbert De Clercq[2†], Laura Leoni[1], Phil Salmon[3], Paul J. Coucke[2], Andy Willaert[2‡] and Antonella Forlino[1‡]*

[1] Biochemistry Unit, Department of Molecular Medicine, University of Pavia, Pavia, Italy, [2] Department of Biomolecular Medicine, Center of Medical Genetics, Ghent University-University Hospital, Ghent, Belgium, [3] Bruker microCT, Kontich, Belgium

Animal models are essential tools for addressing fundamental scientific questions about skeletal diseases and for the development of new therapeutic approaches. Traditionally, mice have been the most common model organism in biomedical research, but their use is hampered by several limitations including complex generation, demanding investigation of early developmental stages, regulatory restrictions on breeding, and high maintenance cost. The zebrafish has been used as an efficient alternative vertebrate model for the study of human skeletal diseases, thanks to its easy genetic manipulation, high fecundity, external fertilization, transparency of rapidly developing embryos, and low maintenance cost. Furthermore, zebrafish share similar skeletal cells and ossification types with mammals. In the last decades, the use of both forward and new reverse genetics techniques has resulted in the generation of many mutant lines carrying skeletal phenotypes associated with human diseases. In addition, transgenic lines expressing fluorescent proteins under bone cell- or pathway- specific promoters enable *in vivo* imaging of differentiation and signaling at the cellular level. Despite the small size of the zebrafish, many traditional techniques for skeletal phenotyping, such as x-ray and microCT imaging and histological approaches, can be applied using the appropriate equipment and custom protocols. The ability of adult zebrafish to remodel skeletal tissues can be exploited as a unique tool to investigate bone formation and repair. Finally, the permeability of embryos to chemicals dissolved in water, together with the availability of large numbers of small-sized animals makes zebrafish a perfect model for high-throughput bone anabolic drug screening. This review aims to discuss the techniques that make zebrafish a powerful model to investigate the molecular and physiological basis of skeletal disorders.

Keywords: zebrafish, skeletal system, x-ray, microCT analyses, imaging techniques, skeletal diseases

OPEN ACCESS

Edited by:
Wim Van Hul,
University of Antwerp, Belgium

Reviewed by:
Christoph Winkler,
National University of Singapore, Singapore
Michaël R. Laurent,
University Hospitals Leuven, Belgium

****Correspondence:***
Antonella Forlino
aforlino@unipv.it

† These authors have contributed equally to this work

‡ These authors share last authorship

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *25 April 2020*
Accepted: *22 June 2020*
Published: *31 July 2020*

Citation:
Tonelli F, Bek JW, Besio R, De Clercq A, Leoni L, Salmon P, Coucke PJ, Willaert A and Forlino A (2020) Zebrafish: A Resourceful Vertebrate Model to Investigate Skeletal Disorders. Front. Endocrinol. 11:489. doi: 10.3389/fendo.2020.00489

INTRODUCTION

Preclinical animal models can be used to elucidate gene and protein function in ways often impossible in humans, by means of genome sequencing, advances in DNA manipulation and high resolution live-imaging (1). Mammals such as mice and non-human primates are traditionally the preferred models for biomedical research due to their close evolutionary relationship with humans.

However, their use is costly and studies at early developmental stages raise ethical concerns. Furthermore, in most countries the adoption of the "Three R's" principles: Replacement, Reduction, and Refinement (2) for animal research is mandatory and encourages the use of alternative models, such as *Danio rerio* (zebrafish), *Xenopus laevis/tropicalis* (clawed toad), *Drosophila melanogaster* (fruit fly), and *Caenorhabditis elegans* (nematode). In these organisms *in vivo* techniques can be applied with the simplicity and versatility of *in vitro* assays and therefore they are frequently used in fundamental and biomedical research to quickly define gene functions and to develop novel therapeutic options (3). Zebrafish, the most frequently employed non-mammalian vertebrate animal model, is a freshwater bony fish, belonging to the Cyprinidae family and to the Teleostei infraclass of ray-finned fish which arose ∼340 million years ago (4). This species was initially described by the Scottish physician and naturalist Hamilton (5) in a survey on South Asian flora and fauna. Starting from the pioneering research of George Streisinger in the 70s−80s, who was the first to clone a zebrafish and in this way demonstrated the easy genetic manipulation of this species (6), zebrafish became a powerful model organism for developmental studies, genetic research, drug and toxicology screenings and for understanding tissue regeneration and repair (7–9). In contrast to other vertebrate models such as mice, fertilization occurs externally, which together with transparency and rapid embryo to larval transition permits easy access and visualization of development (10) (**Figure 1**). Moreover, due to its rapid growth, a recognizable and complete vertebrate body

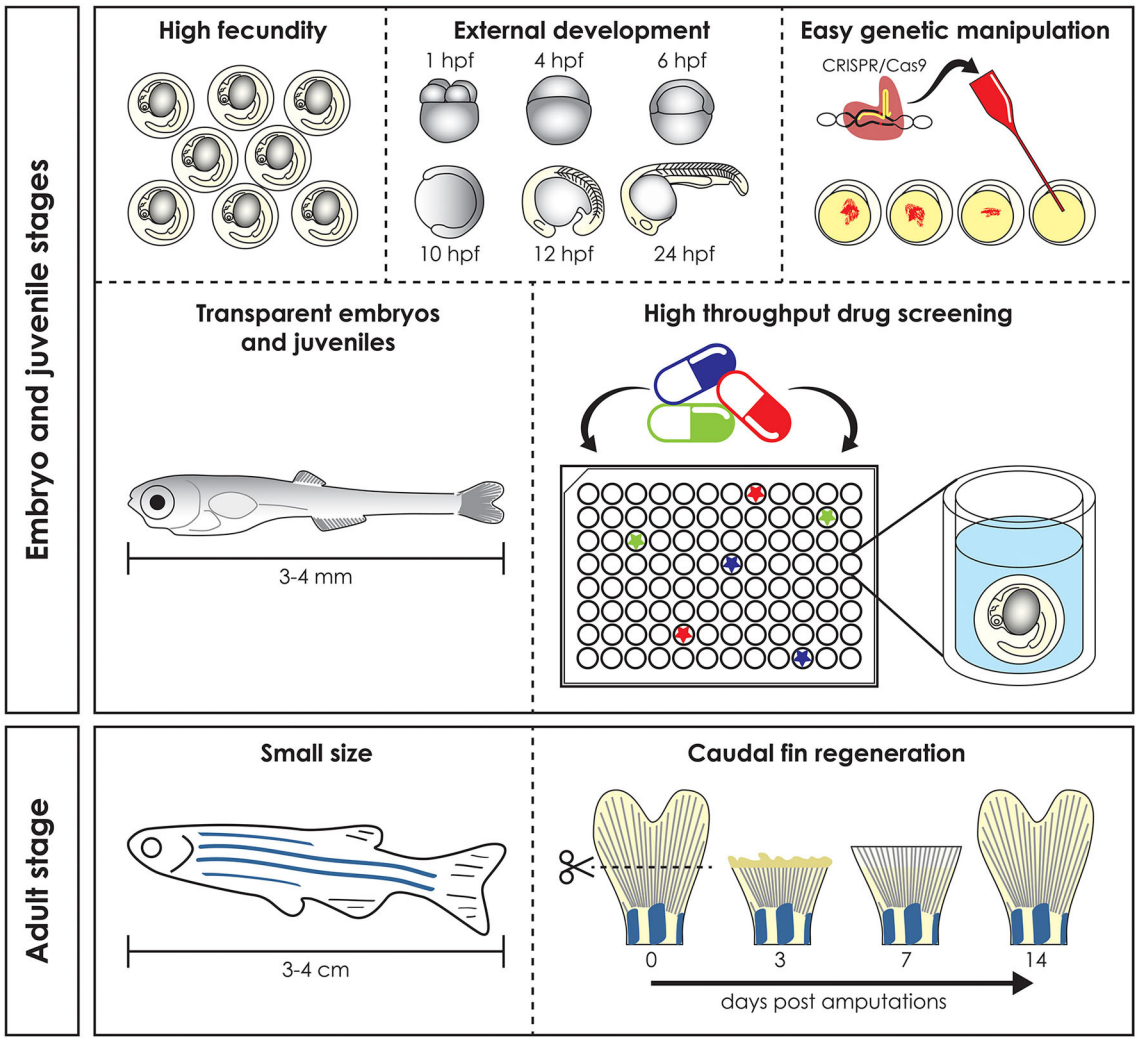


FIGURE 1 | Advantages of the zebrafish model. Zebrafish has several advantages compared to mammal models. High fecundity and external fertilization and development allow easy genomic manipulation, transparent early life stages guarantee *in vivo* imaging and skin permeability makes them suitable for high throughput drug screening (top). Adult zebrafish reaches a maximum size of 3–4 cm and this make it easy and cheap to keep it in large numbers, reducing the husbandry cost (bottom left). Finally, zebrafish is used as a vertebrate model to study regeneration, due to its ability to regenerate different organs, such as the caudal fin, which is completely regenerated 14 days post amputation (bottom right). hpf, hours post fertilization.

plan is already in place by 1 day post fertilization (dpf) and embryogenesis is complete by 3 dpf (11). In contrast to other vertebrate models such as rodents, the small size and large number of offspring of zebrafish allow for increased sample numbers, thereby increasing the statistical power of experiments (3). Finally, the relatively low husbandry cost further contributed to the increasing popularity of the zebrafish as a model for research (11).

Besides developmental studies, the zebrafish is an established research model in many other research fields. During the last 20 years, the zebrafish has proven itself as a useful model to study disease mechanisms (1). This is due to its physiological relevance and genetic tractability to model genetic variation in humans. Compared to mammalian model organisms, the zebrafish genome underwent an extra (third) whole duplication about 350 million years ago, with the result that for many genes in humans, there may be two copies (paralogues) in zebrafish. Despite this there is a relatively high level of genome conservation between zebrafish and humans with more than 70% of human protein-coding genes having at least one zebrafish ortholog. The haploid zebrafish genome has 25 chromosomes containing 1.7 billion base pairs (4). Various forward and reverse genetic approaches have been applied to generate mutant lines that mimic many different human diseases, including skeletal diseases ranging from secondary osteoporosis (OP) to rare disorders such as osteogenesis imperfecta (OI) (12–20). A major benefit of zebrafish is the simplicity of combining mutant and transgenic lines that express fluorescent reporter proteins under the control of responsive elements for signaling pathways or promoters of cell-type-specific markers. This in turn allows for *in vivo* investigation of the effect of a disease mutation on the spatio-temporal expression of specific genes, and on cell differentiation and signaling pathways.

Zebrafish larvae have been intensively used for pharmacological and toxicological screens, because of their small size (easy distribution in microtiter well plates), high abundance and their ability to absorb small compounds from the water through the skin and gills (21). Together with the availability of many different disease models, the zebrafish is a unique tool to develop novel targeted pharmacological approaches (**Figure 1**) (21).

Finally, their ability to regenerate some cells and tissues, such as fins and scales, makes the zebrafish a valuable model for understanding organ repair mechanisms during healthy and pathological conditions (**Figure 1**) (22).

This review, after providing a brief overview of zebrafish bone biology, will focus on the description and use of the various techniques and approaches which make *Danio rerio* a powerful model organism to investigate the molecular and physiological basis of skeletal disorders.

ZEBRAFISH BONE BIOLOGY

The Skeleton

Skeletal development and gene expression and the general inventory of bone types are conserved between zebrafish and mammals, nevertheless few differences need to be considered when using this animal as model for skeletal study. Osteocytes are not present in all bones and/or at all developmental stages, endochondral ossification is rare in zebrafish and vertebral body do not build on a cartilaginous anlage (23, 24). The common perception of mammals being more complex than "lower" organisms, such as teleosts, is false, especially concerning the skeleton. Certain characteristics of the teleost skeleton are more advanced and elaborate compared to mammals, such as the zebrafish skull that contains at least twice the number of bones (24). At the tissue level, the mammalian skeleton mostly consists of cellular bone and hyaline cartilage. While other types of bone, such as hyperostotic and acellular bone and cartilage (i.e., fiber cartilage), can be present in mammalian skeletons, they are often associated with pathological processes. However, in teleosts many different bone and cartilage types with different cellularity and matrix composition exist in wild type conditions not related to disease (25). The zebrafish skeleton consists of a dermal skeleton and an endoskeleton. Scales, polarized structures of the exoskeleton, teeth, and fin rays are part of the dermal skeleton and are distinctive as skeletal structures in their ability to regenerate (25–27). In fish, teeth, scales, and fin rays can all be traced back in evolution to a single structure, called the odontode (28), and they arise at the epithelial-mesenchymal border (29, 30). It has been shown that the mesenchymal tissues that engender these skeletal elements have a neural crest origin (29, 31, 32).

The endoskeleton consists of cranial, axial, and appendicular skeletal elements (33). As in all vertebrates, the zebrafish cranial skeleton arises mostly from the cranial neural crest, while the appendicular skeleton develops from somite-derived paraxial mesoderm (31, 33). In contrast with tetrapods, in which vertebral centrum formation is controlled by somites patterned along the vertebral column, in teleosts the notochord has an instructive role in vertebral centrum patterning as the centra start out as mineralization foci in the notochord sheath (34, 35).

Skeletal Cells

The teleost and mammalian skeletal systems share similar cell types (**Figure 2A**). In cartilage there are (i) chondroblasts as the cartilage forming cells and (ii) chondrocytes maintaining the cartilage matrix. In bone there are (i) osteoblasts as the bone forming cells, (ii) osteocytes that act as the mechanosensors regulating osteoblast and osteoclast activity and (iii) osteoclasts which are the bone resorbing cells (24, 37). Similar to mammals, teleost skeletal histogenesis involves the differentiation of chondroblasts and osteoblasts, that secrete the collagen extracellular matrix, from mesenchymal stem cells (38, 39). Both in mammals and fish, skeletal cells are formed by a complex interplay of intracellular molecular pathways and secreted factors that regulate the timing, location, and pathway by which bone cells differentiate (40–42). Although not investigated in mammals before, in zebrafish osteoblasts are present in clusters at the end of growing bones and can be classified in two different groups (type I and type II) based on cell cluster size, location, and nuclei shape, although they have overlapping functions (36). Type I osteoblasts are located at the edges of growing flat bones, such as the dentary, maxillary, and frontal bone, in large clusters with more than 25 cells with a wide oval, round, or

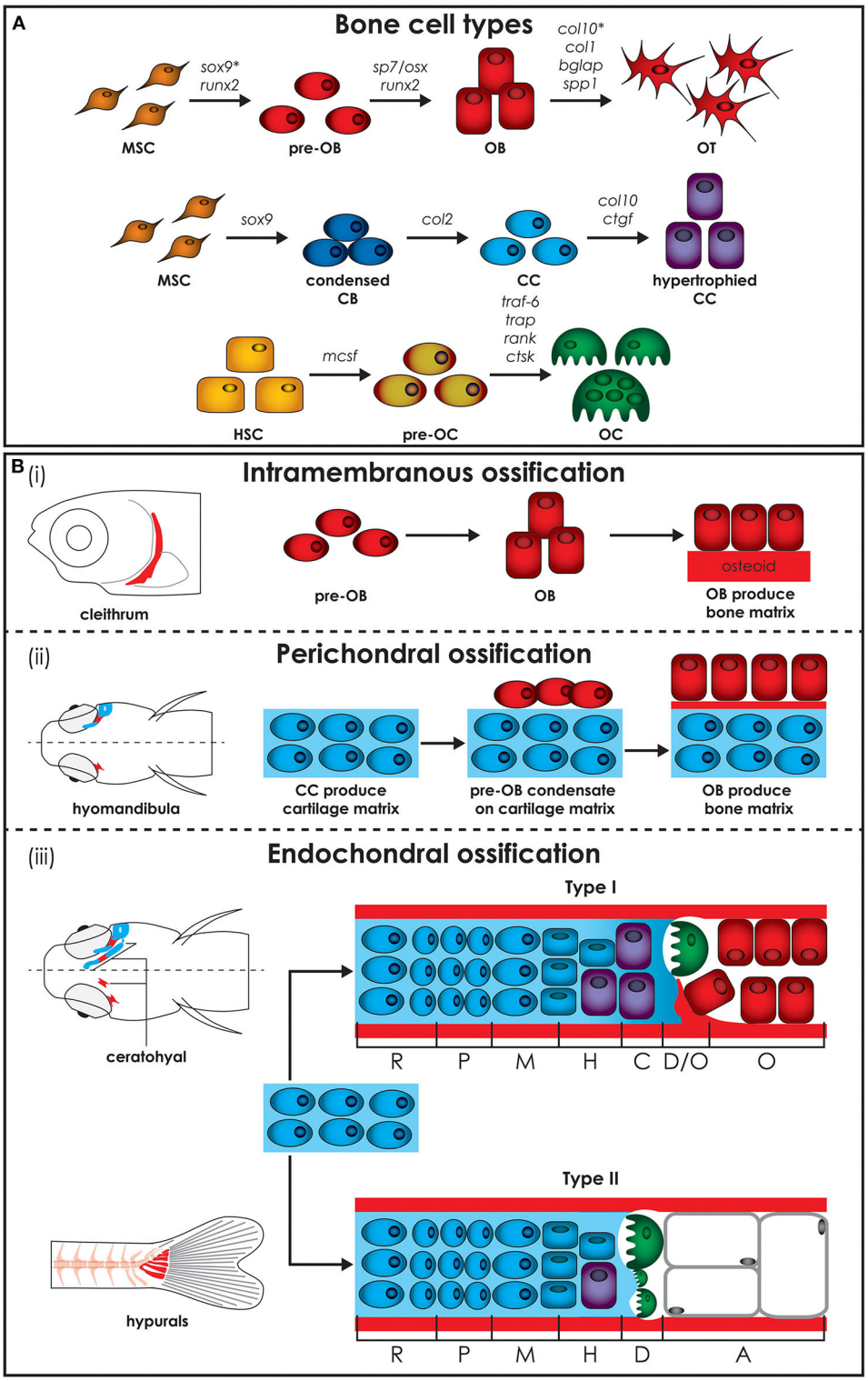


FIGURE 2 | Zebrafish bone cells and ossification types. **(A)** Bone is formed by osteoblasts and osteocytes, while cartilage is formed by chondroblasts and chondrocytes, and both bone and cartilage are degraded by osteoclasts. All bone cell types develop from progenitors similar to the mammalian counterpart and share similar gene expression profiles (genes are indicated above arrows). Note however that HSCs in zebrafish are not present in the bone marrow but in the head kidney. In addition, the genes for collagen X, encoded by *col10*, and SRY-box transcription factor 9 (indicated by*), encoded by *sox9*, are expressed during osteoblasts differentiation in zebrafish, but not in humans. **(B)** Three types of ossification are present in zebrafish: (i) intramembranous ossification, (ii) perichondral ossification,

(Continued)

FIGURE 2 | present in teleosts but not in humans, and (iii) endochondral ossification. (i) During intramembranous ossification mesenchymal stem cells condensate and differentiate into pre-osteoblasts and finally into mature osteoblasts that deposit bone matrix (osteoid) that subsequently mineralizes. (ii) Perichondral ossification starts at the surface of a cartilaginous template where osteoblasts deposit bone matrix without replacing the cartilage. (iii) Endochondral ossification is the process by which growing cartilage is replaced by bone to allow the skeleton to grow. For ossification to start, matrix surrounding the chondrocytes must calcify so that osteoclasts can break down the cartilage. In teleost two types of endochondral ossification exist. Type I endochondral ossification, typical in the ceratohyal, resembles the mammalian endochondral ossification process. This is characterized by a hypertrophic zone, where the cartilage matrix calcifies, followed by a degradation zone where osteoclasts (also referred to as chondroclasts) degrade the cartilaginous matrix, and a bone formation zone. Type II ossification, in the hypurals, is characterized by a lack of the calcification and ossification zones, leading to tubular concave bones filled with adipose tissue. Schematics based on detail description in Weigele and Franz-Odendaal (36). A, adipose zone; C, calcification zone; CB, chondroblasts; CC, chondrocytes; D, degradation zone; H, hypertrophic zone; HSC, hematopoietic stem cell; M, maturation zone; MSC, mesenchymal stem cell; O, ossification zone; OB, osteoblasts; OC, osteoclasts; OT, osteocytes; P, proliferation zone; R, rest zone.

irregularly shaped nucleus. Laterally to these cells there is a zone of differentiating osteoblasts where cells are smaller and more elongated, assuming the typical spindle shape of osteoblast-like cells, which cover all zebrafish bones with a monolayer at the level of the perichondrium (36). Type II osteoblast clusters are smaller (4–12 cells) and are distributed throughout the skeleton. These osteoblasts have a reduced size, elongated nucleus and are present throughout the bony trabecular network of spongy bones. Type II osteoblast clusters can also be detected at the edges of cartilage break down zones and lateral to the epiphysial growth plate, at the outer surface of tubular bones (36).

Most skeletal elements in the adult zebrafish skeleton contain osteocytes, but with a smaller volume and less canaliculi compared to mice and humans (36). The mechanosensing ability of osteocytes in zebrafish is not fully understood yet, but it was shown that osteocytes have a preferred orientation in adult zebrafish vertebrae (36). Acellular bone, without trapped osteocytes, can be found in many zebrafish cranial bones. Contrary to expectations, acellular bone does not appear to be stiffer due to the lack of osteocyte lacunae, making the role of acellular bone unclear (43). It is important to note that both cellular and acellular bone can occur within the same bony element. Osteon-like structures in zebrafish have been reported (for the lateral ethmoid bone) but these structures, composed of a central Haversian canal and bone lamella, do not have osteocytes (36).

In mammals, bone resorbing cells are multinucleated macrophages originating from the fusion and maturation of peripheral blood monocytes differentiated from hematopoietic bone marrow cells (44). Multinucleated osteoclasts can also be found in teleosts, especially in basal teleosts, such as salmonids and cyprinids (45). Nevertheless, in teleosts, smaller and mononucleated osteoclasts are predominant, but they retain the molecular regulators of mammalian osteoclast function (37). Examples include receptor activator of nuclear factor kappa-B (Rank) and Rank-ligand (Rankl) which are important for osteoclast maturation. Mature osteoclasts become tartrate-resistant acid phosphatase (Trap) and cathepsin K (CtsK) positive, which are both required for the cells to be able to degrade bone matrix components (37, 46). Zebrafish are characterized by an ontogenic change at 30 dpf when mononucleated osteoclasts evolve to multinucleated osteoclasts, which perform lacunar resorption and bone remodeling (37).

Each cell type achieves and performs its function by involving specific genes, acting as molecular fingerprints. All three bone cell types develop from similar progenitors as their mammalian counterpart and share similar profiles of gene expression (**Figure 2A**) (36). Gene expression of zebrafish collagen and transcription factor in skeletal cells of cartilage and bone are not completely conserved with mammals. Unlike mammals, zebrafish osteoblasts express collagen type X and various teleosts have been shown to have collagen type II in their bone matrix (47, 48). In addition, *Sox9* expression, which is required for differentiation of chondrocytes, but not of osteoblasts in mammals, has been reported to be involved in bone development in teleosts (49). Unlike tetrapods, zebrafish type I collagen, the most abundant protein in bone, has three instead of two different α chains, namely α1, α3, and α2 encoded by *col1a1a*, *col1a1b,* and *col1a2,* respectively (50). Based on the amino acid sequence, the α3 chain is phylogenetically similar to α1, supporting the common origin of their coding genes, which derive from a genome duplication that occurred at an early stage in teleost evolution (51). Importantly, all amino acid residues involved in human/mouse collagen type I cross-links are conserved in zebrafish, suggesting the existence of similar extracellular assembly (50).

Bone Ossification

Bone formation starts in zebrafish around 4–5 dpf. The bony elements can have three modes of ossification: intramembranous, perichondral, or endochondral. Intramembranous ossification starts with mesenchymal cell condensation and differentiation into osteoblasts, without the need of a cartilage template (**Figure 2B**i) (45). This type of ossification occurs in the skull, for example in the cranial roof and opercular bones, in the vertebral column, where most of the vertebral body is formed by this type of ossification, in scales and in the fin rays (45). In mammals, this ossification is mostly restricted to bones of the cranial vault and the dentary (52).

Perichondral ossification, characterized by bone formation in the perichondrium, is more common in the teleost compared to the mammalian skeleton, where it has been considered as a form of intramembranous ossification (45). In teleosts perichondral ossification is present in the hyomandibula and Meckel's cartilage, where osteoblasts aggregate on the surface of the cartilaginous template and deposit bone matrix into the perichondrium (**Figure 2B**ii).

Endochondral ossification, which is the main type of ossification in mammals, is uncommon in teleosts. In this type of ossification, mesenchymal cells condense and differentiate

into chondroblasts and chondrocytes, which then secrete an extracellular cartilage matrix that functions as a template that is replaced by bone matrix (**Figure 2B**iii). In teleosts, two types of endochondral ossification exist. In a few bones, such as the ceratohyal and the radials in the pectoral fin, type I endochondral ossification takes place at the level of epiphysis and of the epiphysial growth plate resembling the mammalian endochondral ossification process. It is characterized by a resting zone, a proliferation zone with columnar cartilage, and a hypertrophic zone followed by a region in which cartilage matrix calcifies (36). Finally, chondroclasts degrade the cartilaginous matrix (degradation zone), allowing osteoblasts to lay down bone matrix (ossification zone). In the hyomandibula, branchial arches, ethmoid and hypuralia type II endochondral ossification takes place. Here, the calcification and ossification zones are absent and the cartilage template is replaced by adipose cells, leading to tubular concave bones filled with adipose tissue (36, 37).

Because the cranial skeleton is often too complex for screening by high throughput methods, the zebrafish vertebral body is the most investigated component of the skeleton both in early and adult life stages. Although the vertebrae in both mammals and teleosts consist of notochord and bone, there are a few key differences. First, the notochord is the *de facto* vertebral column in early teleost life stages and persists throughout life, while it only forms the intervertebral disc in mammals (53, 54). The notochord consists of a core of large and vacuolated chordocytes which is surrounded by an epithelial layer of chordoblasts that secrete the notochord sheath. This sheath is a stratified structure, composed of a thin external membrane containing elastin, covering a thicker layer of mainly collagen type II (54). Second, while the vertebrae in mammals have a cartilaginous precursor which endochondrally ossifies, zebrafish vertebrae form initially through direct mineralization of the notochord sheath, called chordacentra, in the absence of a cartilaginous precursor (55, 56). To this day, the exact cellular involvement of this notochord sheath mineralization remains unresolved. Third, the teleost vertebra is subsequently built via intramembranous ossification outside the notochord onto the chordacentrum, consisting of a compact autocentrum and trabecular arcocentrum, which forms the neural and haemal arches (56, 57). The osteoblasts produce collagen type I bone matrix and start to ossify the autocentrum at the level of the intervertebral disc, which acts as the growth center of the vertebral centrum (34).

GENERATION OF KNOCK-OUT AND KNOCK-IN ZEBRAFISH MODELS

Forward Genetic Approach

Different methods to generate zebrafish models of human disorders have been explored over the last decades. Initially, a number of large-scale forward genetic screens, based on random mutagenesis with radiation, chemicals, or insertional mutagenesis, revealed zebrafish mutants affecting different aspects of embryonic development and biological processes (58–60). This phenotype-driven approach was also applied to screen for genes involved in skeletal development and diseases (**Table 1**). Several mutants with defects in craniofacial cartilage elements and with mineralized tissue phenotypes (119), or with changes in the shape of the skeleton (96) were identified in large scale forward genetic screens. Mapping of the causative change established some of these mutants as models for human skeletal disorders. For instance, in a study by Gistelinck et al. (120), several type I collagen zebrafish mutants, previously discovered in a forward genetic screen (96), were established as representative models for the brittle bone disorder osteogenesis imperfecta.

Reverse Genetic Approach: Morpholino Knockdown and Gene Editing

Although forward genetics brought great progress to the field of disease modeling, still, for many causal human disease genes, this approach did not reveal corresponding zebrafish mutants, as there is incomplete genome coverage of mutagenesis. Consequently, the need to investigate the function of relevant candidate genes for specific diseases or developmental pathways, sparked the expansion of reverse genetic approaches in the zebrafish field.

The assessment of candidate gene function was initially enabled via knockdown through the use of antisense morpholinos (MO). Their ease of use made this approach increasingly popular for gene function analysis, and several early studies demonstrated that MO-mediated knockdown ("morphants") recapitulated known mutant phenotypes (121, 122). Over the past years, MOs have also been used in zebrafish modeling of skeletal disorders (**Table 1**). An example includes the monogenetic form of X-linked osteoporosis, caused by loss-of-function variants in *PLS3* encoding for plastin 3, a cytoskeletal protein involved in bone homeostasis. MO-mediated knockdown of *pls3* in zebrafish (18) induced malformations of the developing craniofacial bone structure, which could be reversed by the administration of human *PLS3* mRNA. Another example by Flores et al. (68) shows that depletion of *runx2b* by MO injection severely compromised craniofacial cartilage formation, phenocopying the human dominantly inherited disorder cleidocranial dysplasia, a condition characterized by impaired ossification and multiple skeletal abnormalities (68). Nevertheless, problems with the application of MOs in zebrafish emerged, such as the frequent occurrence of p53-dependent apoptosis (123–125), and off-target effects resulting in so-called "pseudophenotypes" (126, 127), but also MO-induced phenotypes that cannot be recapitulated in existing mutants (128). The latter issue has recently been studied in more detail leading to the insight that, at least for some genes, the phenotypic differences between morphants and mutants can be due to genetic compensation in the latter, but not in the former (129).

Definitive reverse genetic approaches in zebrafish recently became available in the form of site-specific nucleases enabling targeted gene modification. Initial work utilized zinc finger nucleases (ZFNs) (130, 131), and transcription activator-like effector nucleases (TALENs) (132). However, CRISPR/Cas9 genome editing is currently the most versatile and frequently

TABLE 1 | Zebrafish models for skeletal disorders.

Disorder	Gene	Type	Origin	References
Acrocapitofemoral dysplasia	*Ihh*	KO	ENU	(40)
Alagille syndrome	*jagd1b*	KO	ENU	(61)
Amelogenesis imperfecta	*slc10a7*	KD	MO	(62)
Auriculocondylar syndrome	*mef2ca*	KO	ENU	(63)
Bruck syndrome	*Plod2*	KO	ENU	(16)
Campomelic dysplasia	*sox9a, sox9b*	KO	ENU	(64)
Cartilage-Hair Hypoplasia	*rmrp*	KO	CR	(65)
Cenani-Lenz syndactyly	*lrp4*	KD	MO	(66)
Chordoma	*HRASV12*	OE	Tol2	(67)
Cleidocranial dysplasia	*runx2b*	KD	MO	(68)
Craniofacial defects	*tgfb2*	KD	MO	(69)
Craniofacial defects	*fgf10a*	KD	MO	(69)
Craniosynostosis	*tcf12*		Tol2	(70)
Craniosynostosis	*cyp26b1*	KO	ENU	(71)
Craniosynostosis	*cyp26b1*	KO	ENU	(72)
Culler-jones syndrome	*gli2*	KO	Tol2	(73)
Delayed mineralization	*Pth4*			(74)
Delayed mineralization			TR	(75)
Ehlers-Danlos syndrome	*b4galt7*	KD	MO/CR	(76)
Fibrodysplasia Ossificans Progressiva	*acvr1*	CE	Tol2	(77)
Gaucher disease	*gba1*	KO	ENU	(78)
Holoprosencephaly	*ptch1*	KO	ENU	(40)
Hyperosteogeny	*n1alcd*	OE	Tol2	(79)
Hyperthyroidism	*tshr*	KO	ENU	(80)
Hypohidrotic ectodermal dysplasia	*eda, edar*	KO	ENU	(81)
Joint disease	*scxa*	KO	CR	(82)
Klippel Feil syndrome	*meox1*		ENU	(83)
Multiple hereditary exostoses	*ext2, papst1*	KO	ENU	(84)
No mineralization	*entpd5*	KO	ENU	(85)
Oculodentodigital dysplasia	*cx43*	KO	ENU	(86)
Orofacial cleft	*tgfβ3*	KD	MO	(87)
Orofacial cleft	*mir140*	KD	MO	(88)
Orofacial cleft	*faf1*	KD	MO	(89)
Orofacial cleft	*wnt9a, irf6*	KO	Tol2	(90)
Osteoarthritis	*col11a2*	KO	ENU	(91)
Osteoarthritis	*prg4a, prg4b*	KO	TA	(92)
Osteogenesis imperfecta	*col1a1a*	MM	ENU	(14, 15, 93)
Osteogenesis imperfecta	*bmp1*	KO	ENU	(94)
Osteogenesis imperfecta	*sp7/osx*	KO	ENU	(95)
Osteogenesis imperfecta	*col1a1a, col1a1b, col1a2*	MM	ENU	(96)
Osteopetrosis	*m-csf*	KO	ENU	(97)
Osteoporosis			TR	(98)
Osteoporosis			TR	(99)
Osteoporosis	*gpr137b*	KO	CR	(100)
Osteoporosis			TR	(101)
Osteoporosis			TR	(102)
Osteoporosis	*atp6v1h*	KO	CR	(20)
Osteoporosis	*lgmn*	KO	TA	(103)
Osteoporosis	*lrp5*	KD	MO	(19)
Osteoporosis	*pls3*	KD	MO	(18)
Osteoporosis			TR	(104)
Pseudoxanthoma elasticum	*enpp1*	KO	ENU	(105)
Pseudoxanthoma elasticum	*abcc6a*	KO	ENU	(106)
Saethre-Chotzen syndrome	*twist, tfc12*	KO	TA	(107)
Saul-Wilson syndrome	*cog4*	KO	CR	(108)
Spine curvature disorders	*kif6*	KO	TA	(109)
Spine curvature disorders	*ptk7*	KO	ZFC	(110)
Spine curvature disorders	*slc39a8*	KO	CR	(111)
Spine curvature disorders	*col8a1a*	KO	ENU	(112)
Spine curvature disorders	*tbx6, her1, her7, hes6*	KO	TA	(35)
Spine curvature disorders	*uts2ra*	KO	TA	(113)
Spine curvature disorders			TR	(114)
Sponastrime dysplasia	*tonsl*	KO	CR	(115)
Stickler/Marshall syndrome	*col11a1a, col11a1b*	KD	MO	(116)
Tumoral calcinosis	*golgb1*	KO	TA	(117)
Vertebral fractures			TR	(118)

KO, Knockout; KD, knockdown; MO, morpholino; CE, cell ablation; MM, missense mutation; ENU, N-ethyl-N-nitrosourea; CR, CRISPR; Tol2, transposon-mediated integration; TR, treatment, meaning OP models induced by microgravity, drugs, aging, physical exercise, iron stress, microRNA, mechanical loading; TA, talen; ZFN, zinc finger nuclease.

employed reverse genetic technology for the creation of both knock-out and knock-in disease models. The CRISPR/Cas9 system induces a double-stranded DNA break (DSB), carried out by the Cas9 nuclease, at a specific target site, recognized by the binding of a single-guide RNA (sgRNA) molecule. Following DSB, different endogenous repair mechanisms can be initiated. On one hand, the error-prone non-homologous end joining (NHEJ) pathway can be activated, often leading to the introduction of *indel* mutations due to imprecise repair, resulting in gene knock-out. The generation of gene knock-outs in zebrafish is relatively straightforward and efficient. In a study

by Zhang et al. (20) for instance, mutations in the *ATP6V1H*, coding for vacuolar ATPase, were identified in patients with short stature and osteoporosis. Loss-of-function mutants in *atp6v1h* were generated in zebrafish through CRISPR/Cas9-mediated gene knock-out (20). These mutants demonstrated loss of bone mass and increased expression of matrix metalloproteases *mmp*9 and *mmp13*. Indeed, pharmacological inhibition of mmp9 and mmp13 rescued the bone phenotype, suggesting that blockade of collagen degradation can be a valid therapeutic target. CRISPR/Cas9 gene editing has been recently used to generate knock-out zebrafish for *crtap* and *p3h1*, two genes that are part of a protein complex which is involved in prolyl 3-hydroxylation and proper folding of collagen type I. Loss-of-function mutations in the human ortholog genes cause recessive forms of OI. These zebrafish models faithfully mimic the human disease and support the defective chaperone role of the 3-hydroxylation complex as the primary cause of the skeletal phenotype (17).

In general, reverse genetic approaches are limited by the time required to generate mutant lines, where stable knock-out zebrafish are mostly obtained and analyzed from the F2 generation on. Therefore, an approach for rapid CRISPR-based reverse genetic screens was developed in which phenotyping is performed directly in F0 (mosaic) founders, which are called "crispants" (133, 134). This enables moderate to rapid throughput reverse genetic screens of candidate genes, contributing to skeletal disease. In a study by Watson et al. (133), the comparison between somatic, CRISPR-generated F0 mutants and homozygous germline mutants for *plod2* and *bmp1*, two genes implicated in recessive OI, revealed phenotypic convergence, suggesting that CRISPR screens of F0 animals may faithfully recapitulate the phenotype of skeletal disease models (133).

As an alternative to NHEJ-mediated repair of CRISPR/Cas9-induced DSB, the homology-directed repair (HDR) pathway can be initiated, but only in the presence of a homologous repair template. In physiological circumstances, HDR occurs between sister chromatids during the G2 and S phase of the cell cycle. The knock-in modeling procedure exploits this mechanism by supplying the CRISPR/Cas9 system with an artificial repair template, homologous to the target sequence and containing a specific variant of interest. For the generation of knock-in models, mostly single-stranded oligodeoxynucleotide (ssODN) repair templates are used (135) mainly because the design and production of ssODNs is easier, cheaper and results in higher HDR efficiencies compared to double-stranded templates such as plasmids (136, 137). The need to complement knock-out models with these more precise knock-in disease models is growing, for various reasons. Firstly, specific point mutations may cause a highly divergent pathobiology compared to loss-of-function mutations modeled by knock-out models. More specifically, certain missense mutations may cause a gain-of-function rather than a loss-of-function, while missense mutations in genes encoding proteins included in protein complexes may exercise a dominant negative effect and change the function of the whole protein complex. For instance, in dominant types of OI caused by mutations in the genes encoding the type I collagen α chains, depending on the type of mutation, either the quantity or the structure of type I procollagen is altered (138). The "quantitative" mutations, mostly resulting in a null *COL1A1* allele, typically cause mild forms of OI, while "qualitative or structural" defects, frequently associated with glycine substitutions, can be responsible for lethal, severe or moderate forms of the disease.

Also, missense mutations in vital developmental genes may be hypomorphic while their loss-of-function counterparts result in early lethality, as reported in the *cdc6* zebrafish mutant for Meier-Gorlin syndrome. Hypomorphic mutations in the *cdc6* gene recapitulate the patient's phenotype, while the knock-out mutants are embryonically lethal. In these cases, the introduction of such point mutations is a prerequisite to faithfully recapitulating human disease. Secondly, as mentioned before, several zebrafish knock-out models failed to generate a phenotype, which can be due to mRNA decay-induced genetic compensation (139), a phenomenon that is not expected to occur in knock-in models.

Nevertheless, several drawbacks mitigate the straightforward use of HDR knock-in zebrafish models. Firstly, HDR pathways have proved highly inefficient for genome editing (140) even despite proposed modifications, such as repair template modification (141, 142), cell cycle arrest (143) and chemical compound administration (144–151). Secondly, CRISPR/Cas9-mediated HDR mechanisms have been shown to be error-prone (152, 153). These issues hindered the development of knock-in zebrafish models and only a limited number have been reported, in contrast to numerous knock-outs. For instance, CRISPR/Cas9-mediated point mutation knock-ins have been generated for genetic variants implicated in inherited cardiac diseases (154–156), although to our knowledge none have been described so far for skeletal diseases. Different recently developed DSB-free alternatives for precise base pair substitution, such as programmable base editing (157–159) and prime editing (160) promise to be more efficient and versatile approaches, but more research is needed to further improve these methods for application to the zebrafish model system.

TRANSGENIC LINES

Transgenic Zebrafish to Trace Bone Cells and Pathways

Despite the development of new approaches in large-scale and more recently single-cell transcriptomics, genomics, epigenomics, and proteomics (161), these techniques are time consuming, expensive and only available in specialized laboratories (162–164). Furthermore, retrospective -omic analyses exclude cells that do not survive to the point of cell harvest, a common and necessary event in growth and regeneration. Therefore, to be able to understand the dynamic nature of tissue development and regeneration, *in vivo* time-lapse imaging is essential.

The recent evolution of genetic engineering has allowed the generation of transgenic animal models, expressing fluorescent proteins under cell- or pathway- specific promoters, enabling *in vivo* imaging of differentiation and signaling (165). However, the generation of transgenic murine models remains technically demanding, time consuming and expensive (166). In addition,

since mice develop *in utero*, it is almost impossible to investigate early developmental processes in real time and the visualization at cellular level usually requires post-mortem analyses (167).

Zebrafish, with its fast external development, transparent early life stages and relative easy genetic manipulation, is rapidly becoming the model of choice for examining developmental processes via time-lapse microscopy. The introduction of reporter genes downstream of a specific promoter makes it possible to produce site-directed indicators in different organs, tissues or cells and permits real time imaging in developing embryos or post-hatch stages; or even in mature zebrafish by fluorescent microscopy on whole mount specimens (168, 169). A variety of transgenic reporter lines have been generated to mark skeletal cell lineages at different stages of differentiation and signal transduction pathways, by using the conserved regulators of skeletal development (**Table 2**). The availability of fluorescent reporter lines, together with the use of powerful techniques such as two or multi-photon or light sheet microscopy, has allowed imaging of tissues and organs at a cellular and subcellular level, especially by exploiting the transparency of early life stages (218).

Transgenic Lines to Trace Bone Cells

The most frequently used lines expressing fluorophores in chondrocytes include Tg*(−4.9sox10:egfp)*^{ba2} and Tg*(Col2a1aBAC:mcherry)*^{hu5910} (**Table 2**). The Tg*(−4.9sox10:egfp)*^{ba2} was employed to detect *sox10* expression in head cartilage during embryo development and to follow migration of neural crest cells during cranium morphogenesis (175). The Tg*(Col2a1aBAC:mcherry)*^{hu5910} reporter line allowed impaired cartilage patterning and loss of chondrocyte organization to be shown in a zebrafish model of a recessive form of Ehlers-Danlos syndrome with partial loss of B4galt7, a transmembrane Golgi enzyme that plays a pivotal role in proteoglycan biosynthesis (76).

In order to trace the differentiation of bone forming cells, transgenic lines for both early and late osteoblast markers, expressing fluorophores under the *osterix/sp7* and *osteocalcin/bglap* promoters, have been generated (**Table 2**). The Tg*(sp7:EGFP)*^{b1212} line allowed osteoblast behavior to be studied during both intramembranous and endochondral ossification. Moreover, this line was used to investigate the abnormal perichondral ossification in the RNA component of the mitochondrial RNA-processing endoribonuclease (*rmrp*) knock-out zebrafish model of cartilage hair hypoplasia (65). *Tg(Ola.sp7:mCherry)*^{zf131} was crossed with the OI type XIII zebrafish model *frilly fins* to elucidate the role of the bone morphogenic protein 1, encoded by *bmp1a* gene, in osteoblast differentiation and localization (94).

The Tg*(Ola.bglap.1:EGFP)*^{hu4008} line was used to understand the fundamental role of osteoblast dedifferentiation during bone healing in response to traumatic injury, and to show that adult zebrafish osteoblasts display an elevated cellular plasticity compared to their mammalian counterpart (195).

Despite the conservation of most of the osteoblastogenic markers, in zebrafish the expression of *col10a* is not limited to chondrocytes as in mammals, but is also present in osteoblasts (203). The transgenic line Tg*(-2.2col10a1a:GFP)*^{ck3}, expressing GFP under *col10a1* promoter, has therefore been used to investigate molecular events driving both chondrocyte and osteoblast development (203).

An interesting application of the transgenic reporter lines is their use in combination with a mineral stain, imaged at different fluorescent wavelengths, enabling the combined study of osteoblast dynamics and bone mineralization (196). For instance, alizarin red staining of the transgenic zebrafish Tg*(Ola.sp7:NLS-GFP)*^{zf132} localized *osterix/sp7* positive osteoblasts in the mineralized bone and revealed the absence of *osterix/sp7* expression in the anterior notochord region at 8 dpf (104). Similarly, mineral staining in combination with Tg(*osx*:Kaede)^{pd64} confirmed the osteoblast independent mineralisation of the notochord (196).

Most of the available osteoclast reporter lines express fluorophores under control of the promoter of cathepsin K (Ctsk), the osteoclast collagenase that mediates bone resorption (**Table 2**) (46). Chatani et al. (97) proved the absence of osteoclasts in the *panther* mutant, which lacks a functional receptor for the macrophage colony stimulator factor, taking advantage of the Tg*(ctsk:mEGFP)* transgenic line. A significantly reduced number of GFP-positive osteoclasts was found in the neural and haemal arches in *panther* larvae, indicating a crucial role of the protein in osteoclast proliferation and differentiation. Additionally, the medaka, another well-characterized teleost bony fish used for developmental and biomedical studies, was used to study osteoclasts by placing the gene encoding for the receptor activator of nuclear factor kappa-B ligand, *rankl*, a key osteoclast differentiation factor, under the control of a heat shock element (23). Increased osteoclast differentiation induced upon Rankl activation in this Tg*(rankl:HSE:CFP)* line resulted in an osteoporotic phenotype (46).

Transgenic Lines to Trace Signal Transduction Pathways

Zebrafish transgenic lines expressing *in vivo* reporter proteins under the control of signaling pathway responsive elements are a powerful tool to dissect dynamically the *in vivo* activation or repression of endogenous signaling pathways in real time (210, 219–221). Calcium, Bmp and Wnt pathways are crucial players during bone formation (222–224). Transgenic lines to further investigate these pathways have been generated (**Table 2**). The Tg*(hsp70:bmp2b-GFP)* line was used to analyze the role of the Bmp2 signaling pathway in an enteric disease, but the transgenic model could be employed to dissect BMP2b signaling in bone (225). To investigate Wnt pathway activation the Tg*(7xTCF-Xla.Siam:GFP)*^{ia4} and Tg*(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} transgenic lines, which contain multimerized *tcf/lef* binding sites for the transcription factor activated by *β-catenin* upstream to a siamois minimal promoter, were generated allowing the dynamics of neural crest-derived cell migration to be traced during development (211). Using the Tg*(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} transgenic line it was also possible to elucidate important regulatory steps in the osteogenic differentiation process of mesenchymal stem cells (73).

Finally, the unfolded protein response (UPR) was shown to play an important role in the modulation of the phenotype in rare

TABLE 2 | Transgenic lines employed to study zebrafish skeleton.

Cell type	Gene/pathway	Transgenic line	References	Applications
Neural crest-derived skeletal cells	*sox10*	Tg*(sox10:GFP)*^{ba5}	(170)	(170)#, (19)*
	sox10	Tg*(sox10:kaede)*^{zf393}	(171)	(90, 171)#
	sox10	Tg*(sox10:mRFP)*^{vu234}	(172)	(78, 172)*
	sox10	Tg*(-4725sox10:Cre)*^{ba74}	(173)	(173, 174)#
	sox10	Tg*(−4.9sox10:egfp)*^{ba2}	(175)	(175–177)#
	fli1	Tg*(fli1:EGFP)*^{y1}	(178)	(19, 78, 89, 178, 179)*
Cartilaginous cells	*foxp2*	Tg*(foxp2-enhancerA:EGFP)*^{zc42}	(180)	(180, 181)#
	col2a1a	Tg*(Col2a1aBAC:mcherry)*^{hu5910}	(40)	(78, 91, 105)*, (40, 182)#, (76)*
	col2a1a	Tg*(-1.7col2a1a:EGFP-CAAX)*^{nu12}	(183)	(183, 184)#, (112)*
	col18a1	Tg*(16Hsa.COL18A1-Mmu.Fos:EGFP)*^{zf215}	(185)	(185)#
Preosteoblasts	*cyp26b1*	Tg*(cyp26b1:YFP)*^{hu5786}	(72)	(72)#
	cyp26b1	*Tg(cyp26b1:YFP)*^{hu7426}	(186)	(186)#
Branchial arches and notochord cells	*cyp26a1*	Tg*(cyp26a1:eYFP)*$^{nju1/+}$	(187)	(187, 188)#
Intervertebral disc cells	*shhb*	Tg*(-5.2shhb:GFP)*^{mb1}	(189)	(189)#
	twist	Tg*(Ola.twist1:EGFP)*^{ca104}	(190)	(190)#
Early osteoblasts	*osx/sp7*	Tg*(sp7:EGFP)*^{b1212}	(181)	(73, 181)#, (112, 179, 191, 192)*, (193)§, (65)*
	osx/sp7	Tg*(Ola.sp7:mCherry)*^{zf131}	(72)	(94)*, (72)#
	osx/sp7	Tg *(Ola.sp7:NLS-GFP)*^{zf132}	(72)	(194)§, (72, 195)#, (78, 85)*, (196)#
	osx/sp7	Tg*(osterix:mCherry-NTRo)*^{pd46}	(197)	(197, 198)§
	osx/sp7	Tg*(osx:Kaede)*^{pd64}	(198)	(196, 199)#, (198)§
	osx/sp7	**Tg*(osx:CFP-NTR)***	(200)	(200)#
	osx/sp7	Tg*(osx:H2A-mCherry)*^{pd310}	(198)	(198)§
	osx/sp7	Tg*(osterix:Lifeact-mCherry)*^{ou2032}	(201)	(201)§
	col10a1	Tg*(Col10a1BAC:mCitrine)*^{hu7050}	(202)	(78, 91, 105)*, (202)#
	col10a1	Tg*(-2.2col10a1a:GFP)*^{ck3}	(203)	(203, 204)#
	runx2	Tg*(Hsa.RUNX2-Mmu.Fos:EGFP)*^{zf259}	(205)	(95, 195)#, (205)§
	runx2	Tg*(RUNX2:egfp)*	(31)	(31)#, (182)*
Mature osteoblasts	*osc/bglap*	Tg*(Ola.bglap.1:EGFP)*^{hu4008}	(205)	(105, 195)*, (205)§
	entpd5a	Tg*BAC(entpd5a:YFP)*^{hu5939}	(85)	(35)#, (85)*
	entpd5a	*TgBAC(entpd5a:Kaede)*^{hu6867}	(195)	(195)*, (35)#
	col1a1	Tg*(col1a1:EGFP)*^{zf195}	(31)	(31)#, (18)*
	rankl	**Tg*(rankl:HSE:CFP)***	(46)	(46)*
	notch1a	Tg*(Ola.sp7:N1aICD)*^{cy31}	(79)	(79)#
Osteoclasts	*ctsk*	Tg*BAC(ctsk:Citrine)*^{zf336}	(206)	(105)*
	ctsk	Tg*(ctsk:YFP)*	(206)	(105)*
	ctsk	Tg*(ctsk:DsRed)*	(207)	(207)#
	ctsk	**Tg*(CTSK-DsRed)***	(97)	(97)#
	ctsk	Tg*(Ola.ctsk:EGFP)*^{zf305}	(97)	(97)#
	ctsk	**Tg*(ctsk:mEGFP)***	(46)	(46, 208)*
	trap	**Tg*(TRAP:GFP)***	(97)	(97)#
	trap	Tg*(trap:GFP-CAAX)*^{ou2031}	(201)	(201)§
Bmp responsive cells	Bmp pathway	Tg*(Bre:GFP)*^{p77}	(209)	(209)#

(Continued)

TABLE 2 | Continued

Cell type	Gene/pathway	Transgenic line	References	Applications
	Bmp pathway	Tg*(bre:egfp)*^{pt510}	(210)	(177, 210)$^{\#}$
	Bmp pathway	Tg*(BMPRE:EGFP)*^{ja18}	(169)	(169)$^{\#}$, (78)*
β-catenin activated cells	Wnt pathway	Tg*(7xTCF-Xla.Siam:GFP)*^{ja4}	(211)	(211)$^{\#}$, (78)*
	Wnt pathway	Tg*(7xTCFXla.Siam:nlsmCherry)*^{ja5}	(211)	(73, 211)$^{\#}$
	Wnt pathway	Tg*(hsp70l:wnt8a-GFP)*^{w34}	(212)	(213)$^{\#}$
	Wnt pathway	Tg*(hsp70l:dkk1-GFP)*^{w32}	(214)	(73)$^{\#}$, (214)§
	Wnt pathway	Tg*(myl7:EGFP)*^{twu34}	(215)	
Stress responsive cells	UPR pathway	Tg*(ef1α:xbp1δ-gfp)*^{mb10}	(216)	(216)$^{\#}$
	UPR pathway	Tg(Hsa.*ATF6RE*:d2GFP)^{mw85}	(217)	(217)
	UPR pathway	Tg(Hsa.*ATF6RE*:eGFP)^{mw84}	(217)	(217)

*Transgenic lines used to characterize mutants with skeletal pathologies, $^{\#}$transgenic lines used to analyse skeletal development and molecular pathways, §transgenic lines used to study skeletal regeneration, Medaka transgenic lines are reported in bold.

skeletal diseases (226, 227). Interestingly, transgenic zebrafish lines allowing different branches of this pathway to be followed are already available (216, 217, 228, 229). For instance, the transgenic zebrafish model Tg*(ef1α:xbp1δ-gfp)*^{mb10} has been used to trace *in vivo* the splicing of *xbp1*, one of the terminal effectors of the UPR (216).

Live Imaging of Bone Regeneration

Tracing bone cells *in vivo* using transgenic lines in adult zebrafish is challenging due to tissue depth and complexity, but is possible in external structures such as fin rays or scales, which are easily accessible and suitable for regeneration studies (198, 230, 231). Indeed, the available panel of transgenic lines expressing fluorescent and photo-switchable reporter genes in bone cells is useful to trace regeneration *in vivo* (198). This strategy has clarified important biological aspects such as the cellular basis of integumentary bone regeneration. *In vivo* imaging of the Tg*(sp7:EGFP)*^{b1212} transgenic line during caudal fin regeneration showed the presence of GFP positive cells at the amputation plane starting from 2 days post amputation (dpa) and their association with the formation of newly mineralized matrix by 5 dpa (181). Osteoblast lineage tracing in the Tg*(osx:Kaede)*^{pd64} clarified migration and dedifferentiation of scleroblasts during fin regeneration (196).

However, the slow rates of regeneration require long-term live imaging to capture dynamic cellular events to improve the understanding of development, homeostasis, and regeneration by stem cell populations (232). Thus, to enable up to 24 h of continuous live imaging, specific protocols for long-term anesthesia of adult zebrafish have been optimized (198). Indeed, the transgenic line Tg*(osx:H2A-mCherry)*^{pd310} allowed spatio-temporally distinct cell division, motility, and death dynamic within a founder osteoblast pool to be imaged as bone regenerates (198).

Transgenic Lines as Tool for Drug Screening

Transgenesis is not only used to analyze bone development over time, to assess a mutant phenotype or track cell signaling, but also to evaluate drug screening effects (98, 104). Huang and colleagues employed the transgenic line Tg(O*la.sp7:NLS-GFP)*^{zf132} to test anti-osteoporosis chemical drugs. This line, that expresses GFP under control of *osterix/sp7*, allowed for a faster *in vivo* evaluation of drug effects on bone mass and density compared to traditional staining methods. In another study, the *osteocalcin/bglap* reporter transgenic line Tg*(Ola.Bglap:EGFP)*^{hu4008} was employed to test chlorpropamide effects on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). The drug negatively regulated osteoblast-like cell dedifferentiation, thus helping to maintain bone forming cells in an active state promoting caudal fin ray regeneration (233).

Tips for Transgenic Lines Selection

For the proper selection of transgenic lines there are some aspects that require consideration. First, the choice of the reporter protein is influenced by differences such as color, brightness, toxicity, tissue penetration, subcellular localization, as well as the stability of the fluorescent protein. For instance, in order to study cell signaling dynamics or when performing prolonged cell lineage tracing, the use of long half-life fluorescent proteins is recommended. Furthermore, differences in signal pattern and intensity can be found among transgenic progeny possibly due to multiple insertions in the same founder, thus complicating the analysis (169). This aspect can be ameliorated by diluting the number of transgenic copies through subsequent generations.

Finally, in order to verify the localization of the reporter protein, the use of dual color analysis in the same transgenic line is recommended (196, 199) by for example complementary secondary techniques such as immunohistochemistry or *in situ* hybridization (169, 199).

X-RAY IMAGING

One of the more frequently used techniques to visualize the human skeleton is x-ray imaging. Classic x-ray systems for human and veterinary purposes need to limit radiation exposure to the patient, and therefore have limited exposure settings, that

is their range of tube accelerating voltage (kV), current (mA), and time of exposure. These parameters are set to optimize the image of the skeleton while keeping the radiation exposure to the patient as low as possible and cannot be easily changed. Consequently, these medical appliances are not appropriate to image the small zebrafish skeletons. Examples of x-ray sources that have a wide range of possible x-ray output settings are small manual units used to scan museum artifacts and fossils, a small animal radiation research platform (SARRP; Xstrahl, Surrey, UK) and the Faxitron© x-ray cabinets. Specifically, these sources can be set to low power but long exposure time parameters, and can be used in combination with high resolution technical film such as mammography film or x-ray film (e.g., AGFA D2) used in aerospace and petroleum factory applications. A Faxitron x-ray cabinet in combination with mammography film was used by Fisher et al. (93) to image the skeleton of WT and *chihuahua* mutant zebrafish to screen for skeletal abnormalities (**Table 2**).

With the revolution of digital sensors capturing the x-ray signal, it has become straightforward to take an x-ray image of a small or large part of the human skeleton. The use of digital x-ray sensors is however more challenging when using zebrafish (24, 234) as the resolution is too low in most cases to capture a quality image of the small zebrafish skeleton. A modern system such as a Faxitron Ultrafocus x-ray cabinet can provide digital x-ray images up to a 5 μm spatial resolution which can be geometrically magnified (Faxitron©) (**Figure 3A**). This technique was used to screen for deformed and fragile bones in *chihuahua* mutant zebrafish (15) and to assess the gross skeletal anatomy of *prg4a*$^{-/-}$; *prg4b*$^{-/-}$ mutant zebrafish (92). Although these digital images may look clean and sharp, the thinner less mineralized bones may not be present in the image, which represents a loss of information about the zebrafish skeleton (234). In contrast, technical film such as AGFA D2 can theoretically capture extremely high-resolution images. Such technical film works well in combination with low energy settings needed for optimal imaging of the zebrafish skeleton. Moreover, this film is able to capture an image of smaller bones, which is not always possible when using a digital sensor.

The main advantage of using x-rays to image the zebrafish skeleton is that it is a cheap and quick methodology. Furthermore, x-ray imaging can be repeated on live organisms and can be used as a preliminary diagnostic tool for skeletal imaging before applying a more specialized method such as micro computed tomography (microCT) or mineral staining (**Figures 3B,C**). For instance, x-ray imaging is frequently used in aquaculture related research where it is a first line tool to assess skeletal deformities (235, 236). Although x-ray imaging can be employed to assess skeletal deformities in adult zebrafish, its use for juvenile zebrafish, where the skeleton is too small to be captured on film or digitally, is not feasible. In addition, x-ray images of zebrafish are not suitable for quantification of tissue or bone mineral densities. MicroCT currently provides a better solution to estimate these bone parameters (80, 120).

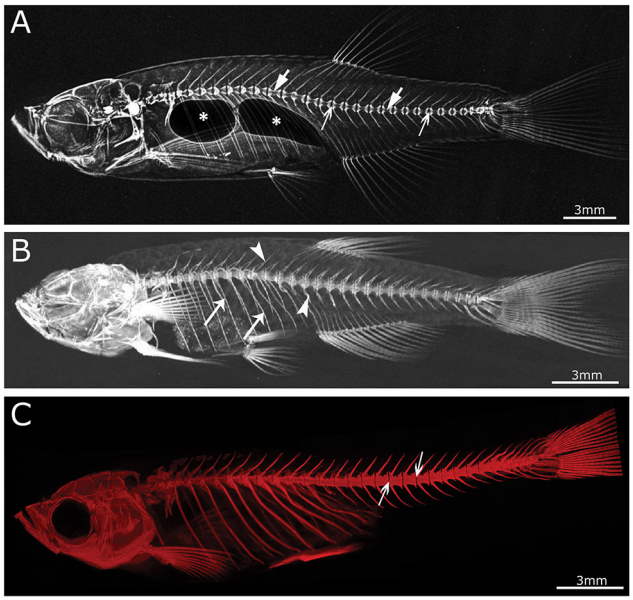


FIGURE 3 | Imaging techniques in zebrafish. **(A)** Lateral x-ray image of a wild type zebrafish acquired with a Faxitron tabletop X-ray cabinet. Notice the outline of the major bones in the skull and vertebral column and the outline of the double chambered swim bladder (indicated by asterisks) in the abdominal cavity. The tissue inside the vertebrae (indicated by block arrows) and intervertebral spaces (indicated by line arrows), i.e., the notochord, can be easily assessed for the presence of mineral. **(B)** Lateral view of a 3D reconstructed microCT scanned adult zebrafish at 21 μm. More details are visible in the skull and especially the vertebral column compared to the x-ray image (neural and haemal arch are indicated by arrow heads and the ribs with a small arrows). **(C)** Lateral image in the fluorescent channel of a zebrafish whole mount cleared and stained with alizarin red for mineralized tissues. Compared to the images above, more details of the skeleton can be observed, especially in the vertebral column where all individual bones and their outlines can be noticed. The alizarin red image also allows to assess the presence of mineral in the intervertebral space (indicated by arrows). All images were taken of wild type zebrafish.

MICRO COMPUTED TOMOGRAPHY

Computed tomography (CT) is a non-invasive technology based on x-ray analysis that allows detailed 3D reconstructions of large specimens. The generation of CT images involves the capturing and recording of x-rays that pass through the sample onto a detector. This process is repeated several times for multiple angles, followed by the virtual reconstruction into a 3D image (237). The required resolution for zebrafish imaging is beyond the capabilities of medical CT machines (≥70 μm), requiring higher resolutions, which can be obtained by microCT (**Figure 3B**) (237). The resolutions that can be achieved with modern microCT scanners vary from relatively low resolutions (≥20 μm), with quick scan times and large sample size, to higher resolutions (≤10 μm), with longer scanning durations and smaller sample size. It is important to note that the magnification, often described as the size of the voxels (3D pixels) is not identical to spatial resolution, which is roughly 2–3 times larger

(238). MicroCT is less time consuming and provides excellent 3D resolution compared to optical microscopy/histology. Although mainly mineralized tissues are recorded, resulting in a loss of information on aspects such as cells and non-mineralized tissues, the use of contrast agents allows visualization of different tissues such as adipose or epithelial tissue and can even enhance the signal of poorly mineralized bone (239, 240). For example, scanning of juvenile stages can be performed by staining the samples with silver nitrate beforehand, allowing for visualization of early bone development where only low amounts of mineral are present (241). However, with this approach only relative mineralization densities can be determined, and not absolute hydroxyapatite levels, which is an important parameter when modeling skeletal disorders. The amount of hydroxyapatite present in samples can be determined by performing a calibration microCT scan of a reference object (phantom) with a known hydroxyapatite concentration. This approach was used in a study of the effect of aging on bone mineral density (BMD) in zebrafish, revealing progressively increased BMD with age, in contrast to humans (101). When interpreting skeletal phenotypes, it is important not to rely on a single method, because certain phenotypes can be better detected using other methods. For example, a mineralized notochord leading to completely solid centra is easier to assess using microCT compared to mineral staining (72). In addition to 3D renderings, microCT data allows the creation and viewing of individual slices throughout the sample, similar to histological sections. Histology of mineralized tissues is notoriously difficult and requires special protocols because samples cannot be demineralized for sectioning. As an example, a complementary approach of both histology and high resolution microCT (6 μm) was used in a zebrafish model for craniosynostosis revealing fusion of the coronal suture (107).

Although low resolution microCT (≥20 μm) does not allow the detection of subtle skeletal changes, such as fusions between adjacent bones, it is perfectly suitable for whole-body scanning and phenotyping of adult zebrafish with a moderate throughput (**Figure 3B**). Such a procedure was applied by Gistelink et al. (120), where individual vertebral bodies (neural/haemal arches and centrum) of different OI zebrafish models were manually segmented. Subsequently, tissue mineral density (TMD), vertebral length, bone volume, and thickness were determined for each component (80). Manual segmentation is a laborious process and possibly introduces human bias into the analysis, which can be overcome by semi-automated segmentation algorithms such as FishCut (80). FishCut enables the measuring of a large number of parameters in the vertebral column, and is supplemented by a statistical approach for analysis (80). Models for Bruck syndrome, osteogenesis imperfecta and hyperthyroidism have been successfully analyzed by this high-throughput pipeline, thereby standardizing zebrafish skeletal analyses (80, 120). High resolution microCT (≤10 μm) on the other hand, allows for more detailed analysis, but is very time consuming and limits the scanning to only small segments of the skeleton (**Figure 4**). MicroCT scans of a vertebral body at 1 μm voxel size revealed osteocyte lacunae, which is beyond the resolution range of whole body microCT scans (**Figures 4B,D**) (242). In a study by Newham et al. (118), high resolution scans of vertebral bodies before and after mechanical compression were analyzed via geometric morphometrics. The obtained measurements were successfully used to determine the deformation zones and subsequently used to predict the deformation and strain during loading (118).

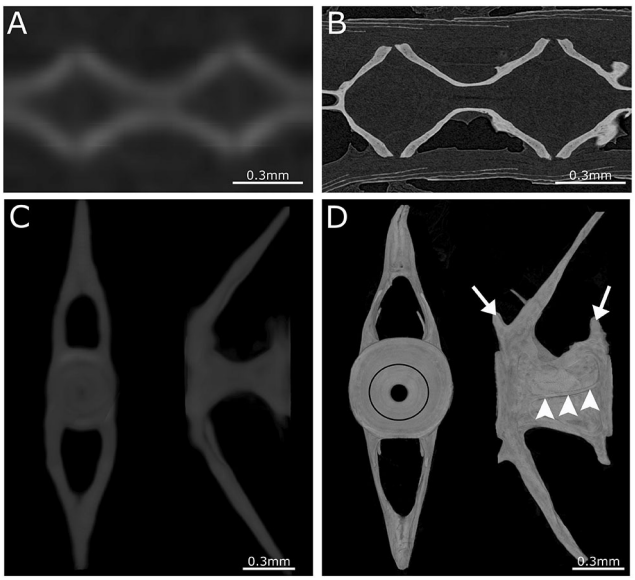


FIGURE 4 | Comparison between low- and high-resolution microCT. **(A)** Image of parasagittal microCT plane at 21 μm. **(B)** Similar structure as in **(A)** but scanned at 0.75 μm. Comparison between low-resolution and high-resolution microCT clearly demonstrates the ability to distinguish separate vertebrae and compact bone only using high-resolution microCT. **(C)** Anterior and lateral view of a 3D maximal projection surface render of a vertebrae scanned at 21 μm. **(D)** Similar structure as in **(C)** but scanned at 0.75 μm. Notice the difference in detail where the growth rings (black circle) are visible in the vertebral endplate on the anterior view. The lateral view of high-resolution microCT shows the outline of the vertebra with the pre- and post-zygapophyses (white arrows), and an antero-posterior running medial vertebral trabecula (white arrowheads).

BONE HISTOLOGY: FROM WHOLE MOUNT TO SECTIONS

Whole mount staining and high-resolution section analysis of the zebrafish skeleton represent complementary techniques, commonly used to describe bone development and structure at tissue and cellular levels.

Whole Mount Mineral and Cartilage Staining

In biomedical research, where the zebrafish is used as a model organism, whole mount staining is generally used to study the morphology of the skeleton (**Table 3**). The most commonly used techniques are staining of mineralized tissues with alizarin red S (ARS), staining of cartilage matrix with alcian blue (AB) or staining both tissues with a combination of both ARS and AB (**Figure 5**). These staining methods are based on well-established protocols, where a specimen is made translucent to transparent

TABLE 3 | Techniques applied to evaluate bone phenotype in zebrafish models.

Disorder	Stage	AR	AB	Dual stain	Calcein	Morphology	Histology	TEM	SEM	ISH	Transgenics	MicroCT	X-Ray	AFM	qBei	Nanoindentation	FTIR	References
Acrocapitofemoral dysplasia	L			x							x							(40)
Alagille syndrome	L			x	x					x								(61)
Amelogenesis imperfecta	L	x	x															(62)
Auriculocondylar syndrome	L			x			x											(63)
Bruck syndrome	L-J-A	x	x				x	x	x				x					(16)
Campomelic dysplasia	L		x		x		x				x							(64)
Cartilage-Hair Hypoplasia	L	x		x			x				x	x						(65)
Cenani-Lenz syndactyly	L		x				x				x							(66)
Chordoma	L							x	x			x						(67)
Cleidocranial dysplasia	L		x					x			x							(68)
Craniofacial defects	L			x				x										(69)
Craniofacial defects	L			x				x										(69)
Craniosynostosis	L-A	x									x	x						(70)
Craniosynostosis	L			x			x				x							(71)
Craniosynostosis	L-A	x		x		x						x						(72)
Culler-jones syndrome	A					x						x						(73)
Delayed mineralization	L			x							x	x						(74)
Delayed mineralization	L-A	x		x			x											(75)
Ehlers-Danlos syndrome	L	x	x				x											(76)
Fibrodysplasia ossificans progressiva	L-A	x					x						x					(77)
Gaucher disease	L			x			x											(78)
Holoprosencephaly	L			x														(78)
Hyperosteogeny	L-A			x														(40)
Hyperthyroidism	A																	(79)
													x					(80)

(Continued)

TABLE 3 | Continued

Disorder	Stage	AR	AB	Dual stain	Calcein	Morphology	Histology	TEM	SEM	ISH	Transgenics	MicroCT	X-Ray	AFM	qBei	Nanoindentation	FTIR	References
Hypohidrotic ectodermal dysplasia	A	x		x		x					x	x						(81)
Joint disease	L-A	x		x		x	x			x	x	x						(82)
Klippel Feil syndrome	L A	x																(83)
Multiple hereditary exostoses	L	x	x							x								(84)
No mineralization	L-A	x	x	x		x				x	x							(85)
Oculodentodigital dysplasia	A								x							x		(86)
Orofacial cleft	L	x	x	x		x												(87)
Orofacial cleft	L			x														(88)
Orofacial cleft	L		x			x				x	x							(89)
Orofacial cleft	L		x			x					x							(90)
Osteoarthritis	L-A			x			x				x	x		x				(91)
Osteoarthritis	L-A		x				x			x	x	x	x					(92)
Osteogenesis imperfecta	L-A			x						x			x					(93)
Osteogenesis imperfecta	L-A	x		x		x	x			x	x							(94)
Osteogenesis imperfecta	L-A	x					x	x		x	x							(95)
Osteogenesis imperfecta	L-A	x				x						x						(96)
Osteogenesis imperfecta	L-A			x	x	x						x	x					(15)
Osteogenesis imperfecta	L-A			x	x							x			x	x	x	(14)
Osteopetrosis	L-A	x			x	x	x	x										(97)
Osteoporosis	L				x	x												(98)
Osteoporosis	L	x																(99)
Osteoporosis	A					x	x					x						(100)
Osteoporosis	A					x						x						(101)
Osteoporosis	L	x	x	x	x						x							(102)
Osteoporosis	L-A	x	x	x	x	x						x						(20)
Osteoporosis	L	x				x												(103)

(Continued)

TABLE 3 | Continued

Disorder	Stage	AR	AB	Dual stain	Calcein	Morphology	Histology	TEM	SEM	ISH	Transgenics	MicroCT	X-Ray	AFM	qBei	Nanoindentation	FTIR	References
Osteoporosis	L			x		x	x				x							(19)
Osteoporosis	L										x							(18)
Osteoporosis	L	x				x					x							(104)
Pseudoxanthoma elasticum	L-J	x		x	x	x					x							(105)
Pseudoxanthoma elasticum	L-J	x				x				x	x							(106)
Saethre-Chotzen syndrome	A	x		x				x				x						(107)
Saul-Wilson Syndrome	L		x															(108)
Spine curvature disorders	L-J-A			x		x				x		x						(109)
Spine curvature disorders	L-J-A				x	x						x						(110)
Spine curvature disorders	J-A	x																(111)
Spine curvature disorders	L-A	x	x			x		x			x	x						(112)
Spine curvature disorders	L-A	x			x					x	x							(35)
Spine curvature disorders	L-A					x				x		x						(113)
Spine curvature disorders	A					x	x					x						(114)
Sponastrime dysplasia	L			x														(115)
Stickler/Marshall syndrome	L		x			x	x	x										(116)
Tumoral calcinosis	A								x		x							(117)
Vertebral fractures	A					x						x						(118)

L, Larval stage; J, Juvenile stage; A, Adult stage; AR, Alizarin red; AB, Alcian blue; TEM, Transmission electron microscopy; SEM, Scanning electron microscopy; AFM, Atomic force microscopy; qBei, Quantitative backscattered electron imaging; FTIR, Fourier-transform infrared spectroscopy.

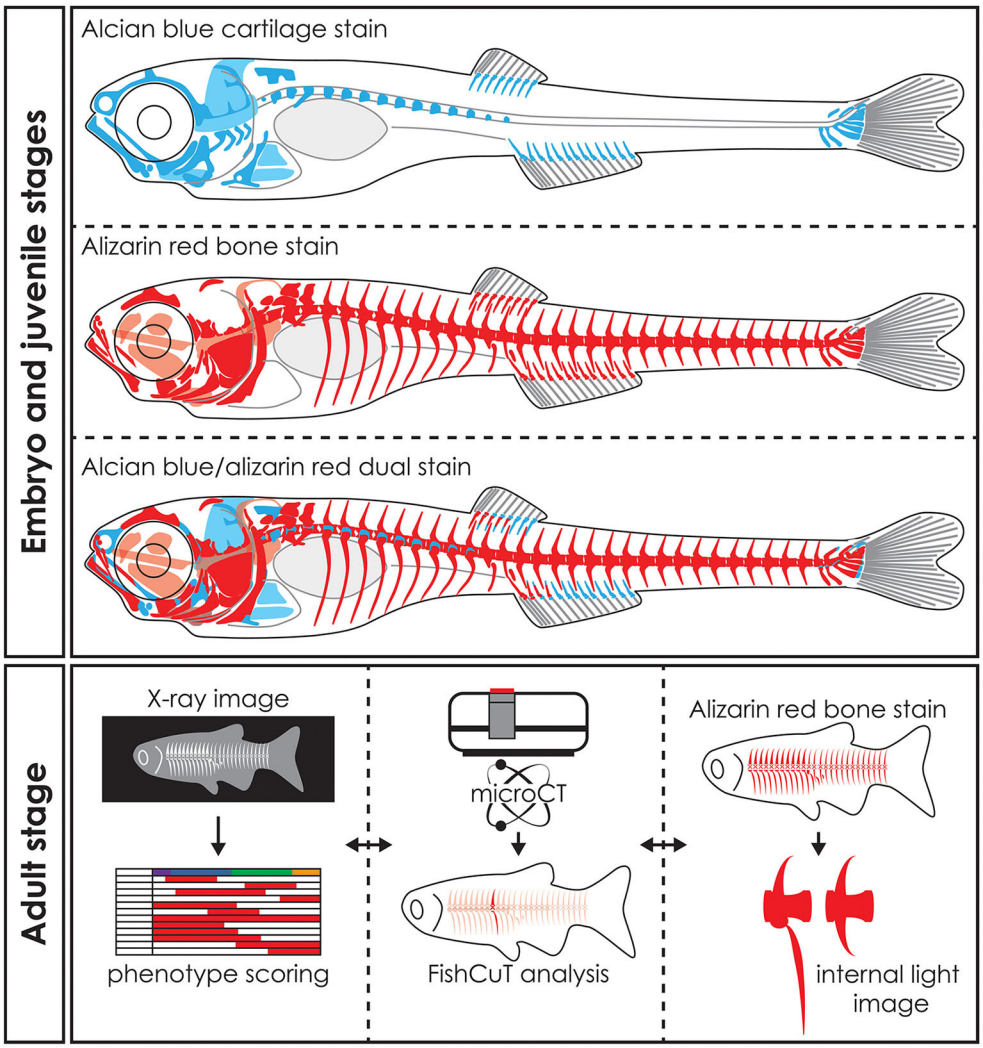


FIGURE 5 | Whole mount staining in early stages and applications of visualization techniques in adult zebrafish. Schematic representation of whole mount cleared and stained early stage zebrafish for cartilage with alcian blue, mineralized tissues (bone) with alizarin red and dual stained for both cartilage and mineralized tissues. Notice that only part of the skull, the basiventrals [for definition see Gadow and Abbott (243)] of the abdominal vertebrae and the fins endoskeleton are pre-formed in cartilage. Many bones in the skull and especially in the vertebral column are formed by direct intramembranous ossification. Images of adult skeletons taken by x-ray can be used to score for skeletal abnormalities, while microCT data can be used in an analysis program such as FishCuT to obtain quantitative data of bone measurements such as size, volume, thickness, and bone mineral density (80, 120). Bright field images or fluorescent images of whole mount cleared and stained zebrafish for mineralized tissues with alizarin red can be used to study skeletal abnormalities in detail. The three techniques are mostly used on euthanized and fixed specimens and thus can be applied on the same specimen sequentially. Moreover, the data procured by these visualization techniques can be integrated into a large data matrix and allows detailed phenotypic descriptions of zebrafish disease models.

and cartilage matrix or mineralized tissues are stained with a dye. Images of whole mount cleared and stained animals, taken with a modern stereo microscope, have an even higher resolution than standard microCT images (**Figures 3B,C**). Therefore, the whole mount clearing and staining technique can be considered as the gold standard for observing the whole zebrafish skeleton in detail.

Alizarin Red S

Many different protocols exist for ARS staining of mineralized tissues, however the main steps are based on (i) removing the pigmentation of the tissue with a bleaching solution (basic pH), (ii) neutralization of depigmentation, (iii) staining the animal with ARS, and (iv) clearing the animal of excess stain (244). The ARS molecule is a dihydroxyanthraquinone, likely binding the Ca^{2+} on the hydroxyapatite surface to form either a salt or a chelate form (245), thus it specifically stains mineralized tissue. In disease models ARS will stain ectopic mineralization in soft tissues. For example, ectopic mineralization was shown surrounding the eye, in the wall of the bulbus arteriosus of the heart and in the ventral skin of the dragon fish ($dgf^{-/-}$), a knock-out zebrafish model for the gene that encodes Enpp1, and modeled for generalized arterial calcification of infancy (GACI)

and pseudoxanthoma elasticum (PXE) (105, 106). Bone collagen in teleosts can also be deposited without being mineralized, as was shown in salmon vertebral bone (246, 247) and in the dentine of replacement teeth of the African bichir (248). It is important to underline that the unmineralized collagen cannot be visualized with ARS, however, mineralization usually quickly follows collagen deposition. Finally, there is also one mineralized collagenous tissue that does not stain with alizarin red S, the hypermineralized enameloid of the tooth cusps (248, 249).

ARS staining for mineralized tissues is frequently used to assess the development of skeletal elements in the head, axial skeleton, and fins at early life stages (**Figure 5**). In addition, investigating the early skeletal phenotype can be focused on a delay or advance in the development or specifically on the mineralization status of early skeletal elements. Because ARS is autofluorescent in the rhodamine channel (red), it can be used in combination with skeletal transgenic zebrafish reporter lines in which the fluorescent signal of the skeletal cells is in a different light spectrum. Alternatively, a Kaede reporter line, where the spectrum of the fluorescent protein can be changed by exposing the specimen to UV-light, can be used in a more flexible way (196). While most studies using ARS for mineralized tissue examined fixed specimens, ARS can also be used as a live stain especially in early stages where pigmentation does not obscure the underlying skeleton yet [reviewed in (250)]. Staining with ARS can also be employed to assess the juvenile and adult skeleton (**Figure 5**) because mineralized bone is the main skeletal tissue present at these life stages and is easy to observe with this technique.

Alcian Blue

Staining cartilage whole mounts with AB 8GX, similar to ARS staining, is based on several basic steps including (i) removing the pigmentation of the tissue with a bleaching solution (basic pH), (ii) staining the specimens with AB (acid pH), (iii) rehydration and clearing the specimens of excess stain, and (iv) dehydration and storing the specimens. The AB molecule is part of the phthalocyanine dyes with most often copper (Cu^{2+}) as the central metallic ion which results in a blue stain. AB has specifically four tetramethylisothiouronium solubility groups with S=C bonds that are easily broken to bind an insoluble AB molecule to the tissue (251). The stain binds as a salt to sulfated and carboxylated acid mucopolysaccharides and glycoproteins present in the cartilage matrix (251). Alcian blue is in most cases dissolved in a dehydrating ethanol/acetic acid solution and brought to a specific low pH. This low pH (1.5–2.5) causes AB to stain very specifically to the cartilage matrix (**Figure 5**).

Cartilage is the main skeletal tissue in early life stages of zebrafish, particularly in the skull (chondrocranium) and fins (252). Therefore, AB staining has been largely used in early life stages, i.e., 2–20 dpf, to study the morphology of the chondrocranium in different skeletal zebrafish models (62, 68) (**Figure 5**). Developing malformations are mainly defined as the irregular shape of skeletal elements, but can also be defined by the absence of skeletal elements or the incorrect morphogenesis of a single skeletal element (66, 84). Relative to the entire skeleton, not much cartilage is present in later life stages (late juveniles, adults) of zebrafish, yet AB staining can be used to assess for example cartilaginous joints (92).

Alcian Blue/Alizarin Red S Double Stain

Staining of cartilage and mineralized tissues can also be combined in a single specimen, as described in several papers by Kimmel et al. (253, 254). In this protocol tissues are stained first with AB followed by ARS staining (**Figure 5**). The dual staining for cartilage and mineralized tissues is similar to the single stain methods, except that AB can also be dissolved in a salt/ethanol solution, where the salts can be sodium acetate or the more commonly used magnesium chloride (244, 255).

The dual staining protocol is mostly used to assess development of malformations of the early skeleton but can also be used to investigate the normal development and developmental sequence of the skeleton (69). More specifically, dual staining has been used to assess ossification and mineralization status of cartilaginous bones (40, 87) and shape morphology of skeletal elements (61, 166).

The main advantage of this staining technique is the visualization of both cartilage and bone in an individual specimen, so that both connective tissues can be studied at the same time. However, this approach has also several disadvantages. First, when an acid/ethanol solution is used for AB staining, this acidic staining solution demineralizes the tissues that are subsequently visualized with ARS. This results in a reduced staining of mineralized tissues compromising the correct phenotypic assessment. This issue was reviewed by Witten et al. (24). Therefore, it is advisable to always use single staining protocols, either as an alternative or as a validation method in parallel to the double staining protocol. Second, dissolving AB in a non-acidic salt/ethanol solution is however challenging because pH higher then 6 decreases the specificity of the staining solution for mucopolysaccharides and glycoproteins (251).

ARS and AB Whole Mount Staining Advantages and Pitfalls

Considering the simplicity and above all the extensive use of the ARS and AB whole mount staining, a brief overview of its general advantages and disadvantages may be useful.

Both the single staining and double staining approaches are cheap and generally fast to use. Specimens that have not developed scales yet, can often be stained in a single day, with observations made the same day or the day after. In contrast, adult specimens can take up to 2 weeks to stain (244). Indeed, staining protocols need to be adapted to the size of the specimens. Therefore, a thorough description of the staining protocol is indispensable for the interpretation and reproducibility of results (251, 256).

Detailed observations of cartilaginous and mineralized connective tissues can be made owing to the high sensitivity and specificity of both the ARS and AB stains. In particular, small mineralized structures such as the initial mineralizations in early life stages and small intermuscular bones or tendons in adult life stages can be visualized by ARS with high fidelity (24, 234), especially when using fluorescent light which greatly enhances the visibility of these small structures (55, 250). Importantly,

ARS stain disappears over time especially in small mineralized structures requiring immediate observation and imaging once the staining procedure is finished. In contrast, when specimens are stored correctly in 100% glycerol, AB staining will remain specific for a longer time (256).

Although AB stains cartilage matrix specifically when the correct pH is used, AB solutions with a pH that is too high or solutions that have a too high or too low salt concentration can result in non-specific staining of non-cartilaginous connective tissue, i.e., collagen type I bone matrix. Non-specific staining can lead to incorrect interpretations of results. Finally, careful interpretation is needed of single AB stained connective tissues in specimens of 15 dpf and older. During the perichondral ossification of cartilaginous bones in zebrafish (**Figure 2B**ii), when a collagenous sheath forms around cartilaginous bone, the AB solution fails to stain the cartilage, and therefore the cartilaginous connective tissue appears absent. The presence of cartilage beneath the collagen can however still be confirmed using oblique light settings.

Histological Stains

Bone histology is often necessary to complement other imaging techniques, such as whole mount imaging, and remains one of the methods of choice to investigate the skeletal phenotype and bone mineralization during developmental stages (**Table 3**). The small size of zebrafish has forced researchers to adapt existing, standard histological procedures performed on human and murine skeletal tissues. High quality histological preparations and extensive knowledge about the zebrafish skeletal anatomy and development are indispensable for a correct skeletal evaluation (36, 45). Since zebrafish share similar bone cell types and cellular markers with mammals, it is possible to apply the standard histological and histomorphometric staining protocols available for mammalian bone, although with some technical optimization. In zebrafish in particular, the cellular composition analysis requires high-magnification imaging because skeletal elements may consist of a very limited number of cells, that are smaller in comparison with mammalian cells (24).

Unlike humans and mice, histology on zebrafish can easily be performed on a whole specimen in different developmental stages. Skeletal development can be followed in early juvenile stages looking at the mineralization of the notochord sheath and of cranial bones, while in adult zebrafish histology is most often performed on the abdominal vertebra (the first 10 vertebrae articulated with ribs, although this number is variable), the scales and the caudal fin rays.

Histological Specimen Preparation

In general, the histological procedure for both whole adult zebrafish and dissected bone samples, involves fixation in 4% paraformaldehyde in phosphate buffer saline (PBS) pH 7.2 overnight at 4°C, decalcification in 10% EDTA pH 7.2 for 7 days at 4°C and dehydration according to standard histological protocols or in a gradient series of acetone solutions (199). Importantly, while no decalcification is required up to 20 dpf, for juvenile to adult life stages the time of decalcification varies and depends on the developmental stage and size. Juveniles from 21 dpf till adulthood are normally decalcified for 4 up to 7 days (257).

According to Oralova et al. (199), paraffin embedding does not provide high quality histological details of zebrafish embryos and of early juvenile stages. In these cases, epoxy, or methacrylate resin embedding media are recommended (258). From epoxy blocks, semi, and ultrathin sections can be obtained for light and transmission electron microscopy, respectively, while methacrylate is more suitable for histochemical reactions (24). When using transgenic zebrafish lines expressing fluorescent reporters, fluorescence is generally lost in paraffin embedded samples. Cryosections preserve fluorescence, but significantly decreases the quality of the morphological structure due to processing artifacts. For this reason, Orolova and colleagues developed a new protocol using glycol methacrylate (GMA) embedding, which preserves both fluorescent labeling, epitopes for immunostaining and morphology, making it a more suitable choice (199).

Staining of Skeletal Sections

Different stains can be applied to histological sections of the zebrafish skeleton. Masson's trichrome and toluidine blue are commonly used and generally allow visualization of collagen and particular aspects of bone. Masson's trichrome, which usually stains muscle fibers red, collagen and bone in blue/green, cytoplasm in light red/pink, and cell nuclei in dark brown to black, reveals much thinner layers of collagen fibrils in a mutant zebrafish model for type I collagenopathies, a heterogenous group of connective tissue disorders caused by genetic defects in type I collagen (120). Toluidine blue is often used to detect bone cells, but is also a powerful dye to visualize proteoglycans, elastin and, when using birefringent light—collagen type I and type II fiber organization. Toluidine blue was used to detect abnormalities in glycosaminoglycan pattern in the pharyngeal skeleton of a zebrafish model for a recessive OI knock-out of *sec24C/sec24D*, two components of the COPII vesicle complex required for collagen secretion (259). Moreover, sections stained with toluidine blue showed compressed and deformed vertebrae, and excessive bone formation and remodeling at the vertebral endplates in the Bruck syndrome *plod2* mutant, characterized by the loss of type I collagen telopeptide lysyl hydroxylation (16).

The most widely used mineral staining assays include ARS, calcein and von Kossa staining, which specifically bind to calcium in the mineralized bone. In a study by Pasqualetti et al. (260), successive staining with ARS and calcein allowed evaluation of bone formation at the level of the circuli of growing scales in wild-type animals (260). In the *panther* fish, characterized by impaired osteoclast proliferation and differentiation, von Kossa staining enabled detection of altered mineralization of the neural arches (97).

Finally, collagen fiber maturation can be investigated by sirius red staining under polarized light, as performed to study the actinotrichia and lepidotrichia pattern in the *chihuahua* zebrafish, carrying a mutation in collagen type I α1 chain (15, 93, 261).

Transmission Electron Microscopy Analysis

Transmission electron microscopy (TEM) has also been used to investigate zebrafish bone. TEM represents a powerful method to analyze ultrastructural features of tissues since it provides much higher magnification and resolution compared to light microscopy, allowing visualization of cellular and matrix structures at a subnanometer scale. For instance, an altered distribution of bone collagen fiber diameter, a frequently described feature in various skeletal pathological conditions, was detected in the *crtap* and *p3h1* knock-out models of OI type VII and VIII by TEM, revealing the crucial role of the collagen post translational modification complex in bone organization (17). TEM was also used to show enlarged endoplasmic reticulum cisterna in these models, reinforcing ER stress as a key element in the OI phenotype and a potential target for new therapeutic approaches (17, 226, 227).

Immunohistochemistry

Immunohistochemistry (IHC) on zebrafish sections is also possible but limited, compared to mammal specimens, given the reduced availability of specific zebrafish antibodies. Nonetheless, with IHC, the spatiotemporal pattern of distribution of several proteins, a key prerequisite for understanding development, have been elucidated in embryos both in physiological and pathological conditions (199). For example, a structural defect in the extracellular matrix (ECM) has been detected in the *fndc3a*$^{wue1/wue1}$ zebrafish where IHC of type II collagen showed a loss of mature actinotrichia in 52 h post fertilization (hpf) embryos and β-catenin staining revealed divergent ECM assembly in the regenerated adult fin (262).

Determining the exact spatial localization of the protein of interest in immunostained whole mount larvae is difficult, especially for more deeply located tissues. To overcome this limitation, it is possible to perform whole-mount IHC followed by GMA embedding and sectioning, as was shown by Oralova et al. (199). In this way, the distribution of labeled cells was mapped and quantified allowing for close investigation of the cellular behavior during tissue development, cell migration, and adhesion events, as well as growth and differentiation. As an example, the use of a pan cytokeratin antibody on Tg(*sox17:egfp*) embryos allowed the authors to localize the protein of interest, Sox17, and the epidermis in the same section (199).

Finally, alkaline phosphatase (Alp), expressed by osteoblasts and required for the mineralization of extracellular matrix, and Trap, expressed by osteoclasts, and important for bone resorption, can both be immunostained to detect active osteoblasts and osteoclasts, respectively, and have been used for example to follow cell differentiation in scales (260).

Histological Analysis of Tissue Regeneration

Zebrafish's ability to repair caudal fin rays and scales has led to the optimization of specific histological protocols for these tissues involving both tissue sectioning as well as whole organ analysis (263). The analysis of histological sections has made clear that during regeneration in the caudal fin rays, cells near the site of injury can dedifferentiate, proliferate and replace the damaged or missing cells (196, 264). Furthermore, histological studies have identified a population of *Runx2/Sp7* positive chondrocytes involved in bone repair, and have helped to elucidate the ability of periosteal cells to generate cartilage in response to injury in *indian hedgehog homolog a* (*Ihha*) mutants (265).

To study mineralization and cellular compositions of caudal fin rays and scales, the tissue can also be isolated and directly stained without the need for dehydration and sectioning. For instance, by using ARS and calcein double staining and ALP immunohistochemistry, the specific mineralization pattern of bone forming cells in different areas of a scale was elucidated (260). Masson's trichrome staining of regenerating ray collagen proved that multiple amputations do not affect the regenerative bone capacity (266).

IS THE MEDAKA AN ALTERNATIVE TOOL IN SKELETAL RESEARCH?

Together with zebrafish, medaka (*Oryzias latipes*) is the other most frequently used small teleost in biomedical research. This species native to East Asia, belongs to the Adrianichthyidae family (order Beloniformes) and had an ancestor living in saltwater (267).

Evolutionarily, zebrafish and medaka are distantly related (268), with the last common ancestor dating back 110–200 million years ago (269). Being a small fish, medaka shares all the advantages already described for zebrafish, although it has a faster generation time, 2 vs. 3 months, shortening genetic experiments (23).

Similar to zebrafish, the medaka shares common skeletal developmental schemes as well as the presence of most of skeletal cells, chondrocytes, osteoblasts, and osteoclasts with tetrapods, but notably is missing osteocytes (23, 24).

The medaka genome, that underwent a whole duplication like that of the zebrafish, is available and easy to manipulate using the same techniques as in zebrafish research allowing easy generation of skeletal disease models and transgenic lines (46, 208, 270–273).

The almost completely conserved phenotypic features between zebrafish and medaka allow researchers to exploit the same imaging techniques to analyze skeletal components in both physiological and pathological conditions, either in terms of x-ray imaging or more specialized methods, such as microCT, whole mount or histological staining methods (23).

LIMITATIONS OF THE ZEBRAFISH MODEL

To take full advantage of the zebrafish as a model of human diseases it is important to be aware of existing drawbacks. Due to the extra whole genome duplication compared to mammals, as mentioned above, about 20% of the zebrafish genes have two functional copies, complicating the generation of knock-out disease models (274). Furthermore, some of the duplicated genes have functionally diverged, thus limiting the use of zebrafish in accurately modeling human diseases (11, 24). Additionally, the limited availability of antibodies against zebrafish proteins and the difficulty in establishing tissue specific primary cell

lines impairs zebrafish use in research. Finally, the generation of conditional knock-outs and knock-ins is still difficult in zebrafish. Although recently a method to integrate *loxP* sequences at specific sites in the zebrafish genome using the CRISPR/Cas9 technology has been developed, and conditional mutants of *tbx20* and *fleer* have been generated employing Cre recombinase technology (275, 276).

CONCLUSIONS

In the last decade the zebrafish has emerged as a unique model to investigate common and rare human skeletal disorders. The advances in gene editing techniques, from the initial insertion of random genomic mutations by exposure to mutagenic substances, to the knockdown expression of specific genes by antisense morpholino oligonucleotides, to the change of the genome at a specific site by nuclease technologies and their simple use in zebrafish, have all allowed research groups to generate new bone disease models. In particular, the versatile and cheap CRISPR/Cas9 system has found a wide use in many laboratories and undergone a series of optimizations allowing an increasingly specific and error-free gene editing. Nevertheless, its use for knock-in mutations still requires further optimization. The combining of zebrafish skeletal disease models with already available or newly generated transgenic lines, has contributed tremendously to the advances made in *in vivo* analysis of bone cells. The advances in confocal microscopy and the emergence of light sheet microcopy allows for better visualization and characterization of larval phenotypes in skeletal disease models, taking advantage of larvae transparency. X-ray and microCT have been optimized for small adult zebrafish bones, allowing analysis of the whole skeleton or small elements at high resolution. On the other hand, traditional skeletal specific dyes, such as alizarin red and alcian blue remain a valuable tool to study bone in larvae and adults. Finally, biomedical research has an urgent need for high throughput drug screening platforms and zebrafish models of skeletal diseases represent a bridge from *in vitro* to *in vivo* approaches.

In conclusion, ongoing technological advances in analytical techniques are making the zebrafish emerge as a unique and powerful model for the investigation and understanding of human skeletal disorders, and additionally as an efficient platform for compound discovery.

AUTHOR CONTRIBUTIONS

FT, JB, RB, AD, AW, and AF: writing—original draft. All authors: review and editing. LL, JB, and AD: figures.

FUNDING

This work was supported by the Italian Ministry of Education, University and Research (MIUR) [Dipartimenti di Eccellenza (2018–2022)] and the Brittle Bone Foundation Grant-2019 to AF. The study was supported by Ghent University (Methusalem Grant BOFMET2015000401).

REFERENCES

1. Gut P, Reischauer S, Stainier DYR, Arnaout R. Little fish, big data: zebrafish as a model for cardiovascular and metabolic disease. *Physiol Rev.* (2017) 97:889–938. doi: 10.1152/physrev.00038.2016
2. Russel WMS, Burch RL. *The Principles of Humane Experimental Technique* Wheathampstead: Universities Federation for Animal Welfare (1959).
3. Carnovali M, Banfi G, Mariotti M. Zebrafish models of human skeletal disorders: embryo and adult swimming together. *Biomed Res Int.* (2019) 2019:1253710. doi: 10.1155/2019/1253710
4. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* (2013) 496:498–503. doi: 10.1038/nature12111
5. Hamilton F. *An Account of the Fishes Found in the River Ganges and Its Branches*. Edimburgh: Edinburgh Printed for Constable A, and Company (1822).
6. Streisinger G, Walker C, Dower N, Knauber D, Singer F. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature.* (1981) 291:293–6. doi: 10.1038/291293a0
7. Kimmel CB. Genetics and early development of zebrafish. *Trends Genet.* (1989) 5:283–8. doi: 10.1016/0168-9525(89)90103-0
8. Poss KD, Keating MT, Nechiporuk A. Tales of regeneration in zebrafish. *Dev Dyn.* (2003) 226:202–10. doi: 10.1002/dvdy.10220
9. Zon LI, Peterson RT. *In vivo* drug discovery in the zebrafish. *Nat Rev Drug Discov.* (2005) 4:35–44. doi: 10.1038/nrd1606
10. Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Dev Dyn.* (2009) 238:2975–3015. doi: 10.1002/dvdy.22113
11. Harper C, Lawrence C. *The Laboratory Zebrafish*. Notre Dame: CRC Press (2016). 274 p.
12. Bergen DJM, Kague E, Hammond CL. Zebrafish as an emerging model for osteoporosis: a primary testing platform for screening new osteo-active compounds. *Front Endocrinol.* (2019) 10:6. doi: 10.3389/fendo.2019.00006
13. Besio R, Chow CW, Tonelli F, Marini JC, Forlino A. Bone biology: insights from osteogenesis imperfecta and related rare fragility syndromes. *FEBS J.* (2019) 286:3033–56. doi: 10.1111/febs.14963
14. Fiedler IAK, Schmidt FN, Wolfel EM, Plumeyer C, Milovanovic P, Gioia R, et al. Severely impaired bone material quality in chihuahua zebrafish resembles classical dominant human osteogenesis imperfecta. *J Bone Min Res.* (2018) 33:1489–99. doi: 10.1002/jbmr.3445
15. Gioia R, Tonelli F, Ceppi I, Biggiogera M, Leikin S, Fisher S, et al. The chaperone activity of 4PBA ameliorates the skeletal phenotype of chihuahua, a zebrafish model for dominant osteogenesis imperfecta. *Hum Mol Genet.* (2017) 26:2897–911. doi: 10.1093/hmg/ddx171
16. Gistelinck C, Witten PE, Huysseune A, Symoens S, Malfait F, Larionova D, et al. Loss of type I collagen telopeptide lysyl hydroxylation causes musculoskeletal abnormalities in a zebrafish model of bruck syndrome. *J Bone Min Res.* (2016) 31:1930–42. doi: 10.1002/jbmr.2977
17. Tonelli F, Cotti S, Leoni L, Besio R, Gioia R, Marchese L, et al. Crtap and p3h1 knock out zebrafish support defective collagen chaperoning as the cause of their osteogenesis imperfecta phenotype. *Matrix Biol.* (2020) 90:40–60. doi: 10.1016/j.matbio.2020.03.004
18. van Dijk FS, Zillikens MC, Micha D, Riessland M, Marcelis CL, de Die-Smulders CE, et al. PLS3 mutations in X-linked osteoporosis with fractures. *N Engl J Med.* (2013) 369:1529–36. doi: 10.1056/NEJMoa1308223
19. Willems B, Tao S, Yu T, Huysseune A, Witten PE, Winkler C. The Wnt co-receptor Lrp5 is required for cranial neural crest cell migration in zebrafish. *PLoS ONE.* (2015) 10:e0131768. doi: 10.1371/journal.pone.0131768
20. Zhang Y, Huang H, Zhao G, Yokoyama T, Vega H, Huang Y, et al. ATP6V1H deficiency impairs bone development

through activation of MMP9 and MMP13. *PLoS Genet.* (2017) 13:e1006481. doi: 10.1371/journal.pgen.1006481
21. Lam PY, Peterson RT. Developing zebrafish disease models for *in vivo* small molecule screens. *Curr Opin Chem Biol.* (2019) 50:37–44. doi: 10.1016/j.cbpa.2019.02.005
22. Gemberling M, Bailey TJ, Hyde DR, Poss KD. The zebrafish as a model for complex tissue regeneration. *Trends Genet.* (2013) 29:611–20. doi: 10.1016/j.tig.2013.07.003
23. Lleras-Forero L, Winkler C, Schulte-Merker S. Zebrafish and medaka as models for biomedical research of bone diseases. *Dev Biol.* (2020) 457:191–205. doi: 10.1016/j.ydbio.2019.07.009
24. Witten PE, Harris MP, Huysseune A, Winkler C. Small teleost fish provide new insights into human skeletal diseases. *Methods Cell Biol.* (2017) 138:321–46. doi: 10.1016/bs.mcb.2016.09.001
25. Witten PE, Huysseune A, Hall BK. A practical approach for the identification of the many cartilaginous tissues in teleost fish. *J Appl Ichthyol.* (2010) 26:257–62. doi: 10.1111/j.1439-0426.2010.01416.x
26. Iwasaki M, Kuroda J, Kawakami K, Wada H. Epidermal regulation of bone morphogenesis through the development and regeneration of osteoblasts in the zebrafish scale. *Dev Biol.* (2018) 437:105–19. doi: 10.1016/j.ydbio.2018.03.005
27. Van der heyden C, Huysseune A. Dynamics of tooth formation and replacement in the zebrafish (*Danio rerio*) (*Teleostei*, *Cyprinidae*). *Dev Dyn.* (2000) 219:486–96. doi: 10.1002/1097-0177(2000)9999:9999<::AID-DVDY1069>3.0.CO;2-Z
28. Reif WE. *Evolution of Dermal Skeleton and Dentition in Vertebrates: The Odontode Regulation Theory Evolutionary Biology*. Boston, MA: Springer (1982).
29. Huysseune A, Sire JY. Evolution of patterns and processes in teeth and tooth-related tissues in non-mammalian vertebrates. *Eur J Oral Sci.* (1998) 106(Suppl. 1):437–81. doi: 10.1111/j.1600-0722.1998.tb02211.x
30. Sire JY, Huysseune A. Formation of dermal skeletal and dental tissues in fish: a comparative and evolutionary approach. *Biol Rev Camb Philos Soc.* (2003) 78:219–49. doi: 10.1017/S1464793102006073
31. Kague E, Gallagher M, Burke S, Parsons M, Franz-Odendaal T, Fisher S. Skeletogenic fate of zebrafish cranial and trunk neural crest. *PLoS ONE.* (2012) 7:e47394. doi: 10.1371/journal.pone.0047394
32. Mongera A, Nusslein-Volhard C. Scales of fish arise from mesoderm. *Curr Biol.* (2013) 23:R338–9. doi: 10.1016/j.cub.2013.02.056
33. Bird NC, Mabee PM. Developmental morphology of the axial skeleton of the zebrafish, *Danio rerio* (*Ostariophysi*: *Cyprinidae*). *Dev Dyn.* (2003) 228:337–57. doi: 10.1002/dvdy.10387
34. Fleming A, Kishida MG, Kimmel CB, Keynes RJ. Building the backbone: the development and evolution of vertebral patterning. *Development.* (2015) 142:1733–44. doi: 10.1242/dev.118950
35. Lleras Forero L, Narayanan R, Huitema LF, VanBergen M, Apschner A, Peterson-Maduro J, et al. Segmentation of the zebrafish axial skeleton relies on notochord sheath cells and not on the segmentation clock. *Elife.* (2018) 7:e33843. doi: 10.7554/eLife.33843.043
36. Weigele J, Franz-Odendaal TA. Functional bone histology of zebrafish reveals two types of endochondral ossification, different types of osteoblast clusters and a new bone type. *J Anat.* (2016) 229:92–103. doi: 10.1111/joa.12480
37. Witten PE, Huysseune A. A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. *Biol Rev Camb Philos Soc.* (2009) 84:315–46. doi: 10.1111/j.1469-185X.2009.00077.x
38. Eames BF, Amores A, Yan YL, Postlethwait JH. Evolution of the osteoblast: skeletogenesis in gar and zebrafish. *BMC Evol Biol.* (2012) 12:27. doi: 10.1186/1471-2148-12-27
39. Schlombs K, Wagner T, Scheel J. Site-1 protease is required for cartilage development in zebrafish. *Proc Natl Acad Sci USA.* (2003) 100:14024–9. doi: 10.1073/pnas.2331794100
40. Hammond CL, Schulte-Merker S. Two populations of endochondral osteoblasts with differential sensitivity to Hedgehog signalling. *Development.* (2009) 136:3991–4000. doi: 10.1242/dev.042150
41. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int J Biochem Cell Biol.* (2008) 40:46–62. doi: 10.1016/j.biocel.2007.06.009
42. Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, et al. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev Cell.* (2010) 19:329–44. doi: 10.1016/j.devcel.2010.07.010
43. Horton JM, Summers AP. The material properties of acellular bone in a teleost fish. *J Exp Biol.* (2009) 212(Pt 9):1413–20. doi: 10.1242/jeb.020636
44. Bar-Shavit Z. The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. *J Cell Biochem.* (2007) 102:1130–9. doi: 10.1002/jcb.21553
45. Hall BK. *Bones and Cartilage: Developmental and Evolutionary Skeletal Biology*. 2nd ed. London: Elsevier/Academic Press (2015).
46. To TT, Witten PE, Renn J, Bhattacharya D, Huysseune A, Winkler C. Rankl-induced osteoclastogenesis leads to loss of mineralization in a medaka osteoporosis model. *Development.* (2012) 139:141–50. doi: 10.1242/dev.071035
47. Eames BF, Singer A, Smith GA, Wood ZA, Yan YL, He X, et al. UDP xylose synthase 1 is required for morphogenesis and histogenesis of the craniofacial skeleton. *Dev Biol.* (2010) 341:400–15. doi: 10.1016/j.ydbio.2010.02.035
48. Li N, Felber K, Elks P, Croucher P, Roehl HH. Tracking gene expression during zebrafish osteoblast differentiation. *Dev Dyn.* (2009) 238:459–66. doi: 10.1002/dvdy.21904
49. Yan YL, Willoughby J, Liu D, Crump JG, Wilson C, Miller CT, et al. A pair of sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development.* (2005) 132:1069–83. doi: 10.1242/dev.01674
50. Gistelinck C, Gioia R, Gagliardi A, Tonelli F, Marchese L, Bianchi L, et al. Zebrafish collagen type I: molecular and biochemical characterization of the major structural protein in bone and skin. *Sci Rep.* (2016) 6:21540. doi: 10.1038/srep21540
51. Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S, Venkatesh B. Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Mol Biol Evol.* (2004) 21:1146–51. doi: 10.1093/molbev/msh114
52. Hirasawa T, Kuratani S. Evolution of the vertebrate skeleton: morphology, embryology, and development. *Zoological Lett.* (2015) 1:2. doi: 10.1186/s40851-014-0007-7
53. Corallo D, Schiavinato A, Trapani V, Moro E, Argenton F, Bonaldo P. Emilin3 is required for notochord sheath integrity and interacts with Scube2 to regulate notochord-derived Hedgehog signals. *Development.* (2013) 140:4594–601. doi: 10.1242/dev.094078
54. Stemple DL. Structure and function of the notochord: an essential organ for chordate development. *Development.* (2005) 132:2503–12. doi: 10.1242/dev.01812
55. Bensimon-Brito A, Cancela ML, Huysseune A, Witten PE. Vestiges, rudiments and fusion events: the zebrafish caudal fin endoskeleton in an evo-devo perspective. *Evol Dev.* (2012) 14:116–27. doi: 10.1111/j.1525-142X.2011.00526.x
56. Arratia G, Schultze HP. Reevaluation of the caudal skeleton of certain actinopterygian fishes: III. Salmonidae. Homologization of caudal skeletal structures. *J Morphol.* (1992) 214:187–249. doi: 10.1002/jmor.1052140209
57. Arratia G, Schultze HP, Casciotta J. Vertebral column and associated elements in dipnoans and comparison with other fishes: development and homology. *J Morphol.* (2001) 250:101–72. doi: 10.1002/jmor.1062
58. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, et al. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development.* (1996) 123:37–46.
59. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, et al. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development.* (1996) 123:1–36.
60. Lawson ND, Wolfe SA. Forward and reverse genetic approaches for the analysis of vertebrate development in the zebrafish. *Dev Cell.* (2011) 21:48–64. doi: 10.1016/j.devcel.2011.06.007
61. Zuniga E, Stellabotte F, Crump JG. Jagged-Notch signaling ensures dorsal skeletal identity in the vertebrate face. *Development.* (2010) 137:1843–52. doi: 10.1242/dev.049056

62. Ashikov A, Abu Bakar N, Wen XY, Niemeijer M, Osorio GRP, Brand-Arzamendi K, et al. Integrating glycomics and genomics uncovers SLC10A7 as essential factor for bone mineralization by regulating post-Golgi protein transport and glycosylation. *Hum Mol Genet.* (2018) 27:3029–45. doi: 10.1093/hmg/ddy213
63. Miller CT, Swartz ME, Khuu PA, Walker MB, Eberhart JK, Kimmel CB. mef2ca is required in cranial neural crest to effect endothelin1 signaling in zebrafish. *Dev Biol.* (2007) 308:144–57. doi: 10.1016/j.ydbio.2007.05.018
64. Yan YL, Miller CT, Nissen RM, Singer A, Liu D, Kirn A, et al. A zebrafish sox9 gene required for cartilage morphogenesis. *Development.* (2002) 129:5065–79.
65. Sun X, Zhang R, Liu M, Chen H, Chen L, Luo F, et al. Rmrp mutation disrupts chondrogenesis and bone ossification in zebrafish model of cartilage-hair hypoplasia via enhanced Wnt/beta-catenin signaling. *J Bone Miner Res.* (2019) 34:2101–16. doi: 10.1002/jbmr.3820
66. Tian J, Shao J, Liu C, Hou HY, Chou CW, Shboul M, et al. Deficiency of lrp4 in zebrafish and human LRP4 mutation induce aberrant activation of jagged-Notch signaling in fin and limb development. *Cell Mol Life Sci.* (2019) 76:163–78. doi: 10.1007/s00018-018-2928-3
67. Burger A, Vasilyev A, Tomar R, Selig MK, Nielsen GP, Peterson RT, et al. A zebrafish model of chordoma initiated by notochord-driven expression of HRASV12. *Dis Models Mech.* (2014) 7:907–13. doi: 10.1242/dmm.013128
68. Flores MV, Lam EY, Crosier P, Crosier K. A hierarchy of Runx transcription factors modulate the onset of chondrogenesis in craniofacial endochondral bones in zebrafish. *Dev Dyn.* (2006) 235:3166–76. doi: 10.1002/dvdy.20957
69. Swartz ME, Sheehan-Rooney K, Dixon MJ, Eberhart JK. Examination of a palatogenic gene program in zebrafish. *Dev Dyn.* (2011) 240:2204–20. doi: 10.1002/dvdy.22713
70. Blumel R, Zink M, Klopocki E, Liedtke D. On the traces of tcf12: investigation of the gene expression pattern during development and cranial suture patterning in zebrafish (*Danio rerio*). *PLoS ONE.* (2019) 14:e0218286. doi: 10.1371/journal.pone.0218286
71. Laue K, Janicke M, Plaster N, Sonntag C, Hammerschmidt M. Restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of osteogenesis during zebrafish development. *Development.* (2008) 135:3775–87. doi: 10.1242/dev.021238
72. Spoorendonk KM, Peterson-Maduro J, Renn J, Trowe T, Kranenbarg S, Winkler C, et al. Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. *Development.* (2008) 135:3765–74. doi: 10.1242/dev.024034
73. Aman AJ, Fulbright AN, Parichy DM. Wnt/beta-catenin regulates an ancient signaling network during zebrafish scale development. *Elife.* (2018) 7:e37001. doi: 10.7554/eLife.37001.020
74. Suarez-Bregua P, Saxena A, Bronner ME, Rotllant J. Targeted Pth4-expressing cell ablation impairs skeletal mineralization in zebrafish. *PLoS ONE.* (2017) 12:e0186444. doi: 10.1371/journal.pone.0186444
75. Franz-Odendaal TA, Edsall SC. Long-term effects of simulated microgravity and vibration exposure on skeletal development in zebrafish. *Stem Cells Dev.* (2018) 27:1278–86. doi: 10.1089/scd.2017.0266
76. Delbaere S, Van Damme T, Syx D, Symoens S, Coucke P, Willaert A, et al. Hypomorphic zebrafish models mimic the musculoskeletal phenotype of beta4GalT7-deficient ehlers-danlos syndrome. *Matrix Biol.* (2019) 89:59–75. doi: 10.1016/j.matbio.2019.12.002
77. LaBonty M, Pray N, Yelick PC. A zebrafish model of human fibrodysplasia ossificans progressiva. *Zebrafish.* (2017) 14:293–304. doi: 10.1089/zeb.2016.1398
78. Zancan I, Bellesso S, Costa R, Salvalaio M, Stroppiano M, Hammond C, et al. Glucocerebrosidase deficiency in zebrafish affects primary bone ossification through increased oxidative stress and reduced Wnt/beta-catenin signaling. *Hum Mol Genet.* (2015) 24:1280–94. doi: 10.1093/hmg/ddu538
79. Liang ST, Chen JR, Tsai JJ, Lai YH, Hsiao CD. Overexpression of notch signaling induces hyperosteogeny in zebrafish. *Int J Mol Sci.* (2019) 20:3613. doi: 10.3390/ijms20153613
80. Hur M, Gistelinck CA, Huber P, Lee J, Thompson MH, Monstad-Rios AT, et al. MicroCT-based phenomics in the zebrafish skeleton reveals virtues of deep phenotyping in a distributed organ system. *Zebrafish.* (2018) 15:77–78. doi: 10.1089/zeb.2017.1540
81. Harris MP, Rohner N, Schwarz H, Perathoner S, Konstantinidis P, Nusslein-Volhard C. Zebrafish eda and edar mutants reveal conserved and ancestral roles of ectodysplasin signaling in vertebrates. *PLoS Genet.* (2008) 4:e1000206. doi: 10.1371/journal.pgen.1000206
82. Kague E, Hughes SM, Lawrence EA, Cross S, Martin-Silverstone E, Hammond CL, et al. Scleraxis genes are required for normal musculoskeletal development and for rib growth and mineralization in zebrafish. *FASEB J.* (2019) 33:9116–30. doi: 10.1096/fj.201802654RR
83. Dauer MVP, Currie PD, Berger J. Skeletal malformations of Meox1-deficient zebrafish resemble human Klippel-Feil syndrome. *J Anat.* (2018) 233:687–95. doi: 10.1111/joa.12890
84. Clement A, Wiweger M, von der Hardt S, Rusch MA, Selleck SB, Chien CB, et al. Regulation of zebrafish skeletogenesis by ext2/dackel and papst1/pinscher. *PLoS Genet.* (2008) 4:e1000136. doi: 10.1371/journal.pgen.1000136
85. Huitema LF, Apschner A, Logister I, Spoorendonk KM, Bussmann J, Hammond CL, et al. Entpd5 is essential for skeletal mineralization and regulates phosphate homeostasis in zebrafish. *Proc Natl Acad Sci USA.* (2012) 109:21372–7. doi: 10.1073/pnas.1214231110
86. Zhang Y, Cui FZ, Wang XM, Feng QL, Zhu XD. Mechanical properties of skeletal bone in gene-mutated stopsel(dtl28d) and wild-type zebrafish (*Danio rerio*) measured by atomic force microscopy-based nanoindentation. *Bone.* (2002) 30:541–6. doi: 10.1016/S8756-3282(02)00676-2
87. Cheah FS, Winkler C, Jabs EW, Chong SS. Tgfbeta3 regulation of chondrogenesis and osteogenesis in zebrafish is mediated through formation and survival of a subpopulation of the cranial neural crest. *Mech Dev.* (2010) 127:329–44. doi: 10.1016/j.mod.2010.04.003
88. Eberhart JK, He X, Swartz ME, Yan YL, Song H, Boling TC, et al. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat Genet.* (2008) 40:290–8. doi: 10.1038/ng.82
89. Ghassibe-Sabbagh M, Desmyter L, Langenberg T, Claes F, Boute O, Bayet B, et al. FAF1, a gene that is disrupted in cleft palate and has conserved function in zebrafish. *Am J Hum Genet.* (2011) 88:150–61. doi: 10.1016/j.ajhg.2011.01.003
90. Dougherty M, Kamel G, Grimaldi M, Gfrerer L, Shubinets V, Ethier R, et al. Distinct requirements for wnt9a and irf6 in extension and integration mechanisms during zebrafish palate morphogenesis. *Development.* (2013) 140:76–81. doi: 10.1242/dev.080473
91. Lawrence EA, Kague E, Aggleton JA, Harniman RL, Roddy KA, Hammond CL. The mechanical impact of col11a2 loss on joints; col11a2 mutant zebrafish show changes to joint development and function, which leads to early-onset osteoarthritis. *Philos Trans R Soc Lond B Biol Sci.* (2018) 373:20170335. doi: 10.1098/rstb.2017.0335
92. Askary A, Smeeton J, Paul S, Schindler S, Braasch I, Ellis NA, et al. Ancient origin of lubricated joints in bony vertebrates. *Elife.* (2016) 5:e16415. doi: 10.7554/eLife.16415.016
93. Fisher S, Jagadeeswaran P, Halpern ME. Radiographic analysis of zebrafish skeletal defects. *Dev Biol.* (2003) 264:64–76. doi: 10.1016/S0012-1606(03)00399-3
94. Asharani PV, Keupp K, Semler O, Wang W, Li Y, Thiele H, et al. Attenuated BMP1 function compromises osteogenesis, leading to bone fragility in humans and zebrafish. *Ame J Hum Genet.* (2012) 90:661–74. doi: 10.1016/j.ajhg.2012.02.026
95. Kague E, Roy P, Asselin G, Hu G, Simonet J, Stanley A, et al. Osterix/Sp7 limits cranial bone initiation sites and is required for formation of sutures. *Dev Biol.* (2016) 413:160–72. doi: 10.1016/j.ydbio.2016.03.011
96. Henke K, Daane JM, Hawkins MB, Dooley CM, Busch-Nentwich EM, Stemple DL, et al. Genetic screen for postembryonic development in the zebrafish (*Danio rerio*): dominant mutations affecting adult form. *Genetics.* (2017) 207:609–23. doi: 10.1534/genetics.117.300187
97. Chatani M, Takano Y, Kudo A. Osteoclasts in bone modeling, as revealed by *in vivo* imaging, are essential for organogenesis in fish. *Dev Biol.* (2011) 360:96–109. doi: 10.1016/j.ydbio.2011.09.013

98. Chen JR, Lai YH, Tsai JJ, Hsiao CD. Live fluorescent staining platform for drug-screening and mechanism-analysis in zebrafish for bone mineralization. *Molecules.* (2017) 22:2068. doi: 10.3390/molecules22122068
99. Huo L, Wang L, Yang Z, Li P, Geng D, Xu Y. Prednisolone induces osteoporosis-like phenotypes via focal adhesion signaling pathway in zebrafish larvae. *Biol Open.* (2018) 7. doi: 10.1242/bio.029405
100. Urso K, Caetano-Lopes J, Lee PY, Yan J, Henke K, Sury M, et al. A role for G protein-coupled receptor 137b in bone remodeling in mouse and zebrafish. *Bone.* (2019) 127:104–13. doi: 10.1016/j.bone.2019.06.002
101. Monma Y, Shimada Y, Nakayama H, Zang L, Nishimura N, Tanaka T. Aging-associated microstructural deterioration of vertebra in zebrafish. *Bone Rep.* (2019) 11:100215. doi: 10.1016/j.bonr.2019.100215
102. Zhang W, Xu J, Qiu J, Xing C, Li X, Leng B, et al. Novel and rapid osteoporosis model established in zebrafish using high iron stress. *Biochem Biophys Res Commun.* (2018) 496:654–60. doi: 10.1016/j.bbrc.2017.12.172
103. Jafari A, Qanie D, Andersen TL, Zhang Y, Chen L, Postert B, et al. Legumain regulates differentiation fate of human bone marrow stromal cells and is altered in postmenopausal osteoporosis. *Stem Cell Rep.* (2017) 8:373–86. doi: 10.1016/j.stemcr.2017.01.003
104. Huang HX, Lin H, Lan F, Wu YF, Yang ZG, Zhang JJ. Application of bone transgenic zebrafish in anti-osteoporosis chemical screening. *Anim Models Exp Med.* (2018) 1:53–61. doi: 10.1002/ame2.12000
105. Apschner A, Huitema LF, Ponsioen B, Peterson-Maduro J, Schulte-Merker S. Zebrafish enpp1 mutants exhibit pathological mineralization, mimicking features of generalized arterial calcification of infancy (GACI) and pseudoxanthoma elasticum (PXE). *Dis Models Mech.* (2014) 7:811–22. doi: 10.1242/dmm.015693
106. Mackay EW, Apschner A, Schulte-Merker S. Vitamin K reduces hypermineralisation in zebrafish models of PXE and GACI. *Development.* (2015) 142:1095–101. doi: 10.1242/dev.113811
107. Teng CS, Ting MC, Farmer DT, Brockop M, Maxson RE, Crump JG. Altered bone growth dynamics prefigure craniosynostosis in a zebrafish model of Saethre-Chotzen syndrome. *Elife.* (2018) 7:e37024. doi: 10.7554/eLife.37024.041
108. Ferreira CR, Xia ZJ, Clement A, Parry DA, Davids M, Taylan F, et al. A recurrent *de novo* heterozygous COG4 substitution leads to saul-wilson syndrome, disrupted vesicular trafficking, and altered proteoglycan glycosylation. *Am J Hum Genet.* (2018) 103:553–67. doi: 10.1016/j.ajhg.2018.09.003
109. Buchan JG, Gray RS, Gansner JM, Alvarado DM, Burgert L, Gitlin JD, et al. Kinesin family member 6 (kif6) is necessary for spine development in zebrafish. *Dev Dyn.* (2014) 243:1646–57. doi: 10.1002/dvdy.24208
110. Grimes DT, Boswell CW, Morante NF, Henkelman RM, Burdine RD, Ciruna B. Zebrafish models of idiopathic scoliosis link cerebrospinal fluid flow defects to spine curvature. *Science.* (2016) 352:1341–4. doi: 10.1126/science.aaf6419
111. Haller G, McCall K, Jenkitkasemwong S, Sadler B, Antunes L, Nikolov M, et al. A missense variant in SLC39A8 is associated with severe idiopathic scoliosis. *Nat Commun.* (2018) 9:4171. doi: 10.1038/s41467-018-06705-0
112. Gray RS, Wilm TP, Smith J, Bagnat M, Dale RM, Topczewski J, et al. Loss of col8a1a function during zebrafish embryogenesis results in congenital vertebral malformations. *Dev Biol.* (2014) 386:72–85. doi: 10.1016/j.ydbio.2013.11.028
113. Zhang X, Jia S, Chen Z, Chong YL, Xie H, Feng D, et al. Cilia-driven cerebrospinal fluid flow directs expression of urotensin neuropeptides to straighten the vertebrate body axis. *Nat Genet.* (2018) 50:1666–73. doi: 10.1038/s41588-018-0260-3
114. Printzi A, Fragkoulis S, Dimitriadi A, Keklikoglou K, Arvanitidis C, Witten PE, et al. Exercise-induced lordosis in zebrafish *Danio rerio* (Hamilton, 1822). *J Fish Biol.* (2019). doi: 10.1111/jfb.14240
115. Burrage LC, Reynolds JJ, Baratang NV, Phillips JB, Wegner J, McFarquhar A, et al. Bi-allelic variants in TONSL Cause SPONASTRIME dysplasia and a spectrum of skeletal dysplasia phenotypes. *Am J Hum Genet.* (2019) 104:422–38. doi: 10.1016/j.ajhg.2019.01.007
116. Baas D, Malbouyres M, Haftek-Terreau Z, Le Guellec D, Ruggiero F. Craniofacial cartilage morphogenesis requires zebrafish col11a1 activity. *Matrix Biol.* (2009) 28:490–502. doi: 10.1016/j.matbio.2009.07.004
117. Bergen DJM, Stevenson NL, Skinner REH, Stephens DJ, Hammond CL. The golgi matrix protein giantin is required for normal cilia function in zebrafish. *Biol Open.* (2017) 6:1180–89. doi: 10.1242/bio.025502
118. Newham E, Kague E, Aggleton JA, Fernee C, Brown KR, Hammond CL. Finite element and deformation analyses predict pattern of bone failure in loaded zebrafish spines. *J R Soc Interface.* (2019) 16:20190430. doi: 10.1098/rsif.2019.0430
119. Andreeva V, Connolly MH, Stewart-Swift C, Fraher D, Burt J, Cardarelli J, et al. Identification of adult mineralized tissue zebrafish mutants. *Genesis.* (2011) 49:360–6. doi: 10.1002/dvg.20712
120. Gistelinck C, Kwon RY, Malfait F, Symoens S, Harris MP, Henke K, et al. Zebrafish type I collagen mutants faithfully recapitulate human type I collagenopathies. *Proc Natl Acad Sci USA.* (2018) 115:E8037–46. doi: 10.1073/pnas.1722200115
121. Draper BW, Morcos PA, Kimmel CB. Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: a quantifiable method for gene knockdown. *Genesis.* (2001) 30:154–6. doi: 10.1002/gene.1053
122. Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet.* (2000) 26:216–20. doi: 10.1038/79951
123. Ekker SC, Larson JD. Morphant technology in model developmental systems. *Genesis.* (2001) 30:89–93. doi: 10.1002/gene.1038
124. Pickart MA, Klee EW, Nielsen AL, Sivasubbu S, Mendenhall EM, Bill BR, et al. Genome-wide reverse genetics framework to identify novel functions of the vertebrate secretome. *PLoS ONE.* (2006) 1:e104. doi: 10.1371/journal.pone.0000104
125. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, et al. p53 activation by knockdown technologies. *PLoS Genet.* (2007) 3:e78. doi: 10.1371/journal.pgen.0030078
126. Amoyel M, Cheng YC, Jiang YJ, Wilkinson DG. Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain. *Development.* (2005) 132:775–85. doi: 10.1242/dev.01616
127. Gerety SS, Wilkinson DG. Morpholino artifacts provide pitfalls and reveal a novel role for pro-apoptotic genes in hindbrain boundary development. *Dev Biol.* (2011) 350:279–89. doi: 10.1016/j.ydbio.2010.11.030
128. Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, van Impel A, et al. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev Cell.* (2015) 32:97–108. doi: 10.1016/j.devcel.2014.11.018
129. El-Brolosy MA, Stainier DYR. Genetic compensation: a phenomenon in search of mechanisms. *PLoS Genet.* (2017) 13:e1006780. doi: 10.1371/journal.pgen.1006780
130. Doyon Y, McCammon JM, Miller JC, Faraji F, Ngo C, Katibah GE, et al. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol.* (2008) 26:702–8. doi: 10.1038/nbt1409
131. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol.* (2008) 26:695–701. doi: 10.1038/nbt1398
132. Cade L, Reyon D, Hwang WY, Tsai SQ, Patel S, Khayter C, et al. Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. *Nucleic Acids Res.* (2012) 40:8001–10. doi: 10.1093/nar/gks518
133. Watson CJ, Monstad-Rios AT, Bhimani RM, Gistelinck C, Willaert A, Coucke P, et al. Phenomics-based quantification of CRISPR-induced mosaicism in zebrafish. *Cell Syst.* (2020) 10:275–86.e5. doi: 10.1016/j.cels.2020.02.007
134. Wu RS, Lam, II, Clay H, Duong DN, Deo RC, Coughlin SR, et al. A rapid method for directed gene knockout for screening in G0 zebrafish. *Dev Cell.* (2018) 46:112–25.e4. doi: 10.1016/j.devcel.2018.06.003
135. Salsman J, Dellaire G. Precision genome editing in the CRISPR era. *Biochem Cell Biol.* (2017) 95:187–201. doi: 10.1139/bcb-2016-0137
136. Mikuni T, Nishiyama J, Sun Y, Kamasawa N, Yasuda R. High-throughput high-resolution mapping of protein localization in mammalian brain by *in vivo* genome editing. *Cell.* (2016) 165:1803–17. doi: 10.1016/j.cell.2016.04.044
137. Leonetti MD, Sekine S, Kamiyama D, Weissman JS, Huang B. A scalable strategy for high-throughput GFP tagging of endogenous human proteins. *Proc Natl Acad Sci USA.* (2016) 113:E3501–8. doi: 10.1073/pnas.1606731113

138. Forlino A, Marini JC. Osteogenesis imperfecta. *Lancet.* (2016) 387:1657–71. doi: 10.1016/S0140-6736(15)00728-X
139. El-Brolosy MA, Kontarakis Z, Rossi A, Kuenne C, Gunther S, Fukuda N, et al. Genetic compensation triggered by mutant mRNA degradation. *Nature.* (2019) 568:193–7. doi: 10.1038/s41586-019-1064-z
140. Coggins NB, Stultz J, O'Geen H, Carvajal-Carmona LG, Segal DJ. Methods for scarless, selection-free generation of human cells and allele-specific functional analysis of disease-associated SNPs and variants of uncertain significance. *Sci Rep.* (2017) 7:15044. doi: 10.1038/s41598-017-15407-4
141. Renaud JB, Boix C, Charpentier M, De Cian A, Cochennec J, Duvernois-Berthet E, et al. Improved genome editing efficiency and flexibility using modified oligonucleotides with TALEN and CRISPR-Cas9 nucleases. *Cell Rep.* (2016) 14:2263–72. doi: 10.1016/j.celrep.2016.02.018
142. Richardson CD, Kazane KR, Feng SJ, Zelin E, Bray NL, Schafer AJ, et al. CRISPR-Cas9 genome editing in human cells occurs via the fanconi anemia pathway. *Nat Genet.* (2018) 50:1132–9. doi: 10.1038/s41588-018-0174-0
143. Lin S, Staahl BT, Alla RK, Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife.* (2014) 3:e04766. doi: 10.7554/eLife.04766.010
144. Jayathilaka K, Sheridan SD, Bold TD, Bochenska K, Logan HL, Weichselbaum RR, et al. A chemical compound that stimulates the human homologous recombination protein RAD51. *Proc Natl Acad Sci USA.* (2008) 105:15848–53. doi: 10.1073/pnas.0808046105
145. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol.* (2015) 33:538–42. doi: 10.1038/nbt.3190
146. Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Teo S, et al. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature.* (2016) 533:125–9. doi: 10.1038/nature17664
147. Singh P, Schimenti JC, Bolcun-Filas E. A mouse geneticist's practical guide to CRISPR applications. *Genetics.* (2015) 199:1–15. doi: 10.1534/genetics.114.169771
148. Song J, Yang D, Xu J, Zhu T, Chen YE, Zhang J. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. *Nat Commun.* (2016) 7:10548. doi: 10.1038/ncomms10548
149. Srivastava M, Nambiar M, Sharma S, Karki SS, Goldsmith G, Hegde M, et al. An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. *Cell.* (2012) 151:1474–87. doi: 10.1016/j.cell.2012.11.054
150. Xiong X, Chen M, Lim WA, Zhao D, Qi LS. CRISPR/Cas9 for human genome engineering and disease research. *Annu Rev Genomics Hum Genet.* (2016) 17:131–54. doi: 10.1146/annurev-genom-083115-022258
151. Yu C, Liu Y, Ma T, Liu K, Xu S, Zhang Y, et al. Small molecules enhance CRISPR genome editing in pluripotent stem cells. *Cell Stem Cell.* (2015) 16:142–7. doi: 10.1016/j.stem.2015.01.003
152. Boel A, De Saffel H, Steyaert W, Callewaert B, De Paepe A, Coucke PJ, et al. CRISPR/Cas9-mediated homology-directed repair by ssODNs in zebrafish induces complex mutational patterns resulting from genomic integration of repair-template fragments. *Dis Model Mech.* (2018) 11. doi: 10.1242/dmm.035352
153. Boel A, Steyaert W, De Rocker N, Menten B, Callewaert B, De Paepe A, et al. BATCH-GE: batch analysis of next-generation sequencing data for genome editing assessment. *Sci Rep.* (2016) 6:30330. doi: 10.1038/srep30330
154. Armstrong GA, Liao M, You Z, Lissouba A, Chen BE, Drapeau P. Homology directed knockin of point mutations in the zebrafish tardbp and fus genes in ALS using the CRISPR/Cas9 system. *PLoS ONE.* (2016) 11:e0150188. doi: 10.1371/journal.pone.0150188
155. Farr GH 3rd, Imani K, Pouv D, Maves L. Functional testing of a human PBX3 variant in zebrafish reveals a potential modifier role in congenital heart defects. *Dis Model Mech.* (2018) 11. doi: 10.1242/dmm.035972
156. Tessadori F, Roessler HI, Savelberg SMC, Chocron S, Kamel SM, Duran KJ, et al. Effective CRISPR/Cas9-based nucleotide editing in zebrafish to model human genetic cardiovascular disorders. *Dis Model Mech.* (2018) 11. doi: 10.1242/dmm.035469
157. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature.* (2017) 551:464–71. doi: 10.1038/nature24644
158. Qin W, Lu X, Lin S. Programmable base editing in zebrafish using a modified CRISPR-Cas9 system. *Methods.* (2018) 150:19–23. doi: 10.1016/j.ymeth.2018.07.010
159. Zhang Y, Qin W, Lu X, Xu J, Huang H, Bai H, et al. Programmable base editing of zebrafish genome using a modified CRISPR-Cas9 system. *Nat Commun.* (2017) 8:118. doi: 10.1038/s41467-017-00175-6
160. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature.* (2019) 576:149–57. doi: 10.1038/s41586-019-1711-4
161. Stuart T, Satija R. Integrative single-cell analysis. *Nat Rev Genet.* (2019) 20:257–72. doi: 10.1038/s41576-019-0093-7
162. Farrell JA, Wang Y, Riesenfeld SJ, Shekhar K, Regev A, Schier AF. Single-cell reconstruction of developmental trajectories during zebrafish embryogenesis. *Science.* (2018) 360. doi: 10.1126/science.aar3131
163. Spanjaard B, Hu B, Mitic N, Olivares-Chauvet P, Janjuha S, Ninov N, et al. Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. *Nat Biotechnol.* (2018) 36:469–73. doi: 10.1038/nbt.4124
164. Wagner DE, Weinreb C, Collins ZM, Briggs JA, Megason SG, Klein AM. Single-cell mapping of gene expression landscapes and lineage in the zebrafish embryo. *Science.* (2018) 360:981–7. doi: 10.1126/science.aar4362
165. Long F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol.* (2011) 13:27–38. doi: 10.1038/nrm3254
166. Miller RL. Transgenic mice: beyond the knockout. *Am J Physiol Renal Physiol.* (2011) 300:F291–300. doi: 10.1152/ajprenal.00082.2010
167. Tampieri A, Sprio S, Sandri M, Valentini F. Mimicking natural bio-mineralization processes: a new tool for osteochondral scaffold development. *Trends Biotechnol.* (2011) 29:526–35. doi: 10.1016/j.tibtech.2011.04.011
168. Gregg CL, Butcher JT. Quantitative *in vivo* imaging of embryonic development: opportunities and challenges. *Differentiation.* (2012) 84:149–62. doi: 10.1016/j.diff.2012.05.003
169. Moro E, Vettori A, Porazzi P, Schiavone M, Rampazzo E, Casari A, et al. Generation and application of signaling pathway reporter lines in zebrafish. *Mol Genet Genomics.* (2013) 288:231–42. doi: 10.1007/s00438-013-0750-z
170. Dutton JR, Antonellis A, Carney TJ, Rodrigues FS, Pavan WJ, Ward A, et al. An evolutionarily conserved intronic region controls the spatiotemporal expression of the transcription factor Sox10. *BMC Dev Biol.* (2008) 8:105. doi: 10.1186/1471-213X-8-105
171. Dougherty M, Kamel G, Shubinets V, Hickey G, Grimaldi M, Liao EC. Embryonic fate map of first pharyngeal arch structures in the sox10: kaede zebrafish transgenic model. *J Craniofac Surg.* (2012) 23:1333–7. doi: 10.1097/SCS.0b013e318260f20b
172. Kirby BB, Takada N, Latimer AJ, Shin J, Carney TJ, Kelsh RN, et al. *In vivo* time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. *Nat Neurosci.* (2006) 9:1506–11. doi: 10.1038/nn1803
173. Rodrigues FS, Doughton G, Yang B, Kelsh RN. A novel transgenic line using the Cre-lox system to allow permanent lineage-labeling of the zebrafish neural crest. *Genesis.* (2012) 50:750–7. doi: 10.1002/dvg.22033
174. Brunt LH, Begg K, Kague E, Cross S, Hammond CL. Wnt signalling controls the response to mechanical loading during zebrafish joint development. *Development.* (2017) 144:2798–09. doi: 10.1242/dev.153528
175. Wada N, Javidan Y, Nelson S, Carney TJ, Kelsh RN, Schilling TF. Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull. *Development.* (2005) 132:3977–88. doi: 10.1242/dev.01943
176. Hochgreb-Hägelen T, Koo DES, Bronner M. Znf385C mediates a novel p53-dependent transcriptional switch to control timing of facial bone formation. *Dev Biol.* (2015) 400:23–32. doi: 10.1016/j.ydbio.2015.01.011
177. Li L, Mao A, Wang P, Ning G, Cao Y, Wang Q. Endodermal pouch-expressed dmrt2b is important for pharyngeal cartilage formation. *Biol Open.* (2018) 7. doi: 10.1242/bio.035444
178. Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol.* (2002) 248:307–18. doi: 10.1006/dbio.2002.0711

179. Flanagan-Steet H, Aarnio M, Kwan B, Guihard P, Petrey A, Haskins M, et al. Cathepsin-mediated alterations in TGFss-related signaling underlie disrupted cartilage and bone maturation associated with impaired lysosomal targeting. *J Bone Miner Res.* (2016) 31:535–48. doi: 10.1002/jbmr.2722
180. Bonkowsky JL, Wang X, Fujimoto E, Lee JE, Chien CB, Dorsky RI. Domain-specific regulation of foxP2 CNS expression by lef1. *BMC Dev Biol.* (2008) 8:103. doi: 10.1186/1471-213X-8-103
181. DeLaurier A, Eames BF, Blanco-Sanchez B, Peng G, He X, Swartz ME, et al. Zebrafish sp7:EGFP: a transgenic for studying otic vesicle formation, skeletogenesis, bone regeneration. *Genesis.* (2010) 48:505–11. doi: 10.1002/dvg.20639
182. Kague E, Witten PE, Soenens M, Campos CL, Lubiana T, Fisher S, et al. Zebrafish sp7 mutants show tooth cycling independent of attachment, eruption and poor differentiation of teeth. *Dev Biol.* (2018) 435:176–84. doi: 10.1016/j.ydbio.2018.01.021
183. Dale RM, Topczewski J. Identification of an evolutionarily conserved regulatory element of the zebrafish col2a1a gene. *Dev Biol.* (2011) 357:518–31. doi: 10.1016/j.ydbio.2011.06.020
184. Filipek-Górniok B, Carlsson P, Haitina T, Habicher J, Ledin J, Kjellén L. The NDST gene family in zebrafish: role of NDST1B in pharyngeal arch formation. *PLoS ONE.* (2015) 10:e0119040. doi: 10.1371/journal.pone.0119040
185. Kague E, Bessling SL, Lee J, Hu G, Passos-Bueno MR, Fisher S. Functionally conserved cis-regulatory elements of COL18A1 identified through zebrafish transgenesis. *Dev Biol.* (2010) 337:496–505. doi: 10.1016/j.ydbio.2009.10.028
186. Pogoda HM, Riedl-Quinkertz I, Lohr H, Waxman JS, Dale RM, Topczewski J, et al. Direct activation of chordoblasts by retinoic acid is required for segmented centra mineralization during zebrafish spine development. *Development.* (2018) 145. doi: 10.1242/dev.159418
187. Hu P, Tian M, Bao J, Xing G, Gu X, Gao X, et al. Retinoid regulation of the zebrafish cyp26a1 promoter. *Dev Dyn.* (2008) 237:3798–808. doi: 10.1002/dvdy.21801
188. Li J, Hu P, Li K, Zhao Q. Identification and characterization of a novel retinoic acid response element in zebrafish cyp26a1 promoter. *Anat Rec (Hoboken).* (2012) 295:268–77. doi: 10.1002/ar.21520
189. Haga Y, Dominique VJ 3rd, Du SJ. Analyzing notochord segmentation and intervertebral disc formation using the twhh:gfp transgenic zebrafish model. *Transgenic Res.* (2009) 18:669–83. doi: 10.1007/s11248-009-9259-y
190. Ma RC, Jacobs CT, Sharma P, Kocha KM, Huang P. Stereotypic generation of axial tenocytes from bipartite sclerotome domains in zebrafish. *PLoS Genet.* (2018) 14:e1007775. doi: 10.1371/journal.pgen.1007775
191. DeLaurier A, Huycke TR, Nichols JT, Swartz ME, Larsen A, Walker C, et al. Role of mef2ca in developmental buffering of the zebrafish larval hyoid dermal skeleton. *Dev Biol.* (2014) 385:189–99. doi: 10.1016/j.ydbio.2013.11.016
192. Eames BF, Yan YL, Swartz ME, Levic DS, Knapik EW, Postlethwait JH, et al. Mutations in fam20b and xylt1 reveal that cartilage matrix controls timing of endochondral ossification by inhibiting chondrocyte maturation. *PLoS Genet.* (2011) 7:e1002246. doi: 10.1371/journal.pgen.1002246
193. Stewart S, Gomez AW, Armstrong BE, Henner A, Stankunas K. Sequential and opposing activities of Wnt and BMP coordinate zebrafish bone regeneration. *Cell Rep.* (2014) 6:482–98. doi: 10.1016/j.celrep.2014.01.010
194. Blum N, Begemann G. Osteoblast de- and redifferentiation are controlled by a dynamic response to retinoic acid during zebrafish fin regeneration. *Development.* (2015) 142:2894–903. doi: 10.1242/dev.120204
195. Geurtzen K, Knopf F, Wehner D, Huitema LF, Schulte-Merker S, Weidinger G. Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull. *Development.* (2014) 141:2225–34. doi: 10.1242/dev.105817
196. Bek JW, De Clercq A, De Saffel H, Soenens M, Huysseune A, Witten PE, et al. Photoconvertible fluorescent proteins: a versatile tool in zebrafish skeletal imaging. *J Fish Biol.* (2020) doi: 10.1111/jfb.14335
197. Singh SP, Holdway JE, Poss KD. Regeneration of amputated zebrafish fin rays from de novo osteoblasts. *Dev Cell.* (2012) 22:879–86. doi: 10.1016/j.devcel.2012.03.006
198. Cox BD, De Simone A, Tornini VA, Singh SP, Di Talia S, Poss KD. In toto imaging of dynamic osteoblast behaviors in regenerating skeletal bone. *Curr Biol.* (2018) 28:3937–47.e4. doi: 10.1016/j.cub.2018.10.052
199. Oralova V, Rosa JT, Soenens M, Bek JW, Willaert A, Witten PE, et al. Beyond the whole-mount phenotype: high-resolution imaging in fluorescence-based applications on zebrafish. *Biol Open.* (2019) 8. doi: 10.1242/bio.042374
200. Willems B, Buttner A, Huysseune A, Renn J, Witten PE, Winkler C. Conditional ablation of osteoblasts in medaka. *Dev Biol.* (2012) 364:128–37. doi: 10.1016/j.ydbio.2012.01.023
201. Kobayashi-Sun J, Yamamori S, Kondo M, Kuroda J, Ikegame M, Suzuki N, et al. Uptake of osteoblast-derived extracellular vesicles promotes the differentiation of osteoclasts in the zebrafish scale. *Commun Biol.* (2020) 3:190. doi: 10.1038/s42003-020-0925-1
202. Mitchell RE, Huitema LF, Skinner RE, Brunt LH, Severn C, Schulte-Merker S, et al. New tools for studying osteoarthritis genetics in zebrafish. *Osteoarthritis Cartilage.* (2013) 21:269–78. doi: 10.1016/j.joca.2012.11.004
203. Kim YI, Lee S, Jung SH, Kim HT, Choi JH, Lee MS, et al. Establishment of a bone-specific col10a1:GFP transgenic zebrafish. *Mol Cells.* (2013) 36:145–50. doi: 10.1007/s10059-013-0117-7
204. Hu Z, Chen B, Zhao Q. Hedgehog signaling regulates osteoblast differentiation in zebrafish larvae through modulation of autophagy. *Biol Open.* (2019) 8. doi: 10.1242/bio.040840
205. Knopf F, Hammond C, Chekuru A, Kurth T, Hans S, Weber CW, et al. Bone regenerates via dedifferentiation of osteoblasts in the zebrafish fin. *Dev Cell.* (2011) 20:713–24. doi: 10.1016/j.devcel.2011.04.014
206. Bussmann J, Schulte-Merker S. Rapid BAC selection for tol2-mediated transgenesis in zebrafish. *Development.* (2011) 138:4327–32. doi: 10.1242/dev.068080
207. Caetano-Lopes J, Henke K, Urso K, Duryea J, Charles JF, Warman ML, et al. Correction: unique and non-redundant function of csf1r paralogues in regulation and evolution of post-embryonic development of the zebrafish. *Development.* (2020) 147. doi: 10.1242/dev.192211
208. To TT, Witten PE, Huysseune A, Winkler C. An adult osteopetrosis model in medaka reveals the importance of osteoclast function for bone remodeling in teleost fish. *Comp Biochem Physiol C Toxicol Pharmacol.* (2015) 178:68–75. doi: 10.1016/j.cbpc.2015.08.007
209. Alexander C, Zuniga E, Blitz IL, Wada N, Le Pabic P, Javidan Y, et al. Combinatorial roles for BMPs and Endothelin 1 in patterning the dorsal-ventral axis of the craniofacial skeleton. *Development.* (2011) 138:5135–46. doi: 10.1242/dev.067801
210. Laux DW, Febbo JA, Roman BL. Dynamic analysis of BMP-responsive smad activity in live zebrafish embryos. *Dev Dyn.* (2011) 240:682–94. doi: 10.1002/dvdy.22558
211. Moro E, Ozhan-Kizil G, Mongera A, Beis D, Wierzbicki C, Young RM, et al. *In vivo* Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. *Dev Biol.* (2012) 366:327–40. doi: 10.1016/j.ydbio.2012.03.023
212. Weidinger G, Thorpe CJ, Wuennenberg-Stapleton K, Ngai J, Moon RT. The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning. *Curr Biol.* (2005) 15:489–500. doi: 10.1016/j.cub.2005.01.041
213. Felber K, Elks PM, Lecca M, Roehl HH. Expression of osterix is regulated by FGF and Wnt/beta-catenin signalling during osteoblast differentiation. *PLoS ONE.* (2015) 10:e0144982. doi: 10.1371/journal.pone.0144982
214. Stoick-Cooper CL, Weidinger G, Riehle KJ, Hubbert C, Major MB, Fausto N, et al. Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development.* (2007) 134:479–89. doi: 10.1242/dev.001123
215. Huang CJ, Tu CT, Hsiao CD, Hsieh FJ, Tsai HJ. Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev Dyn.* (2003) 228:30–40. doi: 10.1002/dvdy.10356
216. Li J, Chen Z, Gao LY, Colorni A, Ucko M, Fang S, et al. A transgenic zebrafish model for monitoring xbp1 splicing and endoplasmic reticulum stress *in vivo*. *Mech Dev.* (2015) 137:33–44. doi: 10.1016/j.mod.2015.04.001
217. Clark EM, Nonarath HJT, Bostrom JR, Link BA. Establishment and validation of an endoplasmic reticulum stress reporter to monitor zebrafish ATF6 activity in development and disease. *Dis Model Mech.* (2020) 13. doi: 10.1242/dmm.041426

218. Ding Y, Lee J, Hsu JJ, Chang CC, Baek KI, Ranjbarvaziri S, et al. Light-sheet imaging to elucidate cardiovascular injury and repair. *Curr Cardiol Rep.* (2018) 20:35. doi: 10.1007/s11886-018-0979-6
219. Collery RF, Link BA. Dynamic smad-mediated BMP signaling revealed through transgenic zebrafish. *Dev Dyn.* (2011) 240:712–22. doi: 10.1002/dvdy.22567
220. Dorsky RI, Itoh M, Moon RT, Chitnis A. Two tcf3 genes cooperate to pattern the zebrafish brain. *Development.* (2003) 130:1937–47. doi: 10.1242/dev.00402
221. Schwend T, Loucks EJ, Ahlgren SC. Visualization of gli activity in craniofacial tissues of hedgehog-pathway reporter transgenic zebrafish. *PLoS ONE.* (2010) 5:e14396. doi: 10.1371/journal.pone.0014396
222. Blair H, Schlesinger P, Huang C, Zaidi M. Calcium signalling and calcium transport in bone disease. *Subcell Biochem.* (2007) 45:539–62. doi: 10.1007/978-1-4020-6191-2_21
223. Cao X, Chen D. The BMP signaling and *in vivo* bone formation. *Gene.* (2005) 357:1–8. doi: 10.1016/j.gene.2005.06.017
224. Houschyar KS, Tapking C, Borrelli MR, Popp D, Duscher D, Maan ZN, et al. Wnt pathway in bone repair and regeneration - what do we know so far. *Front Cell Dev Biol.* (2019) 6:170. doi: 10.3389/fcell.2018.00170
225. Huang S, Wang Y, Luo L, Li X, Jin X, Li S, et al. BMP2 is related to hirschsprung's disease and required for enteric nervous system development. *Front Cell Neurosci.* (2019) 13:523. doi: 10.3389/fncel.2019.00523
226. Besio R, Garibaldi N, Leoni L, Cipolla L, Sabbioneda S, Biggiogera M, et al. Cellular stress due to impairment of collagen prolyl hydroxylation complex is rescued by the chaperone 4-phenylbutyrate. *Dis Model Mech.* (2019) 12. doi: 10.1242/dmm.038521
227. Besio R, Iula G, Garibaldi N, Cipolla L, Sabbioneda S, Biggiogera M, et al. 4-PBA ameliorates cellular homeostasis in fibroblasts from osteogenesis imperfecta patients by enhancing autophagy and stimulating protein secretion. *Biochim Biophys Acta Mol Basis Dis.* (2018) 1864(5 Pt A):1642–52. doi: 10.1016/j.bbadis.2018.02.002
228. Howarth DL, Lindtner C, Vacaru AM, Sachidanandam R, Tsedensodnom O, Vasilkova T, et al. Activating transcription factor 6 is necessary and sufficient for alcoholic fatty liver disease in zebrafish. *PLoS Genet.* (2014) 10:e1004335. doi: 10.1371/journal.pgen.1004335
229. Yeh KY, Lai CY, Lin CY, Hsu CC, Lo CP, Her GM. ATF4 overexpression induces early onset of hyperlipidaemia and hepatic steatosis and enhances adipogenesis in zebrafish. *Sci Rep.* (2017) 7:16362. doi: 10.1038/s41598-017-16587-9
230. Cardeira J, Gavaia PJ, Fernandez I, Cengiz IF, Moreira-Silva J, Oliveira JM, et al. Quantitative assessment of the regenerative and mineralogenic performances of the zebrafish caudal fin. *Sci Rep.* (2016) 6:39191. doi: 10.1038/srep39191
231. Chen CH, Puliafito A, Cox BD, Primo L, Fang Y, Di Talia S, et al. Multicolor cell barcoding technology for long-term surveillance of epithelial regeneration in zebrafish. *Dev Cell.* (2016) 36:668–80. doi: 10.1016/j.devcel.2016.02.017
232. Park S, Greco V, Cockburn K. Live imaging of stem cells: answering old questions and raising new ones. *Curr Opin Cell Biol.* (2016) 43:30–37. doi: 10.1016/j.ceb.2016.07.004
233. Mishra R, Sehring I, Cederlund M, Mulaw M, Weidinger G. NF-kappaB signaling negatively regulates osteoblast dedifferentiation during zebrafish bone regeneration. *Dev Cell.* (2020) 52:167–82.e7. doi: 10.1016/j.devcel.2019.11.016
234. Bruneel B, Witten PE. Power and challenges of using zebrafish as a model for skeletal tissue imaging. *Connect Tissue Res.* (2015) 56:161–73. doi: 10.3109/03008207.2015.1013193
235. Witten PE, Gil Martens L, Huysseune A, Takle H, Hjelde K. Towards a classification and an understanding of developmental relationships of vertebral body malformations in Atlantic salmon (*Salmo salar Aquaculture* L). *Aquaculture.* 295:6–14. doi: 10.1016/j.aquaculture.2009.06.037
236. Witten PE, Obach A, Huysseune A, Baeverfjord G. Vertebrae fusion in Atlantic salmon (*Salmo salar*): development, aggravation and pathways of containment. *Aquaculture.* (2006) 258:164–72. doi: 10.1016/j.aquaculture.2006.05.005
237. du Plessis A, Broeckhoven C, Guelpa A, le Roux SG. Laboratory x-ray micro-computed tomography: a user guideline for biological samples. *Gigascience.* (2017) 6:1–11. doi: 10.1093/gigascience/gix027
238. Rawson SD, Maksimcuka J, Withers PJ, Cartmell SH. X-ray computed tomography in life sciences. *BMC Biol.* (2020) 18:21. doi: 10.1186/s12915-020-0753-2
239. Babaei F, Hong TL, Yeung K, Cheng SH, Lam YW. Contrast-enhanced X-ray micro-computed tomography as a versatile method for anatomical studies of adult zebrafish. *Zebrafish.* (2016) 13:310–6. doi: 10.1089/zeb.2016.1245
240. Descamps E, Sochacka A, De Kegel B, Van Loo D, Van Hoorebeke L, Adriaens D. Soft tissue discrimination with contrast agents using micro-CT scanning. *Belgian J Zool.* (2014) 144:20–40. doi: 10.26496/bjz.2014.63
241. Charles JF, Sury M, Tsang K, Urso K, Henke K, Huang Y, et al. Utility of quantitative micro-computed tomographic analysis in zebrafish to define gene function during skeletogenesis. *Bone.* (2017) 101:162–71. doi: 10.1016/j.bone.2017.05.001
242. Suniaga S, Rolvien T, Vom Scheidt A, Fiedler IAK, Bale HA, Huysseune A, et al. Increased mechanical loading through controlled swimming exercise induces bone formation and mineralization in adult zebrafish. *Sci Rep.* (2018) 8:3646. doi: 10.1038/s41598-018-21776-1
243. Gadow H, Abbott EC. On the evolution of the vertebral coloumn of fishes. *Philos Trans R Soc Lond.* (1985) 186:163–221. doi: 10.1098/rstb.1895.0004
244. Sakata-Haga H, Uchishiba M, Shimada H, Tsukada T, Mitani M, Arikawa T, et al. A rapid and nondestructive protocol for whole-mount bone staining of small fish and *Xenopus*. *Sci Rep.* (2018) 8:7453–60. doi: 10.1038/s41598-018-25836-4
245. Moriguchi T, Yano K, Nakagawa S, Kaji F. Elucidation of adsorption mechanism of bone-staining agent alizarin red S on hydroxyapatite by FT-IR microspectroscopy. *J Colloid Interface Sci.* (2003) 260:19–25. doi: 10.1016/S0021-9797(02)00157-1
246. Fraser TWK, Witten PE, Albrektsen S, Breck O, Fontanillas R, Nankervis L, et al. Phosphorus nutrition in farmed Atlantic salmon (*Salmo salar*): life stage and temperature effects on bone pathologies. *Aquaculture.* (2019) 511:1–12. doi: 10.1016/j.aquaculture.2019.734246
247. Witten PE, Fjelldal PG, Huysseune A, McGurk C, Obach A, Owen MAG. Bone without minerals and its secondary mineralization in Atlantic salmon (*Salmo salar*): the recovery from phosphorus deficiency. *J Exp Biol.* (2019) 222:1–15. doi: 10.1242/jeb.188763
248. Vandenplas S, De Clercq A, Huysseune A. Tooth replacement without a dental lamina: the search for epithelial stem cells in *Polypterus senegalus*. *J Exp Zool B Mol Dev Evol.* (2014) 322:281–93. doi: 10.1002/jez.b.22577
249. Woltmann I, Shkil FN, De Clercq A, Huysseune A, Witten PE. Supernumerary teeth in the pharyngeal dentition of slow developing zebrafish. *J Appl Ichthyol.* (2018) 34:455–64. doi: 10.1111/jai.13668
250. Bensimon-Brito A, Cardeira J, Dionísio G, Huysseune A, Cancela ML, Witten PE. Revisiting *in vivo* staining with alizarin red S - a valuable approach to analyse zebrafish skeletal mineralization during development and regeneration. *BMC Dev Biol.* (2016) 16:1–9. doi: 10.1186/s12861-016-0102-4
251. Kiernan JA. *Histological and Histochemical Methods: Theory and Practice*. 5th ed. Banbury: Scion Publishing. (2015)
252. Cubbage CC, Mabee PM. Development of the cranium and paired fins in the zebrafish *Danio rerio* (*Ostariophysi*, *Cyprinidae*). *J Morphol.* (1996) 229:121–60. doi: 10.1002/(SICI)1097-4687(199608)229:2<121::AID-JMOR1>3.0.CO;2-4
253. Kimmel CB, Miller CT, Moens CB. Specification and morphogenesis of the zebrafish larval head skeleton. *Dev Biol.* (2001) 233:239–57. doi: 10.1006/dbio.2001.0201
254. Kimmel CB, Walker MB, Miller CT. Morphing the hyomandibular skeleton in development and evolution. *J Exp Zool B Mol Dev Evol.* (2007) 308:609–24. doi: 10.1002/jez.b.21155
255. Walker M, Kimmel C. A two-color acid-free cartilage and bone stain for zebrafish larvae. *Biotech Histochem.* (2007) 82:23–28. doi: 10.1080/10520290701333558
256. Philip NS, Green DM. Recovery and enhancement of faded cleared and double stained specimens. *Biotech Histochem.* (2000) 75:193–6. doi: 10.3109/10520290009066500
257. Copper JE, Budgeon LR, Foutz CA, van Rossum DB, Vanselow DJ, Hubley MJ, et al. Comparative analysis of fixation and embedding techniques for

optimized histological preparation of zebrafish. *Comp Biochem Physiol C Toxicol Pharmacol.* (2018) 208:38–46. doi: 10.1016/j.cbpc.2017.11.003
258. Witten PE. Enzyme histochemical characteristics of osteoblasts and mononucleated osteoclasts in a teleost fish with acellular bone (*Oreochromis niloticus, Cichlidae*). *Cell Tissue Res.* (1997) 287:591–9. doi: 10.1007/s004410050782
259. Sarmah S, Barrallo-Gimeno A, Melville DB, Topczewski J, Solnica-Krezel L, Knapik EW. Sec24D-dependent transport of extracellular matrix proteins is required for zebrafish skeletal morphogenesis. *PLoS ONE.* (2010) 5:e10367. doi: 10.1371/journal.pone.0010367
260. Pasqualetti S, Banfi G, Mariotti M. The zebrafish scale as model to study the bone mineralization process. *J Mol Histol.* (2012) 43:589–95. doi: 10.1007/s10735-012-9425-z
261. Duran I, Mari-Beffa M, Santamaria JA, Becerra J, Santos-Ruiz L. Actinotrichia collagens and their role in fin formation. *Dev Biol.* (2011) 354:160–72. doi: 10.1016/j.ydbio.2011.03.014
262. Liedtke D, Orth M, Meissler M, Geuer S, Knaup S, Koblitz I, et al. ECM alterations in Fndc3a (fibronectin domain containing protein 3A) deficient zebrafish cause temporal fin development and regeneration defects. *Sci Rep.* (2019) 9:13383. doi: 10.1038/s41598-019-50055-w
263. Sehring IM, Weidinger G. Recent advancements in understanding fin regeneration in zebrafish. *Wiley Interdiscip Rev Dev Biol.* (2019) 9:e367. doi: 10.1002/wdev.367
264. Stewart S, Stankunas K. Limited dedifferentiation provides replacement tissue during zebrafish fin regeneration. *Dev Biol.* (2012) 365:339–49. doi: 10.1016/j.ydbio.2012.02.031
265. Paul S, Schindler S, Giovannone D, de Millo Terrazzani A, Mariani FV, Crump JG. Ihha induces hybrid cartilage-bone cells during zebrafish jawbone regeneration. *Development.* (2016) 143:2066–76. doi: 10.1242/dev.131292
266. Azevedo AS, Grotek B, Jacinto A, Weidinger G, Saude L. The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations. *PLoS ONE.* (2011) 6:e22820. doi: 10.1371/journal.pone.0022820
267. Kinoshita M, Murata K, Naruse K, Tanaka M, Kamei Y. *Medaka: Biology, Management, Experimental Protocols*. Vol. 2. Wiley-Blackwell (2019).
268. Furutani-Seiki M, Wittbrodt J. Medaka and zebrafish, an evolutionary twin study. *Mech Dev.* (2004) 121:629–37. doi: 10.1016/j.mod.2004.05.010
269. Wittbrodt J, Shima A, Schartl M. Medaka–a model organism from the far East. *Nat Rev Genet.* (2002) 3:53–64. doi: 10.1038/nrg704
270. Azetsu Y, Inohaya K, Takano Y, Kinoshita M, Tasaki M, Kudo A. The sp7 gene is required for maturation of osteoblast-lineage cells in medaka (Oryzias latipes) vertebral column development. *Dev Biol.* (2017) 431:252–62. doi: 10.1016/j.ydbio.2017.09.010
271. Irie K, Kuroda Y, Mimori N, Hayashi S, Abe M, Tsuji N, et al. Histopathology of a wavy medaka. *J Toxicol Pathol.* (2016) 29:115–8. doi: 10.1293/tox.2015-0070
272. Mantoku A, Chatani M, Aono K, Inohaya K, Kudo A. Osteoblast and osteoclast behaviors in the turnover of attachment bones during medaka tooth replacement. *Dev Biol.* (2016) 409:370–81. doi: 10.1016/j.ydbio.2015.12.002
273. Yu T, Witten PE, Huysseune A, Buettner A, To TT, Winkler C. Live imaging of osteoclast inhibition by bisphosphonates in a medaka osteoporosis model. *Dis Model Mech.* (2016) 9:155–63. doi: 10.1242/dmm.019091
274. Meyer A, Van de Peer Y. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *Bioessays.* (2005) 27:937–45. doi: 10.1002/bies.20293
275. Hadjantonakis AK, Pirity M, Nagy A. Cre recombinase mediated alterations of the mouse genome using embryonic stem cells. *Methods Mol Biol.* (2008) 461:111–32. doi: 10.1007/978-1-60327-483-8_8
276. Utomo AR, Nikitin AY, Lee WH. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol.* (1999) 17:1091–6. doi: 10.1038/15073

Conflict of Interest: PS is a Bruker employee.

The remaining 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 Endocrinology

REVIEW
published: 19 February 2021
doi: 10.3389/fendo.2020.628946

Massively Parallel Sequencing for Rare Genetic Disorders: Potential and Pitfalls

Aideen M. McInerney-Leo[1] *and Emma L. Duncan*[2*]

[1] Dermatology Research Centre, University of Queensland Diamantina Institute, The University of Queensland, Brisbane, QLD, Australia, [2] Department of Twin Research & Genetic Epidemiology, Faculty of Life Sciences and Medicine, School of Life Course Sciences, King's College London, London, United Kingdom

There have been two major eras in the history of gene discovery. The first was the era of linkage analysis, with approximately 1,300 disease-related genes identified by positional cloning by the turn of the millennium. The second era has been powered by two major breakthroughs: the publication of the human genome and the development of massively parallel sequencing (MPS). MPS has greatly accelerated disease gene identification, such that disease genes that would have taken years to map previously can now be determined in a matter of weeks. Additionally, the number of affected families needed to map a causative gene and the size of such families have fallen: *de novo* mutations, previously intractable by linkage analysis, can be identified through sequencing of the parent–child trio, and genes for recessive disease can be identified through MPS even of a single affected individual. MPS technologies include whole exome sequencing (WES), whole genome sequencing (WGS), and panel sequencing, each with their strengths. While WES has been responsible for most gene discoveries through MPS, WGS is superior in detecting copy number variants, chromosomal rearrangements, and repeat-rich regions. Panels are commonly used for diagnostic purposes as they are extremely cost-effective and generate manageable quantities of data, with no risk of unexpected findings. However, in instances of diagnostic uncertainty, it can be challenging to choose the right panel, and in these circumstances WES has a higher diagnostic yield. MPS has ethical, social, and legal implications, many of which are common to genetic testing generally but amplified due to the magnitude of data (*e.g.*, relationship misattribution, identification of variants of uncertain significance, and genetic discrimination); others are unique to WES and WGS technologies (*e.g.*, incidental or secondary findings). Nonetheless, MPS is rapidly translating into clinical practice as an extremely useful part of the clinical armamentarium.

Keywords: gene discovery, massively parallel sequencing, skeletal dysplasias, whole exome sequencing, rare genetic bone disorder

OPEN ACCESS

Edited by:
Wim Van Hul,
University of Antwerp, Belgium

Reviewed by:
Cristina Sobacchi,
National Research Council (CNR), Italy
Alberto Falchetti,
Istituto Auxologico Italiano (IRCCS), Italy

***Correspondence:**
Emma L. Duncan
emma.duncan@kcl.ac.uk

Specialty section:
This article was submitted to Bone Research, a section of the journal Frontiers in Endocrinology

Received: *13 November 2020*
Accepted: *21 December 2020*
Published: *19 February 2021*

Citation:
McInerney-Leo AM and Duncan EL (2021) Massively Parallel Sequencing for Rare Genetic Disorders: Potential and Pitfalls. Front. Endocrinol. 11:628946. doi: 10.3389/fendo.2020.628946

THE RECOGNITION OF RARE GENETIC DISORDERS

In starting this paper exploring massively parallel sequencing (MPS) technologies for rare genetic disorders with particular reference to skeletal diseases, it is extremely fitting that the first description of any monogenic disorder was black bone disease (now known as alkaptonuria). Archibald Garrod, a UK physician, commented in 1902 that the constellation of symptoms constituting alkaptonuria "was apt to make its appearance in two or more brothers and sisters" (1). Increased occurrence in siblings does not necessarily indicate a genetic disorder (increased familiality may also reflect environmental sharing); but crucially Garrod also noted that they were commonly "the offspring of marriages of first cousins who did not themselves exhibit this anomaly … and among whose forefathers there is no record of its having occurred". Through the world-wide dissemination of Gregor Mendel's gardening experiences (2), the modern reader would rapidly recognize this "peculiar mode of incidence….well known in connexion with some other conditions" as a classic description of a recessive monogenic disorder.

Monogenic disorders arise due to carriage of highly penetrant variants affecting a single gene. The presence or absence of disease can be predicted from the presence or absence of the variant(s) of interest. With some allowance for differential penetrance and expressivity, the mathematical and predictable inheritance patterns of monogenic disorders enable meaningful genetic counseling to affected individuals and known carriers and to parents with a child affected by a *de novo* dominant mutation. Monogenic disorders are individually rare but cumulatively affect 1% of the worldwide population (3) and include many (currently, 461 defined) skeletal disorders (4).

MAPPING RARE GENETIC DISORDERS: EARLY DAYS

It took many decades to move from the recognition of monogenic disorders to the mapping of the first gene. Initially, such genes were mapped by linkage—the co-segregation [or linkage] of a genetic region with a disease phenotype within a family. The first disease to be linked to the inheritance of any genetic marker was the dominant disorder of Huntington's disease, initially mapped to the short arm of chromosome 4 in 1983 (5). However, it took another decade until the gene itself (*huntingtin*, located on chromosome 4p16.3) was finally determined, which effort took 58 researchers from six research groups and the participation of 75 large Venezuelan families (6). By this time, though, the first gene to be identified for any human disease had been cloned [*CYBB*, for X-linked chronic granulomatous disease (IM 300640)] (7). Linkage was often aided by recognition of chromosomal aberrations, such as translocation or uniparental disomy, in an affected individual —for example, contributing to the mapping of the gene for cystic fibrosis (8, 9). By 1995 a review article enthused about the dizzying number of genes which had been identified for human diseases—42!—marking the only time the authors have seen the phrase, "Bingo!" used in a scientific paper (10).

Gene mapping by linkage, irrespective of the chosen marker [whether chromosomal banding patterns, restriction fragment length polymorphisms, microsatellites, or single nucleotide polymorphisms (SNPs)] is critically restricted by the number of informative meioses within contributing family pedigrees. Cross-over events and recombination at meiosis incrementally limit the genetic region shared by affected individuals within the family; ergo, large multi-generational families with many affected individuals (equating to multiple meiotic events between distantly related affected individuals) represent the ideal pedigree for gene mapping *via* linkage. It would be unusual for a single pedigree to have sufficient affected individuals and sufficient informative meioses for definitive statistical evidence of linkage; thus, methods of summing genetic information from multiple families were developed. Many monogenic diseases were mapped by linkage, by 2001, 1,336 monogenic disorders [personal correspondence from Dr Victor McKusick, quoted in (11)].

There are some obvious difficulties with gene mapping by linkage. The first is that diseases with late onset or incomplete penetrance are harder to map, as correct disease attribution is more difficult. Large family pedigrees are inherently unlikely in diseases that adversely affect reproductive fitness (which includes many skeletal dysplasias, for example). The success of pooling genetic information from disparate families assumes that all affected individuals, irrespective of which family they come from, have a mutation in the same causative gene and not, for example, mutations in many different genes along a common pathway resulting in a common end phenotype. Here it is relevant to add that within any one family all affected individuals need to carry the same mutation (and, by definition, share the same haplotype of genetic markers); however, when pooling genetic information from multiple families, each family can have a different causative mutation—as long as it is in the same gene. Diseases with significant gene/environment interaction will be difficult to map—unless all family members are exposed equally to the requisite environment, essentially removing its contribution to variable affection status. Lastly, novel mutations are intractable by linkage, as by definition linkage requires the presence of a shared genetic haplotype among affected family members.

MAPPING RARE GENETIC DISORDERS: A COMPLETE FRAMESHIFT

In 2014, in a paper celebrating the 10th anniversary of the release of the Human Genome (12) and using the example of gene mapping for fibrodysplasia ossificans progressiva (FOP; MIM 135100), we wrote that, "if massively parallel sequencing [MPS] technologies had been available when the search for the FOP gene began, the answer could have been found in 15 weeks, not 15 years." At first glance, this statement might seem excessively hubristic even for a celebratory piece. However, to illustrate the point: at this time we had just published a review of MPS in skeletal dysplasias (13) which

at the time of submission (April 2013) listed 22 skeletal dysplasias mapped using MPS with a total of 26 publications; at the time of acceptance just twelve weeks later (July 2013) ten more papers had added another six skeletal dysplasia genes to the list. The *Nosology and Classification of Genetic Skeletal Disorders: 2010 Revision* identified "456 conditions…316 [of which] were associated with mutations in one or more of 226 different genes." (14) By the 2019 revision, pathogenic variants in 437 genes had been identified for 425 of 461 disorders now categorized (92%) (4)—*i.e.* after the decades needed to identify the first 226 genes for rare skeletal disorders, it took less than 10 years to double this number. As for skeletal dysplasias, so for many other monogenic disorders, as the mode of gene discovery rapidly transitioned from positional cloning and other traditional gene mapping methods to MPS (15, 16). Currently, the catalog Online Mendelian Inheritance in Man (https://omim.org/) lists 6,751 phenotypes for which the molecular basis is known and 4,339 genes with a phenotype-causing mutation—these numbers have increased even during the short time this paper was in review.

The key developments underpinning the extraordinary recent progress in gene mapping in rare disorders are:

a. the publication of the human genome project in 2003 (17) (https://www.genome.gov/human-genome-project), providing the reference genome for comparison with sequence data.
b. the development of massively parallel sequencing (MPS) technologies—both undifferentiated genome sequencing and sequencing targeted to the exome or a defined set of genes—allowing sequencing of multiple genomic regions simultaneously.
c. easy accessibility of large databases of genetic variability (such as the UK10K (https://www.uk10k.org/), 1,000Genomes (https://www.internationalgenome.org/), Human Variome Project (https://www.humanvariomeproject.org/), gnomAD (https://gnomad.broadinstitute.org/) and dbSNP (https://www.ncbi.nlm.nih.gov/snp/), so that rare/novel disease-causing variants could be differentiated from more common polymorphisms within ethnically appropriate populations.
d. international collaboration and cooperation, between clinicians and researchers, with interaction through platforms such as the National institute of Health Centers for Mendelian Genomics (http://mendelian.org/), Orphanet (https://www.orpha.net/consor/cgi-bin/index.php), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), the International Rare Diseases Research Consortium (https://irdirc.org/) and Leiden Open Variation Database (https://www.lovd.nl/), informing and encouraging collaborative new gene discovery.

Each of the above websites has detailed information about their formation and governance.

TYPES OF MASSIVELY PARALLEL SEQUENCING

MPS technologies can be divided into pre-defined gene panels and the more agnostic approaches of whole genome and whole exome sequencing (with abbreviations WGS and WES respectively). The authors acknowledge that, strictly speaking, WGS and WES are misnomers, as neither technology has perfect coverage of its eponymous target; however, these common abbreviations will be used in this review. There are many excellent review articles on the technical aspects of the various types of MPS (18, 19). The strengths and weakness of different MPS technologies for new gene discovery and for clinical utility are discussed below.

ANALYSIS OF MPS DATA

Human genetic variability is huge. On average, each individual harbors 3 million SNPs (5,000 private to that individual); 700,000 indels (295 private), 215 large deletions (one private), and 576 genes with either homozygous or compound heterozygous predicted loss-of-function variants (20). Sifting so much data to determine the causal variant for a disease can be, at the risk of understatement, challenging. After stringent quality control of the sequencing data, a typical common-sense and empiric approach adopted by ourselves and many others has been to filter for rare variants (with minor allele frequency thresholds informed by disease frequency and mode of inheritance) of likely deleterious effect (*e.g.*, nonsense, missense, affecting canonical splice-sites, frameshift), affecting highly evolutionarily conserved bases and predicted damaging by one or more *in silico* prediction algorithms [*e.g.*, SIFT (21), Polyphen (22), MutationTaster (23)] that segregate appropriately with disease within a family (24); or, if looking at unrelated individuals, are present in the same gene in multiple unrelated cases (25). Obviously this description is somewhat simplistic, and simply finding variants that fulfil these criteria does not prove they are disease-causing. However, these steps usually lead to a tractable list of variants that can then be assessed for functional consequence and/or compared with data from other unrelated individuals with a common phenotype.

The use of ethnically appropriate populations to determine allele frequencies for variants and inform their categorization as novel, rare, infrequent, or common, is critical. The reference data in most sequencing databases are not populated from all ethnic groups equally, with over-representation of western European Caucasian populations; more recent sequencing efforts have aimed to address this imbalance. Cohorts such as gnomAD (26) provide ethnicity-specific minor allele frequencies; but the robustness of these understandably depends on the size of the sequenced population contributing to the data.

HOW MANY CASES ARE NEEDED TO MAP A MONOGENIC DISORDER?

The success rate of MPS to map novel causative genes depends on the mode of inheritance of the condition. We have focused on examples drawn from skeletal dysplasias here, but the principles apply to other disease groups also.

Autosomal recessive disorders are generally easier to 'solve' as the list of genes with rare homozygous or compound

heterozygous variants is usually relatively short. It is possible to identify the likely causative gene from initial sequencing a single affected individual (27–29)—though, as above, such evidence would need confirmation by identifying pathogenic variants in the same gene in other unrelated individuals and/or functional support.

For *de novo* dominant disorders, the causative gene may be mapped by sequencing a single affected child and parents (30) or by sequencing several unrelated probands (as few as three) and filtering the data for either a common variant shared by all affected individuals (31, 32) or with unique mutations but within a common gene (25, 33). Mapping inherited (as opposed to *de novo*) autosomal dominant diseases is more difficult due to co-inheritance of multiple unimportant variants within a family. The most parsimonious design is to sequence most distantly related affected individuals: as discussed above, these have the largest number of meioses (and, by implication, greatest number of recombination events) separating the affected cases. With n meioses between individuals, the chance of any given variant segregating is $(½)^n$; and use of MPS data from both affected and unaffected individuals can help filter down variants according to disease status. Examples of autosomal dominant skeletal dysplasias mapped within a single family include spondylocostal dysostoses, mapped through MPS of five members of a family (three affected, two unaffected), with pathogenicity subsequently confirmed with functional data (34); and KBG syndrome [MIM 148050] (named after the initials of early affected individuals, in whom skeletal features include macrodontia, craniofacial abnormalities, and short stature), initially mapped through MPS of two affected family members and confirmed through MPS of one unrelated person (35).

Examples of X-linked skeletal dysplasias mapped by WES include the identification of mutations in *FLNA* as the cause of Terminal Osseous Dysplasia (36); and two forms of osteogenesis imperfecta, due to mutations in *PLS3* (37) and *MBTBS2* (38).

Somatic disorders can be mapped through paired analysis, with MPS of affected and unaffected tissues, subtracting the variants in the latter from the former—indeed, this approach is commonly employed in paired tumor/germline sequencing in cancer. This approach has been successful in skeletal dysplasias also—for example, identification of postzygotic somatic mutations in *PIK3CA* as the cause of Congenital Lipomatous Overgrowth with Vascular, Epidermal, and Skeletal anomalies (CLOVES), identified through WES of affected lipomatous tissue from six individuals compared with their germline DNA (39); and of *AKT1* as the cause of Proteus Syndrome (40) through WES of affected *vs.* unaffected tissue biopsies in 29 individuals. Depth of coverage will critically affect the ability to detect mosaicism, in that the allelic 'mix' in somatic disorders will vary both between individuals and between different tissues within an individual. The acceptable depth of MPS for calling germline heterozygous carriage of a variant is relatively modest: 10× is usually regarded as sufficient to 'call' a heterozygous variant and 15× for a homozygous variant (41); at these depths of coverage WES would be unlikely to detect low level mosaicism.

STRENGTHS AND WEAKNESS OF DIFFERENT MPS TECHNOLOGIES FOR NEW GENE DISCOVERY AND FOR CLINICAL UTILITY

In keeping with early predictions that 85% of Mendelian disorders would arise from coding mutations (42) and with the logic inherent in Sutton's law (*viz*., that one robs banks because that's where the money is), it is neither surprising that WES has been the most frequently employed modality to map novel genes, nor how successful this approach has been. Most of the examples provided above used WES as their mode of gene discovery, and the figure given above may well prove an underestimate. WES is not simply much cheaper than WGS for a given coverage: the large databases detailing exonic variation that informs analysis of WES data do not as yet exist for the whole genome (though this is rapidly changing with initiatives such as the UK Biobank 500K Sequencing Project and gnomAD), and proving causality for non-coding variants is difficult.

WES has proven similarly fruitful in diagnostic yield when translated from the research setting to clinical delivery [recently reviewed extensively (43)] with high diagnostic rates reported in both developed and developing countries (44), including sequencing in consanguineous families (44–46) and singleton sequencing (47) [noting that yield is approximately two-fold higher when sequencing parent–child trios compared with singletons (43)]. WES may also lead to a revision of a diagnosis—which may be confronting to both patient and clinician (discussed further below) but hopefully direct more appropriate clinical care (45, 47). A recent study reporting 155 novel causal genes identified during clinical sequencing (WES) in a consanguineous cohort comprising 2,200 families highlighted not only the use of WES for diagnostic purposes but also the benefits of these data in completing the virtuous circle of clinical discovery and clinical delivery, through feedback of these data for ongoing research and gene discovery (44). However, WES is not ideal for detection of copy number variation (48) including detection of large indels.

Very few monogenic disorders due to non-coding/splice-site variants have been identified to date (49). Ironically, a notable exception to this is the skeletal disorder of van Buchem's disease, a high bone mass disorder due to a 52 kb deletion downstream of *SOST* (50), though this disorder was not identified through MPS approaches. Thus, the usefulness of WGS in gene discovery in monogenic disorders, compared to WES, has not yet been established. Certainly WGS captures the exome more evenly (as well, obviously, as the genome) than does WES. WGS is also superior for the detection of large (>50 bp) indels, copy number variation, and chromosomal rearrangements. The higher costs of WGS and analysis are rapidly falling (51); and thus choosing between sequencing technologies from a purely fiscal perspective may soon be redundant. Nonetheless, to date WGS has not demonstrated superiority to WES in diagnostic utility (43); and the extent to which WGS may ultimately provide a diagnosis in cases for which WES has failed to identify a cause is not known.

By definition, a targeted panel approach cannot be used for new gene discovery, as such panels consist of already identified genes. Nonetheless, panel sequencing has an established place within clinical delivery as a cheap, sensitive, and specific means of sequencing known disease genes, with excellent coverage due to the limited targeted region, and minimization of some of the concerns raised with the agnostic approaches such as WES or WGS such as incidental or secondary findings (discussed below). However, the first-line use of WES, rather than panel approaches —even when up to three panels were chosen by expert clinical geneticists—shortens the diagnostic odyssey and is more cost-effective (52).

Considering clinical utility of MPS technologies for bone diseases specifically, both WES (53) and panel sequencing (54) approaches have been reported. There are no inherently unique issues pertaining to clinical use of MPS in skeletal diseases compared to other disorders.

INCORRECT ATTRIBUTION OF PATHOGENICITY

A variant is only rare when considered against the population; within a family, a rare variant is not rare—it has a 50% chance of transmission from a parent to a child; similarly siblings will share a variant identical-by-descent on average 50%. It is extremely easy to be tempted into attributing causality to a rare variant that segregates within a small family just because it is rare [discussed in depth in (55) and (56)]. However, *a priori* one can predict the chance that any particular variant will segregate with disease within a family according to the number of meiosis between affected individuals and within a small family that probability may be higher than the typical threshold for declaring scientific significance (*i.e.* $p < 0.05$). Unsurprisingly, in a review article on this topic, MacArthur et al. wrote that of "406 published severe disease mutations....122 (27%) were either common polymorphisms or lacked direct evidence for pathogenicity" (56).

Efforts to refine criteria for attributing pathogenicity to an identified variant led to the publication of guidelines for classifying the likely pathogenicity of identified variants (*e.g.* 'pathogenic', 'likely pathogenic', 'variants of uncertain significance', *etc.*) according to the strength of evidence (57). These guidelines recommend using multiple criteria and resources to guide classification of an individual variant into a particular category, including population, disease-specific, and sequence databases, the published literature, the type of variant (nonsense, frameshift, initiation codon, canonical splice-sites, large deletions, *etc.*), and *in silico* prediction algorithms. However, considering the evidential basis even within these criteria demonstrates the imperfections. There are multiple *in silico* prediction methods, each with differing criteria (gene-level, variant level, evolutionary conservation, amino acid change, *etc.*) trained on varying datasets—not surprisingly, they vary in performance [recently discussed and compared in (58)]. Replication—observing the same mutation with the same phenotype in an unrelated family—depends on cooperation and collaboration of researchers, and for rare diseases this needs to happen at an international level—which depends on clinical networks. Clinical variation databases (*e.g.*, ClinVar, Online Mendelian Inheritance in Man, Leiden Open Variation Database, Human Gene Mutation Database) rely on curation expertise. Altruism is a key component for the success of any database [including PubMed (https://pubmed.ncbi.nlm.nih.gov/)] —however, clinical reporting of affected cases requires awareness, motivation, confidence, and time. Thus, functional studies, in either *in vitro* or *in vivo* models, are often necessary for definitive classification. To this end, CRISPR technology (for which discoverers Emmanuelle Charpentier and Jennifer Doudna were recently awarded the 2020 Nobel Prize for Chemistry) has proven a boon.

ETHICAL, LEGAL, AND SOCIAL IMPLICATIONS IN MASSIVELY PARALLEL SEQUENCING TECHNOLOGIES

Whatever type of genetic testing is performed—whether MPS or other technologies—pre-test discussion is crucial to ensure the individual is aware of all possible outcomes and their implications, both for the individual personally and for their family members. Some considerations are universally long-recognized risks associated with any type of genetic test (discussed further below). However, MPS can add to the magnitude of risk and/or complexity of results, as well as generating issues specific to the technology, such as secondary findings.

Relationship Misattribution

For decades, clinical genetics professionals have faced the challenge of misattributed relationships identified through genetic testing, especially non-paternity. Most genetics clinicians only disclose this information when clinically necessary (59, 60). Moreover, in accordance with the Institute of Medicine Guidelines (61), non-paternity results [estimated to be present in up to 30.0% of livebirths (62)] are only disclosed to the mother alone. With genetic tests ordered in many more settings and much more frequently, the risk of uncovering misattributed relationships is extremely likely to increase (63). In addition, misattributed relationship results generated by single-gene tests are often associated with some degree of uncertainty, which allows for some degree of clinical discretion. In contrast, the simultaneous identification of both common and rare variants inherent in any MPS technology generates unequivocal results (63).

Disclosure of Genetic Status Through Relationships With Other Family Members

The shared nature of genetic material means that a positive test result in one individual can reveal the genetic status of other family members by inference. This may be due to their affection

status (*e.g.*, a *BRCA1* result in a woman with breast cancer implies mutation carriage in her mother with ovarian cancer) or the nature of inheritance (*e.g.*, the obligate carrier status of parents whose child is diagnosed with a recessive condition).

Unexpected Results Related to the Disease in Question

Genetic tests have the potential to yield information about the future health of an individual, who may be clinically unaffected at the time of testing. In single gene testing for carrier status, careful predisposition testing protocols were developed, particularly for neurodegenerative (64) and cancer susceptibility syndromes (65), to ensure individuals were prepared for the clinical, psychological, and logistical sequelae of learning such information. Preparing an individual for testing by MPS is challenging from a counseling perspective, if only for the large number of genes being tested simultaneously. However, more subtle issues may arise—for example, a causal gene may be identified that differs from the expected gene (66); and the results may confer an increased risk for conditions not previously described in the family or not previously recognized to be significant (*e.g.*, a *TP53* mutation in a family with a strong history of breast cancer).

Variants of Uncertain Significance

Variants of uncertain significance (VUSs) are variants for which there is insufficient evidence to classify them as benign or pathogenic. As additional information becomes available over time, they are sometimes re-classified as pathogenic/likely pathogenic or, more commonly, benign/likely benign (67–69). VUSs have been a long-standing challenge in genetic testing for hereditary cancer generally (68) and *BRCA1/2* specifically (67). The larger the number of genes interrogated, the higher the probability of generating a VUS: 36 and 73% in multigene panels (70) and exome sequencing (71) respectively. A recent systematic review found VUSs are associated with genetic test-specific concern and affects clinical management (72).

Incidental or Secondary Findings

Incidental or secondary findings are genetic test results unrelated to the primary condition. Incidental findings are generally regarded to be inadvertent or accidental discoveries emerging during data analysis. In contrast, secondary findings emerge from the deliberate interrogation of 'actionable' genes in individuals undergoing WES or WGS, with the goal of prevention or early detection of treatable conditions. To overcome the challenge of terminology, these are cumulatively referred to as incidental and secondary findings (ISFs) (73).

In 2013, the American College of Medical Genetics published guidelines recommending that all individuals having WES/WGS have automatic analysis of 56 actionable genes, associated with 24 hereditary cardiac or cancer predisposition syndromes (74). Among other statements, the guidelines stated that neither patient age nor patient preferences should be taken into account because this would be "logistically challenging" for laboratories (74). The paper stimulated multiple articles in response. Concerns raised included the lack of scientific evidence to support screening of all 56 genes, with insufficient information about phenotype and penetrance (75, 76). The potential for large numbers of VUSs was also recognized as was the challenge of interpreting variants in ethnic minorities (77). The potential for iatrogenic harm or false reassurance was raised. Multiple papers stated that the guidelines disregarded individual autonomy (78, 79) and contravened the ACMG's own guidelines on genetic testing in children (75)—with overlapping concerns of lack of informed consent (75). The second version of the guidelines removed the wording around any obligation to interrogate these genes whenever WES/WGS and acknowledged that all patients should have the right to opt out—and modified the medically actionable genes to a slightly different list with the overall number increased to 59 (80). At present, some laboratories offer secondary screening of the ACMG 59^{TM} (81); however, the extent to which it has been adopted by clinical laboratories world-wide is unclear. Additionally, there is ongoing debate about whether the ACMG 59^{TM} should be offered and reported in the prenatal period (82). The ACMG Board of Directors recently released a policy statement stating that they do not support the use of ACMG 59^{TM} as a screening tool in the general population (83).

Genetic Discrimination

Fear of genetic discrimination, particularly as it pertains to insurance underwriting, is a deterrent in the pursuit of clinically indicated genetic testing (84–86). Several papers suggest these fears are not ill-founded, with incidences of proven or alleged genetic discrimination reported in carriers of recessive conditions (87–89) and—perhaps surprisingly—individuals receiving a negative (*i.e.* good news) result in predictive testing for familial mutations (88, 89) and healthy carriers of dominant variants who pursued surgical/medical interventions and/or screening to mitigate their risk (89–96). Policies and legislation have been introduced in many countries (including the UK, US, Canada, Australia, and European countries) to limit or prohibit the use of genetic test results in insurance underwriting (97), but initial studies suggest that awareness of such legislation among non-genetics clinicians (98) and members of the public (99, 100) is low. For example, a UK study found that *BRCA1/2* carriers had difficulty obtaining insurance even after the introduction of the Concordat and Moratorium on Genetics and Insurance (95).

Equity

Personalized (or precision) medicine aims to improve care by customizing management to the individual and the profile of their disease. Genetic testing is an integral component of personalized medicine and encompasses a gamut of approaches, from tumor sequencing [*e.g.*, improving survival through targeted chemotherapy (101)] to common variant genotyping [*e.g.*, use of polygenic risk scores, usually determined through microarray technology (102)] to rare variant detection by MPS technologies (as discussed above). Access to genetic services is limited by racial, ethnic, and social factors; and disproportionate access has potential to widen, rather than reduce, health disparities both within developed countries (103, 104) and

between developed and developing countries (105) [though here we would highlight increasing use of MPS technologies clinically in communities with higher rates of intrafamilial marriage (44–46)].

FINAL THOUGHTS: ACCESS TO SEQUENCING AND FUTURE DIRECTIONS

In 2016, one of the current authors wrote "Conventional sequencing is commercially available for a finite number of mutations in clear-cut monogenic diseases—but these conditions represent a minority of genetic disorders. In Australia, genetic testing is available for 597 genes which cause <500 different syndromes and conditions, a small subset of the ~5,000" [McInerney-Leo, PhD thesis; data drawn from the Royal College of Pathologists, Australia, accessed 2016 (http://geneticstesting.rcpa.edu.au)]. Just four years later, the situation is very different, with both public and private access to testing for multiple conditions in Australia and in many countries around the world. A recent review article led with an arresting title of *A Diagnosis for All Rare Genetic Diseases: the Horizon and the Next Frontiers*, (49) and presented a vision that all families with a rare genetic disorder would ultimately receive a genetic diagnosis through sequencing technologies and novel data analyses approaches. This aim is not only exciting but with ongoing international cooperation and collaboration—even mid-coronavirus—it also seems achievable (49).

AUTHOR CONTRIBUTIONS

Both AM-L and ED prepared and reviewed this manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

AM-L is funded by a National Health and Medical Research Council (NHMRC) Early Career Fellowship (ID 1158111).

REFERENCES

1. Garrod AE. The incidence of alkaptonuria: a study in chemical individuality. 1902. *Mol Med (Cambridge Mass)* (1996) 2(3):274–82. doi: 10.1007/BF03401625
2. Mendel JG. "Versuche über Pflanzenhybriden", Verhandlungen des naturforschenden Vereines in Brünn, Bd. IV für das Jahr, 1865, Abhandlungen: 3–47. For the English translation, see: Druery, C.T.; Bateson, William (1901). "Experiments in plant hybridization". *J R Hortic Society* (1866) 26:1–32.
3. World Health Organisation. *Human Genomics in Global Health: Genes and human diseases* (2020). Available at: https://www.who.int/genomics/public/geneticdiseases/en/index2.html.
4. Mortier GR, Cohn DH, Cormier-Daire V, Hall C, Krakow D, Mundlos S, et al. Nosology and classification of genetic skeletal disorders: 2019 revision. *Am J Med Genet A* (2019) 179(12):2393–419. doi: 10.1002/ajmg.a.61366
5. Gusella JF, Wexler NS, Conneally PM, Naylor SL, Anderson MA, Tanzi RE, et al. A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* (1983) 306(5940):234–8. doi: 10.1038/306234a0
6. Group THsDCR. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* (1993) 72(6):971–83. doi: 10.1016/0092-8674(93)90585-E
7. Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, Baehner RL, et al. Cloning the gene for an inherited human disorder–chronic granulomatous disease–on the basis of its chromosomal location. *Nature* (1986) 322(6074):32–8. doi: 10.1038/322032a0
8. Voss R, Ben-Simon E, Avital A, Godfrey S, Zlotogora J, Dagan J, et al. Isodisomy of chromosome 7 in a patient with cystic fibrosis: could uniparental disomy be common in humans? *Am J Hum Genet* (1989) 45 (3):373–80. doi: 10.1016/0168-9525(89)90184-4
9. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, et al. Identification of the cystic fibrosis gene: genetic analysis. *Science* (1989) 245(4922):1073–80. doi: 10.1126/science.2570460
10. Collins FS. Positional cloning moves from perditional to traditional. *Nat Genet* (1995) 9(4):347–50. doi: 10.1038/ng0495-347
11. Glazier A, Nadeau J, Aitman T. Finding genes that underlie complex traits. *Science* (2002) 298:2345–50. doi: 10.1126/science.1076641
12. Duncan E, Brown M, Shore EM. The revolution in human monogenic disease mapping. *Genes* (2014) 5(3):792–803. doi: 10.3390/genes5030792
13. Lazarus S, Zankl A, Duncan EL. Next-generation sequencing: a frameshift in skeletal dysplasia gene discovery. *Osteoporosis Int J established as result cooperation between Eur Foundation Osteoporosis Natl Osteoporosis Foundation USA* (2014) 25(2):407–22. doi: 10.1007/s00198-013-2443-1
14. Warman ML, Cormier-Daire V, Hall C, Krakow D, Lachman R, LeMerrer M, et al. Nosology and classification of genetic skeletal disorders: 2010 revision. *Am J Med Genet A* (2011) 155A(5):943–68. doi: 10.1002/ajmg.a.33909
15. Chong JX, Buckingham KJ, Jhangiani SN, Boehm C, Sobreira N, Smith JD, et al. The Genetic Basis of Mendelian Phenotypes: Discoveries, Challenges, and Opportunities. *Am J Hum Genet* (2015) 97(2):199–215. doi: 10.1016/j.ajhg.2015.06.009
16. Boycott KM, Rath A, Chong JX, Hartley T, Alkuraya FS, Baynam G, et al. International Cooperation to Enable the Diagnosis of All Rare Genetic Diseases. *Am J Hum Genet* (2017) 100(5):695–705. doi: 10.1016/j.ajhg.2017.04.003
17. Collins FS, Green ED, Guttmacher AE, Guyer MS. A vision for the future of genomics research. *Nature* (2003) 422(6934):835–47. doi: 10.1038/nature01626
18. McCombie WR, McPherson JD, Mardis ER. Next-Generation Sequencing Technologies. *Cold Spring Harbor Perspect Medicine* (2019) 9(11):a036798. doi: 10.1101/cshperspect.a036798
19. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. *Trends Genet TIG* (2014) 30(9):418–26. doi: 10.1016/j.tig.2014.07.001
20. Walter K, Min JL, Huang J, Crooks L, Memari Y, McCarthy S, et al. The UK10K project identifies rare variants in health and disease. *Nature* (2015) 526(7571):82–90. doi: 10.1038/nature14962
21. Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC. SIFT missense predictions for genomes. *Nat Protoc* (2016) 11(1):1–9. doi: 10.1038/nprot.2015.123
22. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods* (2010) 7(4):248–9. doi: 10.1038/nmeth0410-248
23. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods* (2014) 11(4):361–2. doi: 10.1038/nmeth.2890
24. McInerney-Leo AM, Le Goff C, Leo PJ, Kenna TJ, Keith P, Harris JE, et al. Mutations in LTBP3 cause acromicric dysplasia and geleophysic dysplasia. *J Med Genet* (2016) 53(7):457–64. doi: 10.1136/jmedgenet-2015-103647

25. Zankl A, Duncan EL, Leo PJ, Clark GR, Glazov EA, Addor MC, et al. Multicentric Carpotarsal Osteolysis Is Caused by Mutations Clustering in the Amino-Terminal Transcriptional Activation Domain of MAFB. *Am J Hum Genet* (2012) 90(3):494–501. doi: 10.1016/j.ajhg.2012.01.003
26. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* (2020) 581(7809):434–43. doi: 10.1530/ey.17.14.3
27. Bredrup C, Saunier S, Oud MM, Fiskerstrand T, Hoischen A, Brackman D, et al. Ciliopathies with skeletal anomalies and renal insufficiency due to mutations in the IFT-A gene WDR19. *Am J Hum Genet* (2011) 89(5):634–43. doi: 10.1016/j.ajhg.2011.10.001
28. Becker J, Semler O, Gilissen C, Li Y, Bolz HJ, Giunta C, et al. Exome sequencing identifies truncating mutations in human SERPINF1 in autosomal-recessive osteogenesis imperfecta. *Am J Hum Genet* (2011) 88 (3):362–71. doi: 10.1016/j.ajhg.2011.01.015
29. McInerney-Leo AM, Schmidts M, Cortés CR, Leo PJ, Gener B, Courtney AD, et al. Short-rib polydactyly and Jeune syndromes are caused by mutations in WDR60. *Am J Hum Genet* (2013) 93(3):515–23. doi: 10.1016/j.ajhg.2013.06.022
30. Semler O, Garbes L, Keupp K, Swan D, Zimmermann K, Becker J, et al. A Mutation in the 5'-UTR of IFITM5 Creates an In-Frame Start Codon and Causes Autosomal-Dominant Osteogenesis Imperfecta Type V with Hyperplastic Callus. *Am J Hum Genet* (2012) 91(2):349–57. doi: 10.1016/j.ajhg.2012.06.011
31. Rauch F, Moffatt P, Cheung M, Roughley P, Lalic L, Lund AM, et al. Osteogenesis imperfecta type V: marked phenotypic variability despite the presence of the IFITM5 c.-14C>T mutation in all patients. *J Med Genet* (2013) 50(1):21–4. doi: 10.1136/jmedgenet-2012-101307
32. Wade EM, Wade EM, Daniel PB, Jenkins ZA, McInerney-Leo A, Leo P, Morgan T, et al. Mutations in MAP3K7 that Alter the Activity of the TAK1 Signaling Complex Cause Frontometaphyseal Dysplasia. *Am J Hum Genet* (2016) 99(2):392–406. doi: 10.1016/j.ajhg.2016.05.024
33. Simpson MA, Irving MD, Asilmaz E, Gray MJ, Dafou D, Elmslie FV, et al. Mutations in NOTCH2 cause Hajdu-Cheney syndrome, a disorder of severe and progressive bone loss. *Nat Genet* (2011) 43(4):303–5. doi: 10.1038/ng.779
34. Sparrow DB, McInerney-Leo A, Gucev ZS, Gardiner B, Marshall M, Leo PJ, et al. Autosomal dominant spondylocostal dysostosis is caused by mutation in TBX6. *Hum Mol Genet* (2013) 22(8):1625–31. doi: 10.1093/hmg/ddt012
35. Sirmaci A, Spiliopoulos M, Brancati F, Powell E, Duman D, Abrams A, et al. Mutations in ANKRD11 cause KBG syndrome, characterized by intellectual disability, skeletal malformations, and macrodontia. *Am J Hum Genet* (2011) 89(2):289–94. doi: 10.1016/j.ajhg.2011.06.007
36. Sun Y, Almomani R, Aten E, Celli J, van der Heijden J, Venselaar H, et al. Terminal osseous dysplasia is caused by a single recurrent mutation in the FLNA gene. *Am J Hum Genet* (2010) 87(1):146–53. doi: 10.1016/j.ajhg.2010.06.008
37. van Dijk FS, Zillikens MC, Micha D, Riessland M, Marcelis CL, de Die-Smulders CE, et al. PLS3 mutations in X-linked osteoporosis with fractures. *N Engl J Med* (2013) 369(16):1529–36. doi: 10.1056/NEJMoa1308223
38. Lindert U, Cabral WA, Ausavarat S, Tongkobpetch S, Ludin K, Barnes AM, et al. MBTPS2 mutations cause defective regulated intramembrane proteolysis in X-linked osteogenesis imperfecta. *Nat communications* (2016) 7:11920. doi: 10.1038/ncomms11920
39. Kurek KC, Luks VL, Ayturk UM, Alomari AI, Fishman SJ, Spencer SA, et al. Somatic mosaic activating mutations in PIK3CA cause CLOVES syndrome. *Am J Hum Genet* (2012) 90(6):1108–15. doi: 10.1016/j.ajhg.2012.05.006
40. Lindhurst MJ, Sapp JC, Teer JK, Johnston JJ, Finn EM, Peters K, et al. A mosaic activating mutation in AKT1 associated with the Proteus syndrome. *N Engl J Med* (2011) 365(7):611–9. doi: 10.1056/NEJMoa1104017
41. Kong SW, Lee IH, Liu X, Hirschhorn JN, Mandl KD. Measuring coverage and accuracy of whole-exome sequencing in clinical context. *Genet Med Off J Am Coll Med Genet* (2018) 20(12):1617–26. doi: 10.1038/gim.2018.51
42. Cooper DN KM, Antonorakis SE. The nature and mechanisms of human gene mutation. In: CBA Scriver, WS Sly, D Valle, editors. *The Metabolic and Molecular Bases of Inherited Disease, 7th ed.* New York: McGraw-Hill (1995). p. 259–91.
43. Clark MM, Stark Z, Farnaes L, Tan TY, White SM, Dimmock D, et al. Meta-analysis of the diagnostic and clinical utility of genome and exome sequencing and chromosomal microarray in children with suspected genetic diseases. *NPJ Genomic Medicine* (2018) 3(1):16. doi: 10.1038/s41525-018-0053-8
44. Monies D, Abouelhoda M, Assoum M, Moghrabi N, Rafiullah R, Almontashiri N, et al. Lessons Learned from Large-Scale, First-Tier Clinical Exome Sequencing in a Highly Consanguineous Population. *Am J Hum Genet* (2019) 104(6):1182–201. doi: 10.1016/j.ajhg.2019.09.019
45. Cheema H, Bertoli-Avella AM, Skrahina V, Anjum MN, Waheed N, Saeed A, et al. Genomic testing in 1019 individuals from 349 Pakistani families results in high diagnostic yield and clinical utility. *NPJ Genomic Medicine* (2020) 5(1):44. doi: 10.1038/s41525-020-00150-z
46. Alfares A, Alfadhel M, Wani T, Alsahli S, Alluhaydan I, Al Mutairi F, et al. A multicenter clinical exome study in unselected cohorts from a consanguineous population of Saudi Arabia demonstrated a high diagnostic yield. *Mol Genet Metab* (2017) 121(2):91–5. doi: 10.1016/j.ymgme.2017.04.002
47. Stark Z, Tan TY, Chong B, Brett GR, Yap P, Walsh M, et al. A prospective evaluation of whole-exome sequencing as a first-tier molecular test in infants with suspected monogenic disorders. *Genet Medicine* (2016) 18(11):1090–6. doi: 10.1038/gim.2016.1
48. Yao R, Zhang C, Yu T, Li N, Hu X, Wang X, et al. Evaluation of three read-depth based CNV detection tools using whole-exome sequencing data. *Mol Cytogenetics* (2017) 10(1):30. doi: 10.1186/s13039-017-0333-5
49. Boycott KM, Hartley T, Biesecker LG, Gibbs RA, Innes AM, Riess O, et al. A Diagnosis for All Rare Genetic Diseases: The Horizon and the Next Frontiers. *Cell* (2019) 177(1):32–7. doi: 10.1016/j.cell.2019.02.040
50. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, et al. Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. *J Med Genet* (2002) 39(2):91–7. doi: 10.1136/jmg.39.2.91
51. Mazzarotto F, Olivotto I, Walsh R. Advantages and Perils of Clinical Whole-Exome and Whole-Genome Sequencing in Cardiomyopathy. *Cardiovasc Drugs Ther* (2020) 34(2):241–53. doi: 10.1007/s10557-020-06948-4
52. Dillon OJ, Lunke S, Stark Z, Yeung A, Thorne N, Gaff C, et al. Exome sequencing has higher diagnostic yield compared to simulated disease-specific panels in children with suspected monogenic disorders. *Eur J Hum Genet* (2018) 26(5):644–51. doi: 10.1038/s41431-018-0099-1
53. McInerney-Leo AM, Marshall MS, Gardiner B, Coucke PJ, Van Laer L, Loeys BL, et al. Whole exome sequencing is an efficient, sensitive and specific method of mutation detection in osteogenesis imperfecta and Marfan syndrome. *BoneKEy Reports* (2013) 2:456. doi: 10.1038/bonekey.2013.190
54. Collet C, Ostertag A, Ricquebourg M, Delecourt M, Tueur G, Isidor B, et al. Primary Osteoporosis in Young Adults: Genetic Basis and Identification of Novel Variants in Causal Genes. *JBMR Plus* (2018) 2(1):12–21. doi: 10.1002/jbm4.10020
55. Minikel EV, Vallabh SM, Lek M, Estrada K, Samocha KE, Sathirapongsasuti JF, et al. Quantifying prion disease penetrance using large population control cohorts. *Sci Transl Med* (2016) 8(322):322ra329. doi: 10.1126/scitranslmed.aad5169
56. MacArthur DG, Manolio TA, Dimmock DP, Rehm HL, Shendure J, Abecasis GR, et al. Guidelines for investigating causality of sequence variants in human disease. *Nature* (2014) 508(7497):469–76. doi: 10.1038/nature13127
57. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med Off J Am Coll Med Genet* (2015) 17(5):405–24. doi: 10.1038/gim.2015.30
58. Tian Y, Pesaran T, Chamberlin A, Fenwick RB, Li S, Gau CL, et al. REVEL and BayesDel outperform other in silico meta-predictors for clinical variant classification. *Sci Reports* (2019) 9(1):12752. doi: 10.1038/s41598-019-49224-8
59. Wertz DC, Fletcher JC. Privacy and disclosure in medical genetics examined in an ethics of care. *Bioethics* (1991) 5(3):212–32. doi: 10.1111/j.1467-8519.1991.tb00161.x
60. Wright L, MacRae S, Gordon D, Elliot E, Dixon D, Abbey S, et al. Disclosure of misattributed paternity: issues involved in the discovery of unsought information. *Semin Dial* (2002) 15(3):202–6. doi: 10.1046/j.1525-139X.2002.00541.x

61. Research PsCftSoEPiMaBaB. *Screening and Counseling for Genetic Conditions: A Report on the Ethical, Social, and Legal Implications of Genetic Screening, Counseling, and Education Programs*. Washington DC: The Government of the Unites States of America (1983).
62. Bellis MA, Hughes K, Hughes S, Ashton JR. Measuring paternal discrepancy and its public health consequences. *J Epidemiol Community Health* (2005) 59(9):749–54. doi: 10.1136/jech.2005.036517
63. Hercher L, Jamal L. An old problem in a new age: Revisiting the clinical dilemma of misattributed paternity. *Appl Transl Genom* (2016) 8:36–9. doi: 10.1016/j.atg.2016.01.004
64. Tibben A. Predictive testing for Huntington's disease. *Brain Res Bull* (2007) 72(2-3):165–71. doi: 10.1016/j.brainresbull.2006.10.023
65. Berg AO, Allan JD, Calonge N, Frame PS. Genetic risk assessment and BRCA mutation testing for breast and ovarian cancer susceptibility: Recommendation statement. *Ann Internal Medicine* (2005) 143(5):355–61. doi: 10.7326/0003-4819-143-5-200509060-00011
66. Christiaans I, Mook ORF, Alders M, Bikker H, Lekanne Dit Deprez RH. Large next-generation sequencing gene panels in genetic heart disease: challenges in clinical practice. *Neth Heart J* (2019) 27(6):299–303. doi: 10.1007/s12471-019-1251-4
67. Mighton C, Charames GS, Wang M, Zakoor KR, Wong A, Shickh S, et al. Variant classification changes over time in BRCA1 and BRCA2. *Genet Med Off J Am Coll Med Genet* (2019) 21(10):2248–54. doi: 10.1038/s41436-019-0493-2
68. Mersch J, Brown N, Pirzadeh-Miller S, Mundt E, Cox HC, Brown K, et al. Prevalence of Variant Reclassification Following Hereditary Cancer Genetic Testing. *JAMA* (2018) 320(12):1266–74. doi: 10.1001/jama.2018.13152
69. Macklin S, Durand N, Atwal P, Hines S. Observed frequency and challenges of variant reclassification in a hereditary cancer clinic. *Genet Med Off J Am Coll Med Genet* (2018) 20(3):346–50. doi: 10.1038/gim.2017.207
70. Buys SS, Sandbach JF, Gammon A, Patel G, Kidd J, Brown KL, et al. A study of over 35,000 women with breast cancer tested with a 25-gene panel of hereditary cancer genes. *Cancer* (2017) 123(10):1721–30. doi: 10.1002/cncr.30498
71. Dixon-Salazar TJ, Silhavy JL, Udpa N, Schroth J, Bielas S, Schaffer AE, et al. Exome sequencing can improve diagnosis and alter patient management. *Sci Transl Med* (2012) 4(138):138ra178. doi: 10.1126/scitranslmed.3003544
72. Mighton C, Shickh S, Uleryk E, Pechlivanoglou P, Bombard Y. Clinical and psychological outcomes of receiving a variant of uncertain significance from multigene panel testing or genomic sequencing: a systematic review and meta-analysis. *Genet Med Off J Am Coll Med Genet* (2020) 23(1):22–33. doi: 10.1038/s41436-020-00957-2
73. Saelaert M, Mertes H, De Baere E, Devisch I. Incidental or secondary findings: an integrative and patient-inclusive approach to the current debate. *Eur J Hum Genet* (2018) 26(10):1424–31. doi: 10.1038/s41431-018-0200-9
74. Green RC, Berg JS, Grody WW, Kalia SS, Korf BR, Martin CL, et al. ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing. *Genet Med Off J Am Coll Med Genet* (2013) 15(7):565–74. doi: 10.1038/gim.2013.73
75. Burke W, Antommaria AH, Bennett R, Botkin J, Clayton EW, Henderson GE, et al. Recommendations for returning genomic incidental findings? We need to talk! *Genet Med Off J Am Coll Med Genet* (2013) 15(11):854–9. doi: 10.1038/gim.2013.113
76. Holtzman NA. ACMG recommendations on incidental findings are flawed scientifically and ethically. *Genet Med Off J Am Coll Med Genet* (2013) 15(9):750–1. doi: 10.1038/gim.2013.96
77. Amendola LM, Dorschner MO, Robertson PD, Salama JS, Hart R, Shirts BH, et al. Actionable exomic incidental findings in 6503 participants: challenges of variant classification. *Genome Res* (2015) 25(3):305–15.
78. Ross LF, Rothstein MA, Clayton EW. Mandatory extended searches in all genome sequencing: "incidental findings," patient autonomy, and shared decision making. *JAMA* (2013) 310(4):367–8. doi: 10.1001/jama.2013.41700
79. Wolf SM, Annas GJ, Elias S. Point-counterpoint. Patient autonomy and incidental findings in clinical genomics. *Science* (2013) 340(6136):1049–50. doi: 10.1126/science.1239119
80. Kalia SS, Adelman K, Bale SJ, Chung WK, Eng C, Evans JP, et al. Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. *Genet Med Off J Am Coll Med Genet* (2017) 19(2):249–55. doi: 10.1038/gim.2017.17
81. Nussbaum RL, Haverfield E, Esplin ED, Aradhya S. Response to "The use of ACMG secondary findings recommendations for general population screening: a policy statement of the American College of Medical Genetics and Genomics (ACMG)". *Genet Med Off J Am Coll Med Genet* (2019) 21(12):2836–7. doi: 10.1038/s41436-019-0572-4
82. Amor DJ, Chitty LS, Van den Veyver IB. Current controversies in prenatal diagnosis 2: The 59 genes ACMG recommends reporting as secondary findings when sequencing postnatally should be reported when detected on fetal (and parental) sequencing. *Prenat Diagn* (2020) 40(12):1508–14. doi: 10.1002/pd.5670
83. Directors ABo. The use of ACMG secondary findings recommendations for general population screening: a policy statement of the American College of Medical Genetics and Genomics (ACMG). *Genet Med* (2019) 21(7):1467–8. doi: 10.1038/s41436-019-0502-5
84. Joly Y, Ngueng Feze I, Simard J. Genetic discrimination and life insurance: a systematic review of the evidence. *BMC Medicine* (2013) 11:25. doi: 10.1186/1741-7015-11-25
85. Wauters A, Van Hoyweghen I. Global trends on fears and concerns of genetic discrimination: a systematic literature review. *J Hum Genet* (2016) 61(4):275–82. doi: 10.1038/jhg.2015.151
86. Quinlivan JA, Battikhi Z, Petersen RW. What Factors Impact upon a Woman's Decision to Undertake Genetic Cancer Testing? *Front Oncol* (2014) 3:325. doi: 10.3389/fonc.2013.00325
87. Geller LN, Alper JS, Billings PR, Barash CI, Beckwith J, Natowicz MR. Individual, family, and societal dimensions of genetic discrimination: a case study analysis. *Sci Eng Ethics* (1996) 2(1):71–88. doi: 10.1007/BF02639319
88. Low L, King S, Wilkie T. Genetic discrimination in life insurance: empirical evidence from a cross sectional survey of genetic support groups in the United Kingdom. *BMJ* (1998) 317(7173):1632–5. doi: 10.1136/bmj.317.7173.1632
89. Barlow-Stewart K, Keays D. Genetic Discrimination in Australia. *J L Med* (2001) 8:250–62.
90. McKinnon W, Banks KC, Skelly J, Kohlmann W, Bennett R, Shannon K, et al, et al. Survey of unaffected BRCA and mismatch repair (MMR) mutation positive individuals. *Fam Cancer* (2009) 8(4):363–9. doi: 10.1007/s10689-009-9248-6
91. Otlowski M, Barlow-Stewart K, Taylor S, Stranger M, Treloar S. Investigating genetic discrimination in the Australian life insurance sector: the use of genetic test results in underwriting, 1999-2003. *J Law Med* (2007) 14(3):367–96.
92. Barlow-Stewart K, Taylor SD, Treloar SA, Stranger M, Otlowski M. Verification of consumers' experiences and perceptions of genetic discrimination and its impact on utilization of genetic testing. *Genet Med Off J Am Coll Med Genet* (2009) 11(3):193–201. doi: 10.1097/GIM.0b013e318194ee75
93. Keogh LA, Otlowski MF. Life insurance and genetic test results: a mutation carrier's fight to achieve full cover. *Med J Aust* (2013) 199(5):363–6. doi: 10.5694/mja13.10202
94. Tiller J, Morris S, Rice T, Barter K, Riaz M, Keogh L, et al. Genetic discrimination by Australian insurance companies: a survey of consumer experiences. *Eur J Hum Genet* (2020) 28(1):108–13. doi: 10.1038/s41431-019-0426-1
95. Watson M, Foster C, Eeles R, Eccles D, Ashley S, Davidson R, et al, et al. Psychosocial impact of breast/ovarian (BRCA1/2) cancer-predictive genetic testing in a UK multi-centre clinical cohort. *Br J Cancer* (2004) 91(10):1787–94. doi: 10.1038/sj.bjc.6602207
96. Foster C, Evans DG, Eeles R, Eccles D, Ashley S, Brooks L, et al. Predictive testing for BRCA1/2: attributes, risk perception and management in a multi-centre clinical cohort. *Br J Cancer* (2002) 86(8):1209–16. doi: 10.1038/sj.bjc.6600253
97. Belisle-Pipon JC, Vayena E, Green RC, Cohen IG. Genetic testing, insurance discrimination and medical research: what the United States can learn from peer countries. *Nat Med* (2019) 25(8):1198–204. doi: 10.1038/s41591-019-0534-z
98. Huizenga CR, Lowstuter K, Banks KC, Lagos VI, Vandergon VO, Weitzel JN. Evolving perspectives on genetic discrimination in health insurance

among health care providers. *Fam Cancer* (2010) 9(2):253–60. doi: 10.1007/s10689-009-9308-y

99. Allain DC, Friedman S, Senter L. Consumer awareness and attitudes about insurance discrimination post enactment of the Genetic Information Nondiscrimination Act. *Fam Cancer* (2012) 11(4):637–44. doi: 10.1007/s10689-012-9564-0
100. Parkman AA, Foland J, Anderson B, Duquette D, Sobotka H, Lynn M, et al. Public awareness of genetic nondiscrimination laws in four states and perceived importance of life insurance protections. *J Genet Couns* (2015) 24(3):512–21. doi: 10.1007/s10897-014-9771-y
101. Tsimberidou AM, Fountzilas E, Nikanjam M, Kurzrock R. Review of precision cancer medicine: Evolution of the treatment paradigm. *Cancer Treat Rev* (2020) 86:102019. doi: 10.1016/j.ctrv.2020.102019
102. Lambert SA, Abraham G, Inouye M. Towards clinical utility of polygenic risk scores. *Hum Mol Genet* (2019) 28(R2):R133–r142. doi: 10.1093/hmg/ddz187
103. Saulsberry K, Terry SF. The need to build trust: a perspective on disparities in genetic testing. *Genet Testing Mol Biomarkers* (2013) 17(9):647–8. doi: 10.1089/gtmb.2013.1548
104. Smith CE, Fullerton SM, Dookeran KA, Hampel H, Tin A, Maruthur NM, et al. Using Genetic Technologies To Reduce, Rather Than Widen, Health Disparities. *Health affairs (Project Hope)* (2016) 35(8):1367–73. doi: 10.1377/hlthaff.2015.1476
105. Helmy M, Awad M, Mosa KA. Limited resources of genome sequencing in developing countries: Challenges and solutions. *Appl Transl Genom* (2016) 9:15–9. doi: 10.1016/j.atg.2016.03.003

Conflict of Interest: 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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership