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Fetal testicular hormones

Edited by
Nathalie Josso, Rodolfo A. Rey and Andrew Pask

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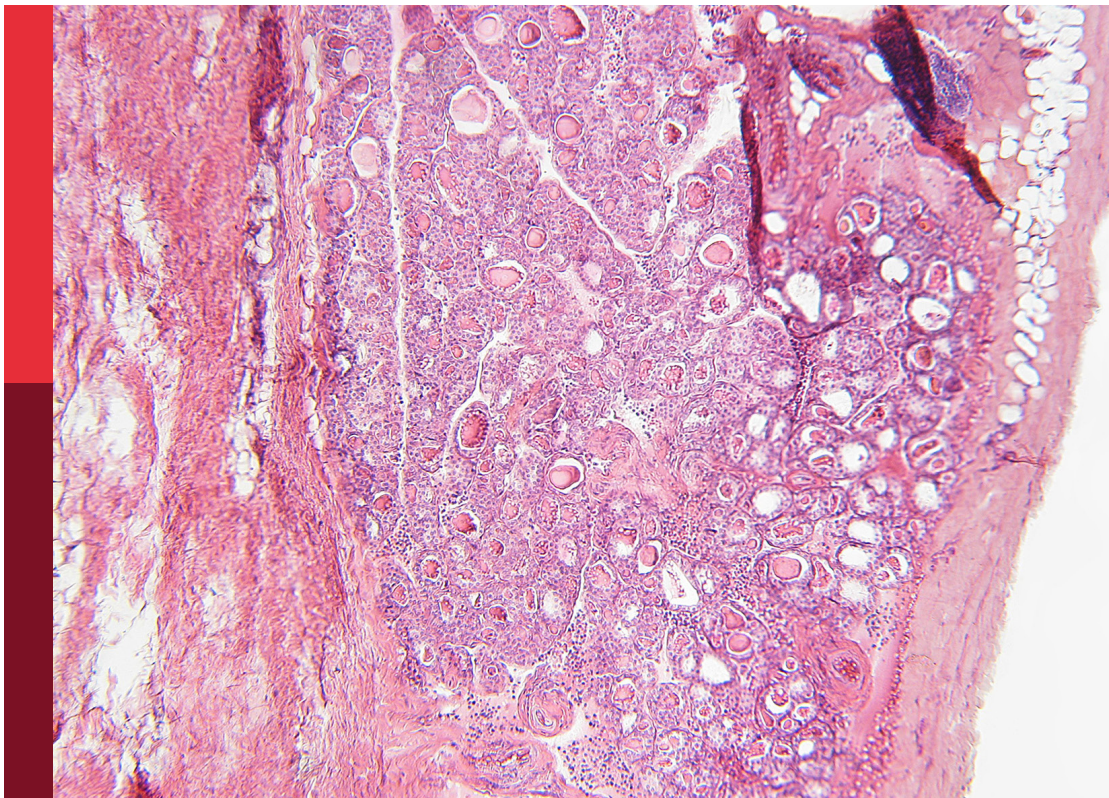

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Fetal testicular hormones

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Editorial: Fetal testicular hormones

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Editorial on the Research Topic
Fetal testicular hormones

Hormones produced by the fetal testis are responsible for shaping the male phenotype, as shown by Alfred Jost during the last century (1, 2). Testosterone, produced by fetal Leydig cells, maintains Wolffian ducts and masculinizes the external genitalia; anti-Müllerian hormone (AMH) secreted by Sertoli cells intiates the regression of the Müllerian ducts while another Leydig-cell peptide, Insulin-like peptide 3 (INSL3), induces testicular descent to the scrotum. Receptors for each of these hormones have been identified in target organs. The tools required to study these genes and pathways have been created, providing a solid foundation on which to build new knowledge in this area.

This Research Topic addresses contemporary questions concerning the synthesis and function of fetal testicular hormones. Amato et al. provide an in-depth analysis on the knowledge of the conserved androgen signaling mechanisms as well as the cell- and organ-specific mechanisms illustrated by the androgen-dependent differentiation processes occurring in the Wolffian duct, leading to the formation of the epididymis, vas deferens and seminal vesicle, and in the primordia of the external genitalia. Ipulan-Colet summarizes the role of androgens in driving sexually dimorphic development of the genital tubercle, and uses this framework to scrutinize the different mechanisms potentially involved in the androgen-dependent sexual dimorphism observed in muscle differentiation. The structure, dynamics of expression and regulation of the more recently discovered Leydig cell hormone INSL3 is reviewed by Ivell et al. The authors deal with the mechanisms through which INSL3 and its G-protein coupled receptor, RXFP2, promote testicular descent during fetal life. They also discuss the novel relevance of INSL3 as a biomarker of Leydig cell function *in utero* to its potential to act as a monitor of exposure to environmental endocrine disruptors that may lead to cryptorchidism.

AMH is a dimeric glycoprotein of the transforming growth factor beta (TGFβ) superfamily (3). Members of this family are typically synthesized as large precursors that, after cleavage, produce a small C-terminal active domain and a larger N-terminal domain. In the case of AMH, the N-terminal domain enhances the C-terminal domain activity on the specific type II AMH receptor. Howard et al. review the most recent discoveries on the regulation of the processing of both AMH and its type II receptor. Cate meticulously dissects the signal transduction mechanisms engaged in the AMH-dependent regression of Müllerian ducts in the male fetus, addressing the canonical AMH signaling pathway that involves the specific type II receptor and shared type I receptors leading to the phosphorylation, nuclear translocation and DNA binding of specific Smad proteins. Few effectors are known to mediate AMH action in Müllerian ducts. Based on transcriptome analyses performed in mesenchymal tissue of Müllerian ducts after the start of AMH expression in mouse male fetuses, Mullen et al. identified *Dlx5* and *Dlx6* as potential AMH target genes. Here, they provide experimental evidence that AMH signaling is essential for a sex dimorphic expression of *Dlx5* and *Dlx6* in Müllerian duct mesenchyme, and that disruption of their expression results in persistence of Müllerian derivatives in male mice. Although AMH action takes place very early in fetal development, Sertoli cells continue to produce high amounts of AMH until puberty. Edelsztein et al. review the recent findings related to the molecular mechanisms involved in the strong androgen-mediated downregulation and the weaker estrogen-induced upregulation of testicular AMH expression during puberty. They underscore the importance of understanding these complex regulatory mechanisms for the interpretation of serum AMH as a biomarker in physiological and pathological conditions in boys and adolescents.

A fine-tuned orchestration of regulatory mechanisms is needed for the testis to differentiate and secrete its fetal hormones in an adequate spatiotemporal manner. Viger et al. review the importance of GATA factors for the differentiation of the testis from the gonadal ridge as well as their relevance in the control of fetal Sertoli and Leydig cell gene expression in normal conditions and in human pathology. O'Donnell et al. address the key role of Sertoli cells and activin A for fetal testicular steroidogenesis, arguing that Sertoli cells synthesize testosterone from androstenedione in the fetal mouse testis, and probably also in the human. Lucas-Herald and Mitchell review the role of two Sertoli cell peptides, AMH and inhibin B, during normal male sexual development in the human fetus and their relevance in congenital disorders of sex development.

Given the rise in differences in sexual development and infertility in humans, this timely collection of articles sheds new light of the role of fetal testicular hormones in sexual development and the potential sources of disruption which may be contributing to reduced reproductive health.

Author contributions

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

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.

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REVIEW
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Expression and Role of INSL3 in the Fetal Testis

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Insulin-like peptide 3 (INSL3) is a small peptide hormone of the insulin-relaxin family which is produced and secreted by the fetal Leydig cells in the testes only. It appears to be undetectable in female fetuses. In the human fetus INSL3 synthesis begins immediately following gonadal sex determination at weeks 7 to 8 post coitum and the peptide can be detected in amniotic fluid 1 to 2 weeks later. INSL3 acts through a unique G-protein-coupled receptor, called RelaXin-like Family Peptide receptor 2 (RXFP2), which is expressed by the mesenchymal cells of the gubernacular ligament linking the testes to the inguinal wall. The role of INSL3 in the male fetus is to cause a thickening of the gubernaculum which then retains the testes in the inguinal region, while the remainder of the abdominal organs grow away in an antero-dorsal direction. This represents the first phase of testis descent and is followed later in pregnancy by the second inguino-scrotal phase whereby the testes pass into the scrotum through the inguinal canal. INSL3 acts as a significant biomarker for Leydig cell differentiation in the fetus and may be reduced by maternal exposure to endocrine disrupting chemicals, such as xenoestrogens or phthalates, leading to cryptorchidism. INSL3 may have other roles within the fetus, but as a Leydig cell biomarker its reduction acts also as a surrogate for anti-androgen action.

Keywords: leydig cell, testis descent, cryptorchidism, endocrine disruption, RXFP2

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INTRODUCTION

Insulin-like peptide 3 (INSL3) was first identified as a Leydig cell-specific gene transcript encoding a putative secretory product from adult testes of boars and mice independently (1, 2) and later confirmed for other species, including human (3, 4). As its name suggests, INSL3 is a small peptide of approximately 6000 Dalton with the insulin-typical A-B heterodimeric structure (**Figure 1**), held together by three internal cysteine bonds. Its expression by the adult testes was subsequently confirmed using immunohistochemistry and immunoassay (6–10). However, it was the development of genetically altered mice lacking INSL3 expression (11, 12), which first suggested a major role for the peptide in the fetus in relation to testicular descent. Furthermore, it was the discovery of an identical phenotype of bilateral cryptorchidism in a natural mutant mouse (*Great*) which identified the receptor (RXFP2; RelaXin-like Family Peptide receptor 2) for INSL3 (13–15).

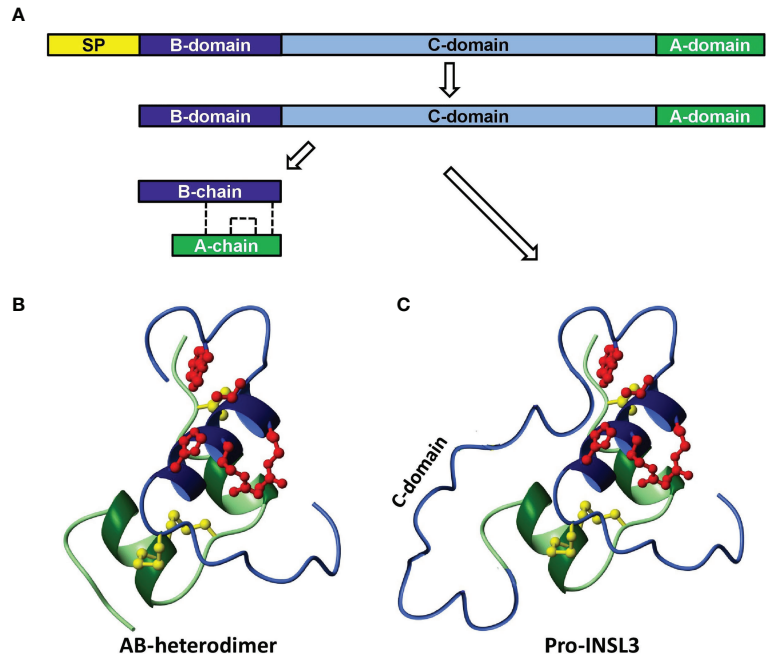

FIGURE 1 | Scheme to illustrate the synthesis and processing of INSL3 from its preliminary precursor form **(A**, prepro-INSL3**)** *via* a possibly secreted pro-form **(A, C**, pro-INSL3**)**, to give rise to the final A-B heterodimer **(B)**. SP, signal peptide. The sulfhydryl bridges formed by cysteine residues are shown as dashed lines **(A)** or as yellow molecular structures **(B, C)** [reproduced from (5)].

Subsequent research confirmed not only the production of INSL3 by the fetal Leydig cells in rodents and the expression of RXFP2 on mesenchymal cells of the gubernacular ligament linking the fetal testes to the inguinal wall (16, 17), but also the unique action of INSL3 to achieve gubernacular thickening (7, 16, 18). Moreover, INSL3 and RXFP2 represent a unique cognate ligand-receptor pair: no other ligand at physiological concentration can activate RXFP2, and no other receptor was able to respond to INSL3 at physiological concentration (14). In retrospect, INSL3 appears to be the molecular identity of the previously mooted hormone 'descendin' which had been partially characterized biochemically as a factor responsible for testicular descent in fetal pigs (19).

INSL3 has now been characterized at the genome, transcript and/or the protein level from the testes of most extant mammal species, including humans. The exceptions are those mammals, such as the Afrotherian tenrec or the manatee, which appear to be primarily testicond, i.e. do not exhibit the descent of testes into a scrotum (20). INSL3 belongs to a small group of peptide hormones which have been referred to as 'neohormones' (21). These are hormones which, while often having ancestry in early vertebrates, have specifically evolved further in mammals to manage the specialist requirements of viviparity and internal fertilization, which required adaptations in both male and female physiology. Neohormones include several members of the relaxin-like family, including INSL3, as well as peptides like oxytocin, and proteins involved in the maternal recognition of pregnancy, such as hCG. Testicular descent became essential to provide a mechanism by which sperm could be stored in the scrotal epididymis at a temperature several degrees below abdominal core temperature. Upon ejaculation into the female tract there is a jump in temperature and environment sufficient to trigger capacitation, hyperactivation, and ultimately apoptosis unless internal fertilization occurs within the oviduct (20).

INSL3 may have roles beyond fetal life, in both adult males and females. These may be relicts of ancestral functions prior to the emergence of mammals and the acquisition of new roles and include support of gametogenesis (8, 22, 23) and bone metabolism (24), and possibly also improvement of kidney function (25). In female mammals INSL3 is expressed by the equivalent cells in the ovary to Leydig cells, the theca interna cells of growing antral follicles. Here, INSL3 is essential for the paracrine regulation of the steroid precursor androstenedione (26). These roles will not be discussed here, where the focus will be primarily on the role of INSL3 in the first phase of testis descent and cryptorchidism in the male fetus.

THE STRUCTURE OF INSL3 AND ITS RECEPTOR

INSL3 is encoded by a single small gene, in the human located on the short arm of chromosome 19 (19p13.11). There is a single intron separating the protein-coding domain. At its 5' end the INSL3 gene is very close to the 3' end of the JAK3 gene and in some species (e.g. the mouse) it even lies within a terminal intron

of that gene (27). Such information suggests that all relevant regulatory sequences are likely to be restricted to only a few kilobases of genomic DNA close to the INSL3 gene. Like the genes for the structurally related peptide hormones insulin and relaxin, the INSL3 gene encodes a precursor polypeptide comprising a signal peptide to aid secretion, followed successively by an A-domain, a connecting C-domain, and finally a B-domain (**Figure 1A**). In the adult, the precursor A-C-B form is mostly processed to yield in the circulation an A-B heterodimer (**Figure 1B**), as for insulin and relaxin, although the precursor A-C-B form can also be secreted (**Figure 1C**). Importantly, both forms of INSL3 are equally bioactive at the receptor, and probably are equally detected by the immunoassays currently in use (10). For INSL3 in the fetus, we have as yet no information regarding INSL3 precursor processing and secretion for any species.

The gene for the INSL3 receptor, RXFP2, is located on the long arm of chromosome 13 (13q13.1) in the human and comprises in its full-length form 18 exons, of which the first 14 encode a long extracellular region of the G-protein-coupled receptor (GPCR), whereas the remaining 4 encode the 7-transmembrane and intracellular regions (15). Importantly, the long extracellular region includes 10 leucine-rich repeat (LRR) elements, as well as an N-terminal low density lipoprotein type a (LDLa) domain. The LRR elements are each encoded by a short 75 bp exon and are involved in the primary recognition of the INSL3 hormone *via* its B-peptide region. The LDLa domain is essential for receptor signaling and it is believed that binding of INSL3 to the receptor causes the LDLa domain to move and interact with sequences in the transmembrane region essential for signal transduction. At the same time, further parts of the INSL3 molecule, particularly in the A-peptide domain, now also appear to interact with extracellular loops of the transmembrane region of the receptor to confirm the actively signaling conformation (28).

Both INSL3 and its receptor RXFP2 are highly homologous between mammalian species, supporting their essential role in testicular descent and reproduction. Both genes, however, at least in the adult, are subject to alternative splicing (9, 29–31). The splice products, which have been identified represent mostly non-functional transcripts; for example, in the rat a rare alternative INSL3 transcript encodes an extended B-peptide only (29, 32). For RXFP2, several variant transcripts have been described; most represent forms lacking one or more of the LRR-encoding exons (31) and which consequently cannot generate a functional receptor. However, since *in vitro* evidence suggests the possibility of hetero- and homo-dimerization of this GPCR (33), it is possible that such splice variants may still be able to modulate normal receptor function.

It is important to add that, as for INSL3, we have no information at all for any species on the structure and expression of RXFP2 transcripts in the fetus. Moreover, the mRNA evidence from the fetus, mostly derived by RT-PCR, has consistently made use of only single PCR primer pairs; the application of a multiplexed PCR matrix as has been used to detect receptor splice variants in adult cells and tissues is absent.

THE DYNAMICS OF INSL3 EXPRESSION IN THE FETAL TESTIS

At least in the adult testis, INSL3 is a constitutive product uniquely of relatively mature, well-differentiated Leydig cells (32). Assuming the same is true for fetal Leydig cells, then INSL3 should be produced as soon as the fetal Leydig cells have acquired their characteristic, presumably steroidogenic phenotype. This occurs shortly after gonadal sex determination and the expression of the SRY and SOX9 genes by the fetal Sertoli cells. In humans, this would be around weeks 7-8 post coitum (pc; equivalent to gestational age (time since last menses) less 2 weeks). It is important to recall that at this time the fetal testes, adrenal glands, kidney and associated tissues are located very close together on each side of the body, such that mutual hormonal influences are largely of a paracrine nature within one side (34). The contralateral organs are further away. For such paracrine systems, only very low concentrations of a hormone are sufficient to activate G-protein-coupled receptors such as RXFP2, well below the 10% effective concentration (EC^{10}) for the receptor ($<10^{-10}$M) and, being locally produced, there may not yet be sufficient hormone to be detected in the fetal bloodstream, or in amniotic fluid. Thus, we can assume that the fetal INSL3/RXFP2 system will be activated before INSL3 becomes measurable in fetal blood or in amniotic fluid. **Figure 2** indicates INSL3 mRNA determined by RNA microarray analysis in samples of human fetal gonads collected during the first trimester (35) and indicates an up-regulation in testes only, and not in ovaries, at weeks 7-8 pc. The upregulation of INSL3 follows precisely (i.e. actually on the same day during fetal development) the concomitant upregulation of the genes necessary for androgen production in the testes (34). If constitutively expressed, we can assume that INSL3 peptide

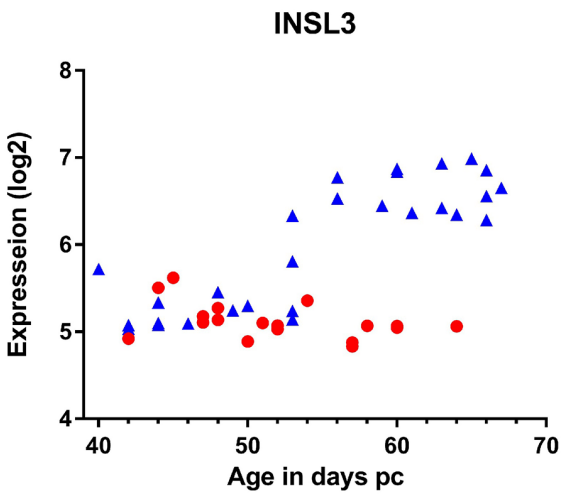

FIGURE 2 | Expression of INSL3 mRNA measured by microarray analysis of gonadal tissue derived from individual male (blue triangles) or female (red circles) human fetuses at the ages indicated (35).

will be produced almost immediately following gene transcription. This probably increases after that, since other studies report highest fetal testis INSL3 mRNA at around gestation weeks 17-18 (36), though it is expressed earlier.

There is limited data available for the human in regard to the INSL3 concentration in fetal blood. Harrison and colleagues reported 0.44-2.04 ng/ml from umbilical cord venous blood in gestation weeks 15-20 (37). Lower concentrations of INSL3 are recorded in cord blood at term of pregnancy [control subjects: France, 0.27 ± 0.18 ng/ml (38); Japan, 0.28 (0.25-0.32 IQR) ng/ml (39); Denmark, 0.13 (<0.05-0.34 range) ng/ml (40); Finland, 0.14 (0.06-0.39 range) ng/ml (40)], with the small differences probably attributable to the different assays being used to measure INSL3.

INSL3 can also be detected in human amniotic fluid that has been routinely collected at amniocentesis for prenatal genetic diagnosis (41, 42). It is measurable only for male fetuses (41) and in the earliest sample available already at gestation week 11, though reaches a maximum at gestation weeks 12-16 (**Figure 3**). It appears to decline to undetectable levels by about week 20. Whilst this might reflect a reduced Leydig cell production at that time, it is also probable that skin keratinization at about gestation week 20 leads to a limitation of peptides and proteins being exuded from fetal blood *via* the fetal skin. After this time, the constitution of amniotic fluid more likely reflects the products of the fetal lungs and kidneys, as well as of the amniotic membranes themselves.

INSL3 has also been measured in fetal fluids from rats (43), pigs (44), and cows (45), where the timing of INSL3 expression largely mirrors the dynamics of testicular descent which, for example, occurs shortly after parturition in rodents, unlike in humans or cows, where it occurs earlier during the second trimester (45). These studies were also able to show that INSL3 from the testes of male fetuses was able to cross the placental barrier either to the maternal circulation [cows (45)] or to neighboring female fetuses [pigs (44)]. The mechanism of such transfer is unknown, but in analogy to insulin would suggest some kind of mediating molecular transport system.

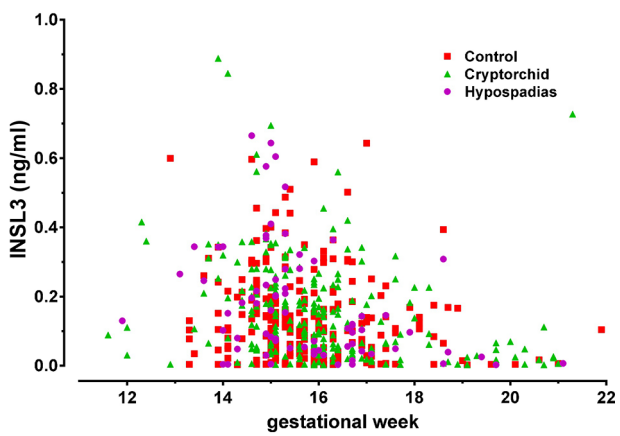

FIGURE 3 | INSL3 concentration measured by specific time-resolved fluorescent immunoassay from human amniotic fluid samples collected at routine amniocentesis at the times indicated. The pregnancies were identified postnatally as normal (control), cryptorchid, or hypospadias [reproduced from (42)].

THE ROLE OF INSL3 IN TESTICULAR DESCENT

Inactivation of the genes encoding INSL3 or its receptor, RXFP2, in mice leads to the same essential phenotype, namely bilateral cryptorchidism with a failure of the first transabdominal phase of testicular descent (11, 12, 46). Similar bilateral cryptorchidism was also achieved in the male offspring of pregnant rats treated with a specific RXFP2 competitive antagonist (47). Genetic evidence in humans is more problematic. Since both genes are autosomally recessive, mutations in single alleles rarely lead to cryptorchidism. Several population studies, however, have indicated an association between heterozygous mutation in either INSL3 or RXFP2 and the incidence of cryptorchidism (usually unilateral) (48, 49). Because cryptorchidism, unless corrected, inevitably leads to male infertility such deleterious mutations are historically at a low frequency.

Testicular descent is a highly dynamic process. Prior to gonadal sex determination, the indeterminate gonad lies adjacent to the fetal kidney and adrenal complex (mesonephros) apposing the dorsal wall of the coelomic cavity. It is attached dorsally to the body wall by the cranial suspensory ligament (CSL) and ventrally to the body wall in the inguinal region by the gubernacular ligament. Initially, both ligaments are relatively short and undeveloped. In the following days, the fetus grows with the kidney moving in an antero-dorsal direction relative to the inguinal region. In the male fetus, testosterone produced by the differentiating Leydig cells causes the CSL to involute, becoming longer and thinner. In contrast, INSL3 produced by the same cells causes the mesenchymal core of the gubernacular ligament to shorten and expand laterally to form the gubernacular bulb, which now retains the fetal testis in the inguinal region as the kidney and adrenal grow away in an antero-dorsal direction (18, 50). Thus, this first phase of testicular descent does not involve any actual ventral movement of the fetal gonads, but merely a retention of the gonad in the inguinal region. Subsequently, in the second phase of descent, the gubernacular bulb everts through an inguinal weakening of the body wall, creating an inguinal canal and causing the fetal testes to relocate into the scrotum (18). This second phase may also require some INSL3 in addition to androgens (16). In the female fetus, the CSL fails to involute, retaining the ovary near the kidney; there is also no INSL3 produced and hence no gubernacular thickening and no gonadal 'descent'. In the male *tfm* (testicular feminization) mouse, there are no functioning androgen receptors, and hence the CSL remains thick and fails to involute, but the INSL3 still induces gubernacular development, with the result that in this mouse the fetal testis is held by the two ligaments within the abdomen in an intermediate location as if on a 'taut bowstring'. In the INSL3 knockout mouse, the opposite occurs, with neither ligament developed and the fetal testes appearing to be loosely swimming within the peritoneal cavity (11). In female fetal mice, which have been genetically

engineered to produce INSL3, it appears that besides causing a slight dislocation of the ovaries, INSL3 also induces abdominal hernia (51), suggesting that the INSL3/RXFP2 system may additionally be involved in aspects of the second inguino-scrotal phase of testis descent in the males. It is to be noted though that RXFP2 expression in female mice is likely to be much reduced because of less androgen production (see below).

Whether the INSL3/RXFP2 hormonal system is involved in later stages of testicular descent is unclear since disruption of the first phase inevitably leads to a disruption of the relative dynamics of testicular descent as a whole, including of subsequent phases. This is a very active stage of fetal development. Anatomical examination of unilateral and bilateral cryptorchidism often indicates that other tissues or ligaments have become interposed possibly because of altered relative timing of their growth trajectories. Certainly, it is understood that androgens as well as neural input from the CGRP-expressing genito-femoral nerve are principally involved in the second inguinal-scrotal phase of testis descent, at least in rodents (18), though *in vitro* studies suggest that both INSL3 as well as AMH may also have a role (52). Cryptorchidism (unilateral or bilateral) is very common in the male population and hints at more than the involvement of one or two simplistic regulatory disorders; it has recently been suggested that it is indeed a neuro-humoral multifactorial syndrome involving a dynamic network of a range of diverse factors (53). However, it should be noted that altogether much less is known about the situation in humans compared to experimental animals. Because the early left and right organ complexes are discrete from one another and each appears to be regulated separately by local paracrine factors during testicular descent, this might explain the preponderance of unilateral cryptorchidism, whereby only one such complex is dynamically disrupted.

Whether INSL3 is also involved in other fetal processes other than testis descent is not known, nor whether RXFP2 is also expressed in other fetal tissues than the gubernaculum. An exception here is provided by horn buds in male ruminants, where RXFP2 is expressed within the horn bud and mutations in the RXFP2 gene are associated with polledness in sheep and cattle (54). More research is needed here; it seems likely that the INSL3/RXFP2 system may well be involved in areas of fetal physiology with significant sex-specific aspects. This might prove relevant, for example, in cases of twinning with male and female fetuses sharing the same uterine environment (see above), although studies of such effects to date are still ambiguous (55).

THE REGULATION OF INSL3 AND RXFP2

As mentioned earlier, the upstream promoter region of the INSL3 gene in most species evaluated appears to be relatively short, encompassing maximally 1000bp. Specifically, it includes three discrete responsive elements for the transcription factor steroidogenic factor 1 (SF1) (32, 56), and *in vitro*, transfected promoter-reporter constructs achieve maximal activity simply by co-transfecting the unmodified transcription factor (27, 32). All three SF1-responsive elements (SFREs) appear to be functional (32, 56). This could be enough to explain the up-regulation of INSL3 in fetal Leydig cells *in vivo* which appears to occur immediately following the expression of SF1 (35) in the same cells, and in these as well as in adult Leydig cells appears to occur constitutively. For example, there is full INSL3 expression in the fetal Leydig cell population of the *hpg* (hypogonadal) mouse in which the HPG axis is disrupted because of an absence of GnRH and hence LH (6). However, whether all three SFREs are normally occupied by SF1 is unclear, as is also whether or not other related transcription factors may also compete for binding to the gene promoter. An SFRE can also bind and respond to the closely related transcription factor Nur77, which at least in adult Leydig cells appears to be principally involved in INSL3 up-regulation (57). It is also recognized that the inhibitory transcription factor COUP-TF may also bind to an SFRE and compete with SF1 to control the up- or down-regulation of a gene (58); and it is now established that COUP-TF may play an important role in the differentiation and development of the fetal testis (59) as well as in regulating the INSL3 gene (60). However, it has also been shown that several nuclear steroid receptors may also influence gene expression using SFREs, probably in a non-classical manner which does not involve direct interaction with the responsive element in the DNA itself (61). Both rodent and bovine INSL3 promoter-reporter constructs can be stimulated *in vitro* by activated estrogen and androgen receptors (56, 62), though whether *via* classical or non-classical mechanisms is not clear. If this is relevant and important also for fetal INSL3 expression is not known. It should also not be forgotten that INSL3 expression is extremely cell-type specific. In the fetus, there is expression only in a subset of fetal Leydig cells (63), and not in any other testicular cell although, for example, fetal Sertoli cells also express SF1. Nor is there any expression in steroidogenic adrenal cells even though these share a similar mesonephric mesenchymal origin as fetal Leydig cells. There are evidently specific elements within the INSL3 gene which determine high cell-type specificity.

Very little is known about the regulation of RXFP2 expression. Indirect evidence from mice in which the LH receptor gene has been disrupted implies that RXFP2 expression requires activated androgen receptors to induce the appropriate level of the RXFP2 receptor (64). What is also evident is that in the male fetus (at least in rodents) its expression is also specifically restricted to certain cell types only (17), such as those of the mesenchymal core of the gubernaculum.

INSL3 AS A MONITOR OF ENVIRONMENTAL ENDOCRINE DISRUPTION

INSL3 represents a major secretory product uniquely from the male fetus and not from either a female fetus or the mother, at a time in the human during the transition from first to second trimester, when most organ systems are developing and differentiating most rapidly. It thus offers to be an excellent biochemical biomarker for such early organogenesis, particularly as this is a period in

pregnancy which is otherwise difficult to monitor (43). Numerous studies in pregnant rats have shown that maternal exposure to a variety of environmental endocrine disrupting chemicals (EDCs) leads to a reduction in INSL3 mRNA or protein expression by the fetal Leydig cells (**Figure 4**) with disruption of the male reproductive phenotype, including cryptorchidism, hypospadias, and reduced anogenital distance (65–67). These chemicals include phthalate esters (**Figure 4**), used as plasticizers or previously in cosmetics, as well as xenoestrogens, such as diethylstilbestrol or bisphenol A (67, 68). The chief mode of action of these substances appears to be to disrupt fetal Leydig cell differentiation leading to a reduction in testosterone production and/or INSL3. Importantly, even after only a brief maternal exposure, these substances also appear to affect Leydig stem cells which reside within the testes even into adulthood, and hence may also influence puberty and adult Leydig cell function (67, 69).

For the human, studies have compared second trimester INSL3 concentration in amniotic fluid with the levels of phthalates and PFOS. Investigations in a large Danish biobank showed at the population level that INSL3, and hence Leydig cell function, was significantly reduced in proportion to the EDC load (42, 70, 71). Similarly, other studies of INSL3 in cord blood at term of pregnancy also indicated a significant negative relationship between phthalate or bisphenol A exposure and INSL3 concentration (39, 72). Similar studies have also indicated that INSL3 concentration in term cord blood is significantly different between control male infants and those exhibiting cryptorchidism (38), implying that at least at a population level these three factors (EDC load, INSL3, cryptorchidism) are mechanistically linked, presumably *via* the common element of fetal Leydig cell function. It is to be noted though that INSL3 in second trimester amniotic fluid does not appear to differ significantly between normal male infants and those born with cryptorchidism or hypospadias (**Figure 3**) (42).

Explanted human fetal testis fragments have also been assessed either using *in vitro* culture (73) or following xenotransplantation to immune-compromised mice (74). Again, EDC exposure suggests a disruption of fetal Leydig cell differentiation and/or functionality. The most commonly used EDC in these experiments is represented by the phthalate esters which are widespread especially in the environment through their inclusion in numerous plastics, coatings, and cosmetics. More recently, however, the focus has shifted to show that also common analgesics, such as acetoaminophen (paracetamol) or ibuprofen, which are widely taken to alleviate pain during pregnancy, appear to have a similar impact on the development of fetal Leydig cells and the expression of INSL3 (74, 75).

What is important in these investigations and is being reinforced through several studies in rats (67, 76–78), is that the impact of these EDCs is less an acute one acting, for example, *via* modulation of steroid receptors, but rather an effect which is altering the differentiation dynamics of Leydig cell precursors. Both in the fetus and during puberty, and possibly also in later life, Leydig cells are developing *via* the two processes of proliferation and differentiation held in fine balance with one another. Perturbation of either of these processes will lead to an altered final Leydig cell functional capacity, and hence to an altered capacity to produce testosterone and other hormones essential to maintain health in later life. INSL3 is an accurate measure of this Leydig cell functional capacity (5) and can monitor the impacts of EDCs and other exogenous factors, besides having endocrine functions in its own right, for example, to improve bone quality (79).

CONCLUSION

INSL3 is a major secreted hormone produced by the Leydig cells of the fetal testis, shortly after its differentiation from the undetermined gonad. This occurs immediately following gonadal sex determination and the expression of the transcription factor SF1. Its main function in the male fetus is to induce thickening of the gubernacular ligament anchoring the testes in the inguinal region, thereby promoting the first transabdominal phase of testicular descent. As a major male fetal hormone in the first and second trimesters of human pregnancy, it likely also has other roles

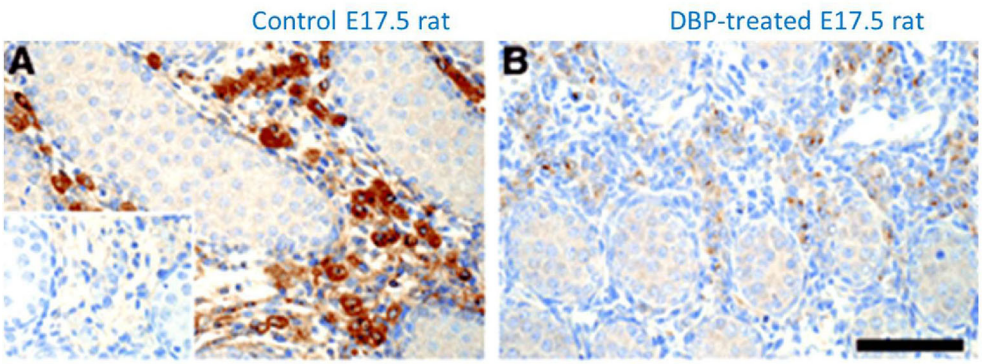

FIGURE 4 | Immunohistochemical staining for INSL3 (brown color) in the fetal testes on gestational day E17.5 of male rats which had been maternally exposed to dibutyl phthalate **(B**; DBP**)** or vehicle **(A)** during the important window for male development (E12-E16). Control section using pre-immune serum is indicated in the bottom left of panel **(A)** [reproduced from (65)].

about which we still have little information. Importantly, it can also act as a biomarker for fetal physiology in this relatively obscure phase of pregnancy, responding to maternal exposures such as to EDCs or to analgesic pharmaceuticals.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to the conception, writing, and editing of the manuscript.

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Activin A and Sertoli Cells: Key to Fetal Testis Steroidogenesis

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The long-standing knowledge that Sertoli cells determine fetal testosterone production levels is not widespread, despite being first reported over a decade ago in studies of mice. Hence any ongoing use of testosterone as a marker of Leydig cell function in fetal testes is inappropriate. By interrogating new scRNAseq data from human fetal testes, we demonstrate this situation is also likely to be true in humans. This has implications for understanding how disruptions to either or both Leydig and Sertoli cells during the *in utero* masculinization programming window may contribute to the increasing incidence of hypospadias, cryptorchidism, testicular germ cell tumours and adult infertility. We recently discovered that activin A levels directly govern androgen production in mouse Sertoli cells, because the enzymes that drive the conversion of the precursor androgen androstenedione to generate testosterone are produced exclusively in Sertoli cells in response to activin A. This minireview addresses the implications of this growing understanding of how *in utero* exposures affect fetal masculinization for future research on reproductive health, including during programming windows that may ultimately be relevant for organ development in males and females.

Keywords: androgens, activin A, Sertoli cell, Leydig cell, fetal steroidogenesis

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DOGMA RELATING TO FETAL TESTIS BIOLOGY: MOVING AND STATIONARY TARGETS

More than 30 years ago, the events that lead to gonad masculinization were shown to be triggered by the expression of *SRY* in pre-Sertoli cells of the fetal testis, in studies of humans (1) and mice (2). Since that time, we have learned just how complicated that process is, including the contribution of signals relating to SOX9 feedback (3), growth factors (4, 5) and retinoic acid (6). These cues direct primordial germ cells to differentiate into the earliest sperm precursors and instruct the somatic cells to multiply, differentiate, and form the testis cords that underpin adult spermatogenesis (7). Our established understanding was that many gonadal cell types were initially bipotential, but subsequently became set in their fate in fetal life. One type became either Sertoli cells in males or granulosa cells in females, providing direct support and instructions to germ cells. However, complementary break-through studies identified that somatic cell fate was actually pliable in both mice and humans. For example, in several studies published over a decade ago, the transcription factor Dmrt1 (Doublesex And Mab-3 Related Transcription Factor 1) was shown to be essential to stabilize the masculinized phenotype of Sertoli and Leydig cells (8), while Foxl2 serves a similar role

in sustaining the ovarian phenotype in granulosa and thecal cell lineages (9). The reality of sex-reversal in somatic cell fate was thereby revealed.

Understanding how somatic cells instruct male or female differentiation has also been important for learning about human conditions which can be linked with sub- or infertility, due to impaired germline differentiation or reduced germ cell survival. The conceptualization of 'Testicular Dysgenesis' as a spectrum of phenotypes, including hypospadias, cryptorchidism, testicular germ cell tumours and infertility, has provided a basis for mechanistic studies of each of these conditions, as each could result from impaired somatic-germline communication in the fetal testis (10). The Testicular Dysgenesis Hypothesis has stood the test of time; the continuously increasing rates of these conditions worldwide highlights the vulnerability of the developing male reproductive tract to lifestyle and environmental exposures (11), in concert with a lesser, but relevant genetic determinant of disease risk. Mechanistically, this hypothesis has focussed on explaining the impact of disruptions to cell-cell communication in the testis *in utero*, particularly with regard to androgen production and signalling (12).

Crucial to understanding the origins of testicular dysgenesis outcomes has been delineation of a masculinization programming window (termed MPW), the period of development when the male fetus is sensitive to exposures that impact on reproductive health. During this window, multiple factors can influence the development of the male gonad and secondary sexual characteristics (**Figure 1**). Virilization of the fetal male is dependent on gonadal function but it is also vulnerable to alteration by maternal- and/or placental-derived factors (**Figure 1**). Best understood in rodent models, endocrine disruptor chemical exposures in the interval following sex determination and prior to birth have repeatedly been shown to increase the incidence of conditions including reduced anogenital distance, hypospadias, cryptorchidism and reduced germ cell numbers (13–15). These outcomes have been explored in data collected from boys and men, and from cultures of human fetal testes (16–18), with a growing understanding that not only exposure to endocrine disruptor chemicals, which can also include common pharmaceuticals, may negatively impact fetal and postnatal development. These agents can alter steroid composition and levels and may thereby influence local events in the testis. For example, ibuprofen alters human fetal Leydig cell function and testosterone production between weeks 7–17 of gestation, indicating that human male fetal steroidogenesis is vulnerable to alterations by a commonly used pharmacological compound (16). Nodal and activin signalling can also influence particular aspects of human fetal steroidogenesis in both the first and second trimesters (19), and as discussed below, these members of the TGFβ signalling pathway are amongst the key candidates for driving lifelong changes in male reproductive health.

Activin A is a member of the TGFβ signalling superfamily that is emerging as an important regulator of male fetal development. Activin A was originally identified as a factor that was produced and acted locally to control release of follicle stimulating hormone from the pituitary gland (20, 21). It is synthesized as a disulfide-linked homodimer of two inhibin beta A subunits, encoded by the *Inhba* gene, and its structure and signalling pathway are characteristic of the more than 30 other members of the TGFβ superfamily of signalling molecules, many of which are simultaneously present in the fetal testis (5). It is now known to be produced throughout the body and serve functions in growth and development of many organs, while activin A overproduction is associated with severe pathologies such as cancer cachexia (22). Physiological regulation of activin signalling is complex, occurring through extracellular, transmembrane and intracellular means which are typically context-dependent. See (21, 23–27) for recent reviews on this topic.

Activin A performs multiple functions in testis development that include governance of Sertoli cell proliferation. This role was first revealed in the adult inhibin α knockout mouse strain with elevated activin A; males succumb to Sertoli cell tumours due to the absence of the potent activin inhibitor, inhibin, which forms as a heterodimer of one inhibin α subunit with an inhibin β A subunit (28). In addition to driving Sertoli cell proliferation *in utero*, activin A production by fetal Leydig cells also promotes the timely entry of fetal germ cells into a quiescent state following sex determination (29–31). Quiescence occurs in an important developmental interval, coincident with important aspects of normal male germ cell differentiation that include epigenetic reprograming and loss of pluripotency, each of which can be linked with reducing the risk of tumour formation (32). We have recently discovered that activin A is an important factor regulating androgen biosynthesis in the fetal testis (33); the mechanisms and significance of these findings will be discussed below.

LEARNING ABOUT ANDROGEN BIOSYNTHESIS IN THE FETAL TESTIS

A Gold Mine of Data From Single Cell Transcriptome Analyses

The application of single cell RNA sequencing to study the mammalian fetal testis has revealed developmental relationships between somatic cells which highlight their common origins. Work in mouse (34) and human (35) have detailed the emergence of distinct Leydig and Sertoli cell populations from a single progenitor, extending findings from earlier lineage mapping studies (36). Important distinctions between fetal and adult Leydig cells have been progressively mapped, revealing that synthesis of the androgen precursor, androstenedione, is different in fetal Leydig cells compared to adults. Whereas luteinizing hormone (LH) stimulates steroid biosynthesis in adult Leydig cells, fetal cells can be stimulated by corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH), and their responsiveness to LH is probably not attained prior to E17 in mice (37, 38). Single cell RNASeq datasets have also shed new light on where, and which, steroids and androgens are produced in the fetal testis, as discussed below.

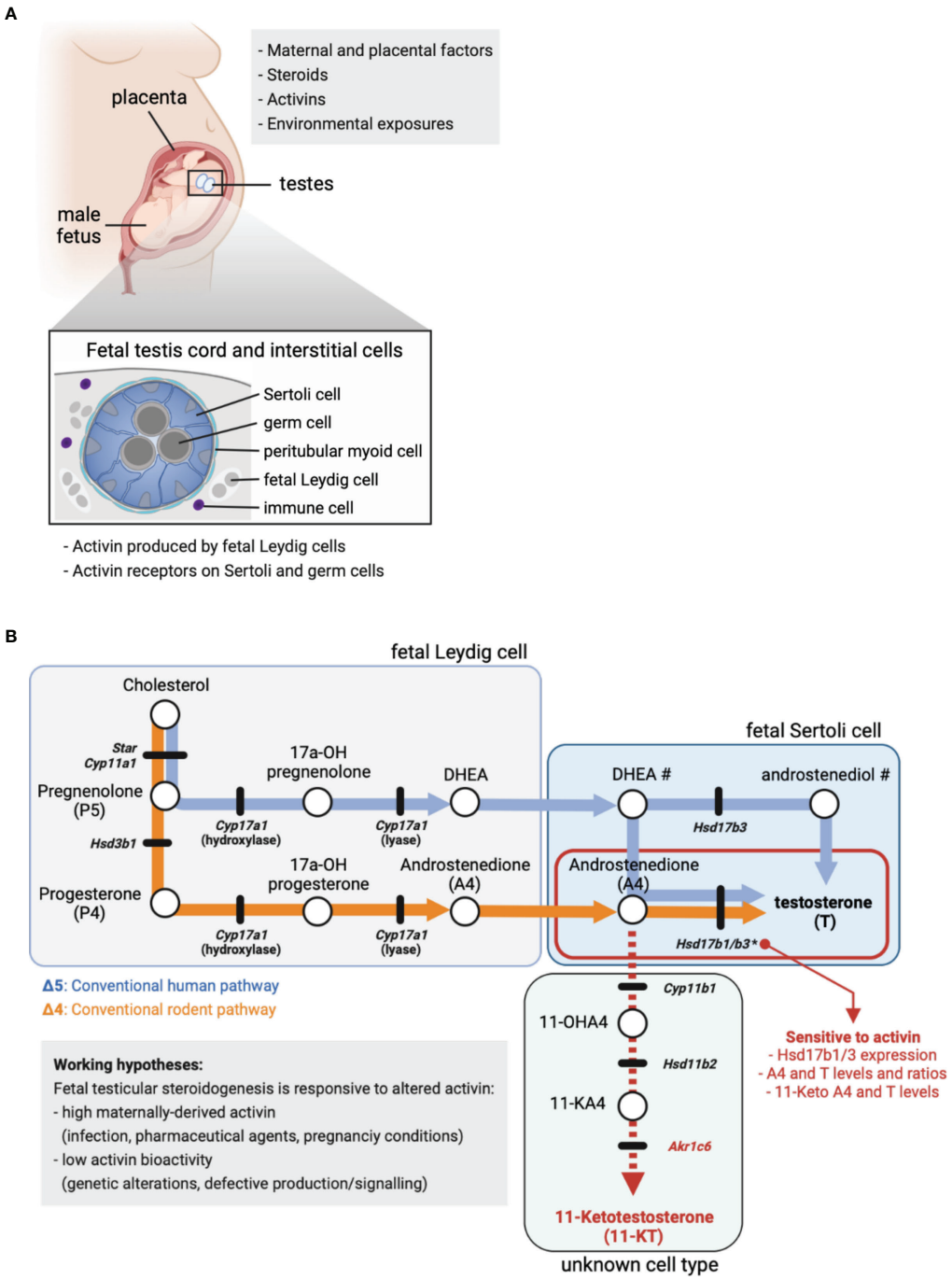

FIGURE 1 | Fetal steroid production. **(A)** Maternal and placental factors, exogenous steroids, activins and environmental exposures each have the potential to alter fetal steroid production. Schematic of placenta, male fetus and testes *in utero*. Cross-section identifies key fetal testis cell types in the cord (germ and Sertoli cells) and in the surrounding interstitium (fetal Leydig, peritubular and immune cells). Activin is produced by fetal Leydig cells and signals *via* specific cell surface receptors present on Sertoli and germ cells. **(B)** Simplified summary of the conventional steroidogenic pathway in human (Δ5, blue line/arrows) and rodent (Δ4, orange line/arrows) fetal testes. The 11-Keto androgen pathway is indicated (red dashed line), as are 11-Hydroxyandrostenedione (11-OHA4), 11-Keto androstenedione (11-KA4) and 11-Ketotestosterone (11-KT). The cellular site of steroid production is indicated, where known. Circles denote cholesterol and steroids, while black lines indicate genes encoding enzymes involved in the conversion of one steroid to the next. Mouse nomenclature has been used throughout; **Table 1** lists human nomenclature. # indicates the local site(s) of DHEA and androstenediol production in human is unclear. * indicates activin A-responsive genes; the ratio of A4 to T in mouse fetal Sertoli cells is dose-dependently reduced by low levels of *Hsd17b1* and *Hsd17b3* expression driven by altered activin A bioactivity. In these testes, excess A4 is associated with higher levels of 11-keto androgens.

Sertoli Cells Are the Site of Testosterone Production in Fetal Mouse Testes

In the rodent testis, testosterone production occurs *via* the Δ4 steroidogenic pathway (**Figure 1B**, orange line) and concludes with conversion of androstenedione (A4) to testosterone (T) by the enzymes HSD17B3 (39) and HSD17B1 (40). The fetal and adult Leydig cell populations develop sequentially and are functionally distinct (41), so while T is produced in Leydig cells in adult mouse testes (42), fetal Leydig cells lack *Hsd17b1* and *Hsd17b3* and cannot convert A4 to T (39, 40). Instead, exclusive expression of *Hsd17b1* and *Hsd17b3* in fetal mouse Sertoli cells (43, 44) identifies Sertoli cells as the site of T synthesis in the fetal testis (33).

Since appropriate levels of steroids and steroidogenic enzymes during embryonic (E) development lay the foundation for the correct development of male sexual characteristics, factors that influence this process may illuminate important areas for research aimed at understanding the foundations of male reproductive disorders. Activin A (encoded by *Inhba*) is produced by fetal Leydig cells and is essential for normal embryonic mouse testis development. *Inhba* transcript levels in male testes increase directly after sex determination (E12.5) and the activin A dimer acts directly on receptors in Sertoli cells influencing their proliferation (30, 31, 45). Recently, our lab identified that activin A also positively regulates expression of *Hsd17b1* and *Hsd17b3* required to convert A4 to T; both are strongly reduced (E13.5 - E15.5) in the absence of activin A (*Inhba* KO) compared with wildtype controls (33). Analysis of intratesticular steroids demonstrated that a sustained decrease in *Hsd17b1* and *Hsd17b3* transcripts reduced conversion of A4 to T within E17.5 *Inhba* KO fetal testes; A4 levels were significantly increased and T tended to decrease. Additionally, the T/A4 ratio revealed a highly significant dose-dependent decrease in androgen production that correlated with reducing *Inhba* gene dosage (33). These data demonstrate that activin A fulfils a central role in determining local steroid levels and androgen production during fetal testis development. Lastly, Whiley et al. measured increased levels of the 11-oxygenated androgens, 11-KA4 (11-keto androstenedione, also named adrenosterone) and 11-KT (11-ketotestosterone) in *Inhba* KO testes (33). Since 11-KT can activate the androgen receptor (46), this finding could have important clinical significance.

Steroidogenesis in the Human Male Fetus: What We Know and What Are It's Vulnerabilities?

Production of the androgen T by the human fetal testis is essential for masculinization. Its release from the testis drives virilization of the Wolffian ducts to form the seminal vesicles and ejaculatory ducts, whereas the 5α-reduction of T to the more potent androgen dihydrotestosterone (DHT) is necessary for virilization of the male external genitalia (47). The first trimester human testes can produce low levels of T *via* the Δ4 pathway prior to a switch to the Δ5 pathway at the beginning of the second trimester (48). Thereafter, the human fetal testis produces high levels of T during the second trimester *via* the classical Δ5 steroidogenic pathway (**Figure 1B**, blue lines) (48, 49). This switch coincides with the maturation of fetal Leydig cells as they acquire their capacity to produce androgen precursors (48). The precursor androstenedione (A4)) is converted to T mainly by the 17β-hydroxysteroid dehydrogenase activity of HSD17B3 (50) (**Figure 1**) and while HSD17B1 can perform this conversion, it is less efficient compared to the mouse enzyme (51). In the human fetal testis, *HSD17B3* levels are comparable in first and second trimesters (48). However, the peak in T synthesis in the second trimester appears due to fetal Leydig cells becoming fully differentiated and acquiring the ability to synthesize androgen precursors (48).

Leydig cells are the major steroidogenic cells of the human fetal testis, expressing a wide range of steroid biosynthetic enzymes, and their differentiation coincides with elevated testicular T levels (49). Thus, these cells are often assumed to be responsible for androgen biosynthesis. However, as described in the section "Sertoli Cells Are the Site of Testosterone Production in Fetal Mouse Testes", although the precursor steroids and A4 are produced by fetal Leydig cells in rodents, the conversion of A4 to T takes place in the Sertoli cells (**Figure 1**). Whether this also occurs in humans is not established (48). To address this possibility, we interrogated published single cell RNASeq datasets to determine in which cell types key steroidogenic enzymes are synthesized in mouse and human testes (35, 44). The expression patterns for *HSD17B3* and *HSD17B1* in human testicular cells provide strong evidence that, as in mice, human Sertoli cells are likely to be the primary site of androgen (T) synthesis in the second trimester fetal testis

TABLE 1 | mRNA expression patterns of key steroidogenic enzymes in fetal mouse and human testis cells[a].

Gene symbol (mouse, human)	Mouse fetal testis cell expression[b]	Human fetal testis cell expression[c]
Cyp11a1, CYP11A1	Leydig cells	Leydig cells > Sertoli cells
Hsd3b1, HSD3B1	Leydig cells	Not available
Cyp17a1, CYP17A1	Leydig cells	Leydig cells
Hsd17b3, HSD17B3	Sertoli cells	Sertoli cells
Hsd17b1, HSD17B1	Sertoli cells	Leydig and Sertoli cells
Cyp11b1, CYP11B1	Leydig cells	Not detected
Hsd11b2, HSD11B2	Stroma, peritubular myoid	Leydig cells
Akr1c6, AKR1C3	Not present	Not detected

[a]*Predominant site of expression as determined by UMAP plots from the relevant dataset (see* ***Supplementary Figure 1****)*
[b]*Data from E18.5 mouse testis cells according to Tan et al. (44)*
[c]*Data from human testis cells at 12, 15 and 16 weeks post-fertilization (14, 17 and 18 weeks gestation) according to Guo et al. (35)*

(**Table 1**; **Supplementary Figure 1**). Interestingly, human scRNASeq data suggest that only a small subset of human fetal Leydig cells produce androgen precursors between weeks 14–18 of gestation [**Supplementary Figure 1** (35)]. This speculation is aligned with evidence that human fetal interstitial and Leydig-like cells are a heterogenous population (49).

Recent studies have revealed that alternative androgen production by the so-called "backdoor pathway" is also important for virilization of the human male fetus (52, 53). This pathway begins with progesterone, probably originating largely from the maternal side of the placenta (**Figure 1A**), followed by sequential biosynthetic steps in the adrenal glands and liver, that ultimately produce androsterone (53). This androgen is present in the human fetal male circulation at levels similar to testosterone (53). Thus, masculinization of the human male fetus depends on androgens produced *via* the classic and backdoor pathways, both within and outside of the testis. It involves a complex interplay of steroidogenic enzymes expressed in multiple tissues to produce testosterone, DHT and androsterone that act within different organs to control human fetal urogenital tract development (49, 52, 53).

Finally, as noted above, certain 11-keto steroids have the capacity to contribute to androgen action in the human fetal testis and other androgen-responsive tissues. Originally identified as androgens in fish, the 11-keto androgens 11-keto testosterone (11-KT) and 11-keto DHT (11-KDHT) are potent agonists of the human androgen receptor (46, 54, 55). They are likely to be synthesized in human peripheral tissues from the abundant adrenal steroid 11b-hydroxyandrostenedione (11OHA4) (**Figure 1B**) (46, 54). CYP11B1 is a key enzyme in 11-keto steroid biosynthesis (**Figure 1B**) and is expressed in mouse and human testis (**Table 1**; **Supplementary Figure 1**). This enzyme can be stimulated by hCG in immature mouse Leydig cells leading to an increase in testicular levels of 11-KT (55). In mice, 11-KT is shows similar androgen activity to T, suggesting it is an important bioactive androgen (55). The enzyme responsible for the biosynthesis of 11-keto androstenedione (11-KA4), HSD11B2 (**Figure 1B**), is expressed in both mouse and human testis cells (**Table 1**; **Supplementary Figure 1**) whereas the enzyme that converts 11-KA4 to 11-KT (AKR1C6 in mice and AKR1C3 in humans (**Figure 1B**), is not expressed in the testis (**Table 1**, **Supplementary Figure 1**). These data suggest that fetal mouse and human testes express the enzymes that produce 11-KA4 and that this precursor can be converted to the androgen 11-KT in peripheral tissues. We have detected both 11-KA4 and 11-KT in fetal mouse testes, indicating that 11-KT could act as bioactive androgen during fetal testis development (33). Importantly, we showed the levels of these keto steroids are responsive to activin A deficiency; reduced conversion of A4 to T led to increased levels of A4 that were accompanied by increased testicular levels of 11-KA4 and 11-KT (33). Thus, in activin A-deficient mice, the reduced capacity to produce the androgen T, as a consequence of reduced *Hsd17b1* and *Hsd17b3* expression in Sertoli cells, is accompanied by an increased capacity to produce the androgen 11-KT (33). Further studies are needed to understand the contribution of 11-KT to androgen action in the human fetal testis and in the masculinization of the male fetus.

CONCLUSIONS: WHY DOES THIS MATTER?

The complexity of human fetal steroidogenesis means that there are multiple cell types and thus sites vulnerable to disruption that could alter androgen levels and activities in different fetal tissues. In the mouse testis, activin A regulation of the T biosynthetic enzymes *Hsd17b1* and *Hsd17b3* in Sertoli cells is now known to alter the ratio of A4 to T and the production of keto-steroids with androgenic bioactivity (**Figure 1B**), and this is likely to be important to some extent in humans (**Table 1**). Changes to activin A bioactivity at the start of gonad development, as in the condition of pre-eclampsia, would be ultimately expected to influence adult fertility and development of other organs; this may also affect the developing ovary. The fact that fetal T is produced by the Sertoli cells and thus T levels are not an accurate measure of fetal Leydig cell function needs to be more widely appreciated. Recent data showing that androgens in the human male fetus testis can originate *via* different biosynthetic pathways adds complexity to the mechanisms *via* which external and intrinsic factors could modulate androgen action during male fetal development. This knowledge will help to identify a roadmap of developmental vulnerability to environmental exposures, focussed appropriately on the key affected cell types. This should ultimately be used to develop strategies that can protect reproductive health.

AUTHOR CONTRIBUTIONS

KL designed content, co-wrote text, and edited text and figure. PW co-wrote and edited text, designed and produced figure. LO'D co-wrote text, and edited text and figure. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Insights Into the Roles of GATA Factors in Mammalian Testis Development and the Control of Fetal Testis Gene Expression

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Defining how genes get turned on and off in a correct spatiotemporal manner is integral to our understanding of the development, differentiation, and function of different cell types in both health and disease. Testis development and subsequent male sex differentiation of the XY fetus are well-orchestrated processes that require an intricate network of cell-cell communication and hormonal signals that must be properly interpreted at the genomic level. Transcription factors are at the forefront for translating these signals into a coordinated genomic response. The GATA family of transcriptional regulators were first described as essential regulators of hematopoietic cell differentiation and heart morphogenesis but are now known to impact the development and function of a multitude of tissues and cell types. The mammalian testis is no exception where GATA factors play essential roles in directing the expression of genes crucial not only for testis differentiation but also testis function in the developing male fetus and later in adulthood. This minireview provides an overview of the current state of knowledge of GATA factors in the male gonad with a particular emphasis on their mechanisms of action in the control of testis development, gene expression in the fetal testis, testicular disease, and XY sex differentiation in humans.

Keywords: transcription, GATA, hormone, testis, fetal, gene expression

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INTRODUCTION

The Vertebrate Family of GATA Transcription Factors

The GATA family of transcriptional regulators comprises six factors (GATA1 to 6) in vertebrates. GATA factors regulate gene transcription by binding to a consensus nucleotide sequence—A/TGATAA/G—called the GATA motif that is scattered throughout the genome. The six GATA proteins are typically classified based on their amino acid sequence and tissue distribution patterns. GATA1/2/3 are hallmarks of hematopoietic cell lineages, and consequently are essential for the differentiation of multiple blood cell types (erythrocytes, leukocytes, megakaryocytes) [reviewed in (1, 2)]. By contrast, the GATA4/5/6 proteins (known as the cardiac family of GATA factors)

populate tissues of mesodermal and endodermal origin such as the heart, organs of the digestive tract, and gonads [reviewed in (1–5)]. Initial insights into the cardiac subfamily of GATA factors came from global gene inactivation studies in mice. Loss of GATA4 precipitates early embryo lethality because of defects in ventral morphogenesis of the embryo and its subsequent inability to form a functional linear heart tube (6, 7). Lack of *Gata6* expression arrests embryo development at an even earlier stage due to defects in extraembryonic development (8). Unlike GATA4 and GATA6, global loss of GATA5 function is not embryonic lethal; rather *Gata5*$^{-/-}$ mice exhibit prominent genitourinary tract abnormalities in females but not males (9). Identification of *GATA* gene mutations associated with clinical disease has helped to highlight the essential nature of GATA function in humans. For the *GATA4* gene alone, more than 100 mutations have been linked to a large spectrum of cardiac abnormalities [reviewed in (2, 10)].

All GATA proteins share a highly conserved zinc finger DNA-binding domain. The specificity of GATA action is conferred in part through interactions with specific partners [reviewed in (2–4)]. The most notable are the Friend of GATA (FOG) proteins [reviewed in (11)]. GATA factors also interact and functionally cooperate with a multitude of other transcription factors that not only amplify GATA activity but also limit their scope of action to select target genes. This is true for the testis where many potential GATA-interacting partners have been identified (12–18). GATA activity can also be positively or negatively modulated by direct post-translational modifications such as sumoylation, acetylation, and phosphorylation. Post-translational modifications alter GATA4 transcriptional activity by changing either its nuclear localization, DNA-binding, stability, and/or cofactor recruitment [reviewed in (10, 19)].

GATA FACTORS IN THE TESTIS

Developmental and Cell Type-Specific Expression Patterns

Three GATA factors (GATA1/4/6) are found in the testis and their expression profiles differ based on testicular cell type and timing of expression. *Gata1* was the first reported to be abundantly transcribed in the testis (20). It is also the only GATA factor limited to a single testicular cell type: the postnatal Sertoli cell (21). GATA1 is absent from the fetal testis and as such is not involved in early testis development (22). The onset of *Gata1* expression in mouse Sertoli cells coincides with the first wave of spermatogenesis; thereafter GATA1 levels remain constant and independent of hormones but become progressively spermatogenic stage-dependent (21, 22). GATA1 is therefore an excellent marker of mature Sertoli cells; however, its functions appear to be dispensable for Sertoli cells, at least in mice (23). GATA4 is one of the earliest markers of gonadal development (22, 24). Expression begins in the coelomic epithelial layer of the presumptive genital ridges of both the mouse and chick (24–26). After testis differentiation, GATA4 levels are upregulated in Sertoli, myoid, and Leydig cells [(22, 26, 27) and reviewed in (28)]. High GATA4 levels persist throughout fetal development and into adulthood (22, 27). Like *Gata4*, the *Gata6* gene is transcribed in Sertoli cells of both fetal and adult testes (29, 30). However, unlike *Gata4*, Sertoli cell *Gata6* transcripts only become upregulated during the later fetal stages (29, 30). While *Gata6* expression has been documented in at least one Leydig cell line (29), data showing that the gene is also expressed in Leydig cells *in vivo* is less robust. A review article published in 2014 citing unpublished observations nonetheless reported that GATA6 is present in both late fetal and postnatal Leydig cells in the mouse (5).

Roles in Early Mammalian Gonad Development and Male Sex Determination

Of the three GATA factors expressed in testis, only GATA4 is involved in early gonad development—in the absence of GATA4, coelomic thickening that permits the subsequent growth of the genital ridge is arrested (24). In the same study, the authors also demonstrated that GATA4 acts early being upstream of LHX9 and SF1, two other critical regulators of genital ridge development (31, 32). GATA4 function in gonadal development does not end at the genital ridge stage as revealed by elegant gene inactivation studies in mice, which showed that GATA4 continues to play multiple essential roles with the timing of *Gata4* deletion being critical to the phenotypic outcome [reviewed in detail in (5)]. *Gata4* deletion prior to sex determination completely blocks testis differentiation and results in XY sex reversal (33). By contrast, *Gata4* deletion after male sex determination allows testis differentiation to proceed unfettered (33). In this same model, testis cord development is nonetheless disrupted due to diminished *Dmrt1* expression (described more below), and males are undervirilized and eventually become infertile (33). GATA4 action is also required in the postnatal testis where it controls male fertility and steroidogenesis (34–39). Its role in male fertility appears to be linked to regulation of blood-testis barrier integrity and Sertoli cell genes critical for this function (35, 40).

GATA4 initiates testis differentiation by regulating at least two genes: Sex determining region Y chromosome (*Sry*) and Sry-related homeobox gene 9 (*Sox9*); both genes are markedly downregulated in newly differentiated testes that lack a fully functional GATA4 protein (41–43). However, as for many of the proposed GATA target genes in the testis, it remains to be seen whether they are indeed direct targets. For the *Sry* gene, evidence suggests that this might be the case. A detailed analysis of *Sry* 5' flanking sequences from 17 mammalian species identified a single broadly conserved 106 bp region termed the *Sry* proximal conserved interval (SPCI). The SPCI contains a conserved binding motif for WT1, a known activator of both mouse and human *SRY* transcription (44–46). WT1 has also been reported to directly cooperate with GATA4 on the mouse, pig, and human *SRY* promoters (15). The requirement for GATA4 in *Sry* and *Sox9* transcription has raised the important question as to whether it also participates in the specification of the Sertoli cell lineage at the time of sex determination. Mice lacking a functional GATA4 protein that can no longer interact

with FOG2 do not express any Sertoli cell markers (41), indicating that the GATA4-FOG2 complex (and by extension GATA4 itself) is required for the differentiation of pre-Sertoli cells. Additional insights have come from studies that have reprogrammed fibroblasts into embryonic Sertoli cells. Initial studies showed that GATA4 along with other transcription factors (SF1, WT1, DMRT1, SOX9, DMRT1) are sufficient to induce the formation of embryonic Sertoli-like cells (47–49). A later study refined the mechanism of GATA4 induction of the Sertoli cell fate by showing that it acts upstream of SF1, LHX9, and the anti-Müllerian hormone (AMH) (50).

GATA FACTORS IN FETAL TESTIS GENE EXPRESSION AND FUNCTION

GATA Control of Fetal Sertoli Cell Gene Expression

Following sex determination, the newly formed testis (by producing key fetal hormones) assumes several important functions in masculinizing the developing male embryo. GATA factors continue to play an important role in regulating genes that participate in this fundamental developmental process. Sertoli cells begin to produce AMH, a glycoprotein hormone belonging to the transforming growth factor beta (TGFβ) superfamily. AMH is most recognized for its role in blocking the Müllerian ducts from developing into female reproductive structures in typical XY males (51). The *AMH* promoter from multiple species contain at least one consensus GATA motif that is activated by GATA4 (22, 52–55). In cell lines, the mechanism of GATA4 action on *AMH* transcription has been proposed to involve synergistic interactions with other transcription factors including SF1 and WT1 (12, 15, 56). A more recent *in vivo* study that inactivated the GATA regulatory motif in the *Amh* promoter in the mouse has validated not only the *Amh* gene as a direct target for GATA4 but also the requirement of a GATA4/SF1 interaction for *Amh* expression in fetal Sertoli cells (57). Endogenous *Amh* expression is also controlled by direct binding of SF1 and SOX9 to the *Amh* promoter (58). Unlike SOX9, however, which is required to initiate the production of *Amh* transcripts in newly differentiated Sertoli cells, GATA4 appears to function like SF1 as an amplifier of *Amh* transcription (57, 58).

While mutagenesis of the *Amh* promoter has highlighted the requirement of GATA binding for *Amh* transcription, simultaneous deletion of *Gata4* and *Gata6* in Sertoli and Leydig cells in the fetal testis has favored the opposite view (38). GATA1 was first proposed to disrupt GATA4 action at the *Amh* promoter thereby decreasing *Amh* expression (59). A separate study reported that AMH levels remained high beyond its normal period of decline in postnatal mouse testis lacking both GATA4 and GATA6 (38). GATA1 was also absent in this model (38). These observations led to the suggestion that GATA factors negatively regulate *Amh* gene transcription, at least in postnatal Sertoli cells (38). However, male gonads lacking GATA4 and GATA6 also have compromised steroidogenic gene expression during the fetal period, and a reported reduction of serum testosterone beyond puberty (38). Testosterone, acting through the androgen receptor, is known to attenuate AMH production in Sertoli cells (60, 61). This might account for the differences in *Amh* expression seen in a GATA4/6-deficient mouse testis model where androgen production is notably reduced (38), in comparison to a mouse model where the GATA binding motif in the *Amh* promoter is mutated and androgen levels are not affected (57).

The *Dmrt1* gene has been proposed to be another GATA4 target in the fetal testis (33). DMRT1 is an evolutionarily conserved transcription factor that regulates testis development in all vertebrates (62). Testes from *Dmrt1*$^{-/-}$ mice, and animals where *Gata4* was conditionally inactivated in testes after sex determination, share a similar testicular cord defect beginning at the mid-fetal stage (33). This suggested that *Dmrt1* gene expression in Sertoli cells is directly regulated by GATA4. In support of this, the *cis*-regulatory elements for the *Dmrt1* gene have been mapped in primary Sertoli cells cultures and shown to contain multiple active GATA binding motifs (63). However, GATA4 is not required to maintain somatic *Dmrt1* expression after fetal development as adult Sertoli cells lacking GATA4 continue to express *Dmrt1* (33). Moreover, while both GATA4 and FOG2 are required for the differentiation of pre-Sertoli cells, *Dmrt1* expression in fetal Sertoli cells continues to require GATA4 but not FOG2 (33).

Several other Sertoli cell-expressed genes have been proposed to be regulated by GATA factors. These include inhibin α (*Inha*), inhibin β B (*Inhbb*), reproductive homeobox 5 (*Rhox5*), and follicle stimulating hormone receptor (*Fshr*) (14, 29, 64–69). The GATA-dependence of these additional putative GATA targets have been limited to promoter activation assays and studies where GATA levels have been manipulated in immortalized cell lines. Whether these genes are in fact genuine GATA targets *in vivo* and what contribution GATA factors have to these genes in terms of fetal Sertoli cell expression remain unanswered.

GATA Control of Fetal Leydig Cell Differentiation and Gene Expression

Leydig cells regulate male sex differentiation during fetal development, and fertility in adults, through the production of two hormones: insulin-like 3 (INSL3) and testosterone. INSL3 initiates the first of two phases of testicular descent during embryogenesis (70, 71). Testosterone is a steroid hormone made from the multistep enzymatic conversion of cholesterol. Testosterone regulates the second phase of testis descent and promotes the masculinization of the Wolffian ducts into organs of the male reproductive tract. In mammals, there are two distinct populations of Leydig cells: fetal Leydig cells (FLCs) present during fetal life and shortly after birth, and adult Leydig cells (ALCs) present in the postnatal testis. FLCs are proposed to derive from progenitor cells originating within the adreno-gonadal primordium and other sources within the mesonephros, neural crest cells, coelomic epithelium [reviewed in (72)]. Many transcription factors have been implicated in FLC differentiation and function [reviewed in (73, 74)]. GATA4 appears to play an integral role in both of these processes. First, XY *Gata4*$^{-/-}$ embryonic stem (ES) cells fail contribute to FLCs in chimeric mouse embryos (75). Second, teratomas derived from XY *Gata4*$^{++}$, but not XY *Gata4*$^{-/-}$

ES cells, express steroidogenic markers when grown in gonadectomized nude mice (75). Together, these two experimental models provided the first evidence that GATA4 is likely required for the cell autonomous differentiation of FLCs. XY fetuses lacking GATA4 in testes at the time of male sex determination are undervirilized with partially descended testes but whose steroidogenic function is apparently intact (33). A major reduction in the number of cells expressing P450 side chain cleavage enzyme (CYP11A1) and 3β-hydroxysteroid dehydrogenase (3βHSD) only occurs in the testicular interstitium of mice lacking both GATA4 and GATA6 (38), suggesting that FLC differentiation and/or their steroidogenic function requires both factors. These mice also have reduced *Insl3* expression; no other *in vitro* or *in vivo* data exists, however, that would suggest the *Insl3* gene to be a direct transcriptional target for GATA factors. In this same model, steroidogenic expression observed in postnatal testes was notably increased but was accompanied by a significant decrease in circulating testosterone (38). The authors of this study further showed that the effects on postnatal testicular steroidogenesis and androgen production were not driven from FLCs or ALCs but rather from an expansion of adrenocortical-like cells residing in the testicular interstitium—thus making the direct contribution of GATA4/6 to Leydig cell gene expression and function in this model difficult to assess.

Independent groups have postulated that GATA4 functions as a master regulator of Leydig cell steroidogenesis based on data generated in steroidogenic cell lines (34, 36, 76). Blockade of *Gata4* expression in Leydig cell lines leads to a suppression of the steroidogenic program and a decrease in hormone production (34, 76). Loss of both GATA4 and GATA6 in primary Leydig cells (77), or disruption of their transcriptional activities in mice (36), has also been shown to suppress steroidogenesis by reducing the expression of several genes involved in androgen biosynthesis, including steroidogenic acute regulatory protein (*Star*), *Cyp11a1*, 3β-hydroxysteroid dehydrogenase 1 (*Hsd3b1*), 17α-hydroxylase (*Cyp17a1*), and 17β-hydroxysteroid dehydrogenase 3 (*Hsd17b3*)—many of these genes have conserved GATA motifs in their *cis*-regulatory regions [reviewed in (4, 18)]. Since some of these genes are also reduced in fetal testes lacking GATA factors but only when the *Gata4* and *Gata6* genes are simultaneously deleted, both factors likely compensate for one another in regulating FLC gene expression. Transcription of several other Leydig cell-expressed genes have been proposed to be targeted by either GATA4 and/or GATA6 [reviewed in (4, 18)]. The GATA-dependence of these additional targets has been mostly limited to characterization of their *cis*-regulatory regions. Therefore, their validation as genuine *in vivo* and/or direct targets of GATA factors remains to be determined.

REGULATION OF GATA FACTORS IN THE FETAL TESTIS

The regulation of *GATA* gene expression and GATA transcriptional activity in the fetal testis remains poorly understood. The transcriptional regulation of the *Gata4* gene was first reported in 2006 (78). In that initial study, activity of the first 124 bp upstream of the mouse *Gata4* transcriptional start site in Leydig and Sertoli cell lines was shown to be dependent on conserved GC-rich and E-box elements (78). Similar findings highlighting the importance of GC-rich and E-box elements were later reported in context of 5 kb of the rat *Gata4* promoter (25). The same 5 kb of rat *Gata4* promoter was also shown to be sufficient to drive GFP expression in the male gonad of transgenic mice from the onset of testis differentiation to adulthood (25). Subsequent analysis showed high transgene expression in both Sertoli and Leydig cells, and at all times (both fetal and mature testis) (79). Targeted mutagenesis of the *Gata4* E-box element in the mouse confirmed the importance of this motif for endogenous *Gata4* expression in both fetal and adult testis (80). Like other *GATA* genes, the rodent and human *GATA4* genes are expressed as multiple transcripts that differ in their 5' untranslated exons, which are driven by alternative promoters (81). Thus, in addition to the typical *Gata4* transcript driven by the E-box dependent proximal *Gata4* promoter, the fetal testis also expresses a second *Gata4* transcript driven by an alternative promoter sequence located nearly 30 kb upstream of the proximal promoter (81, 82). The distal *Gata4* promoter appears to be autoregulated by GATA4 itself in a regulatory mechanism proposed to ensure sufficient *Gata4* expression in the testis at critical times such as during testis differentiation (82). Characterization of the *cis*-regulatory regions required for *Gata6* expression in the testis have not been reported.

Despite their profound roles in testis physiology, the hormonal regulation of GATA factors in the testis have received comparably little attention. Nonetheless, treatment of Sertoli and Leydig cell lines with gonadotropins causes a modest increase in *Gata4* expression (29, 83). Gonadotropins and androgens, however, do not appear to be required for basal *Gata4* or *Gata6* expression in the testis as their expression is retained in different models that perturb these hormones (29). In contrast to the paucity of information on hormones that regulate GATA factors in the testis, much more is known about the signaling pathways activated following hormone stimulation and their impacts on the transcriptional activity of GATA factors, especially GATA4. Two GATA4 phosphorylation sites have been well described: serine 105 (S105) targeted by mitogen-activated protein kinases (MAPK) and serine 261 (S261) targeted by protein kinase A (PKA) (84–87). In the testis, GATA4 phosphorylation by PKA is particularly informative since it helps to better understand the acute gonadotropic regulation of multiple testis-expressed genes that lack classic cAMP response elements (CREs), but contain GATA regulatory motifs, in their respective promoters. For both S105 and S261, *in vitro* studies have shown that GATA4 phosphorylation stimulates GATA4 transcriptional activity on multiple testis target genes (87, 88). GATA4 S105 phosphorylation by MAPK has also been proposed to be essential for GATA4 action in the upregulation of *Sry* expression during testis differentiation (89, 90). A study published in 2019, addressed the *in vivo* role of GATA4 phosphorylation through the detailed characterization of two mouse models (GATA4 S105A and GATA4 S261A) that carry mutations that specifically block GATA4 phosphorylation at either site. One of these mutations (S105A) was associated

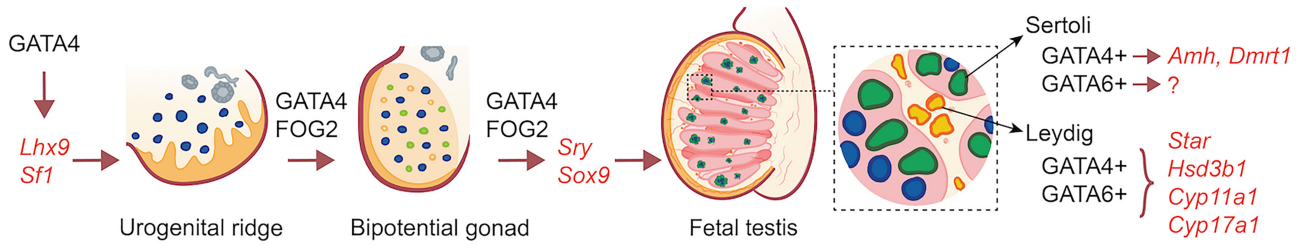

FIGURE 1 | Overview of the action of GATA factors in XY gonad development and fetal testis gene expression.

with a male hormonal defect characterized by a deficiency in testosterone production in adult but not fetal males (36). Interestingly, male sex determination was unaffected in GATA4 S105A mutants (36), which questions the requirement for GATA4 phosphorylation (at least on S105) in the testis differentiation process.

GATA FACTORS IN HUMAN TESTIS AND TESTICULAR PATHOLOGIES

Several studies have investigated the expression and potential role of GATA factors in normal human testis development and function, as well as the pathogenesis of testicular tumors [(91–94) and reviewed in (95)]. Much like rodents and other mammalian species, GATA4 and GATA6 appear to be the predominant GATA factors of the human fetal testis. In Sertoli cells, GATA4 is present during both fetal and postnatal development (91). *GATA4* is also expressed in fetal and postpubertal Leydig cells which correlates with its proposed role in steroidogenesis (91). Interestingly, *GATA4* expression is significantly higher in both Sertoli and Leydig cell tumors (91), suggesting that GATA4 might influence cell proliferation during tumorigenesis in human testicular somatic cells. Unlike rodents, GATA4 is a marker of a subset of early fetal gonocytes (91), as well as multiple testicular germ cell neoplasia that include both precursor carcinoma *in situ* (CIS) testis cells and definite testicular germ cell tumors (seminomas, yolk sac tumors, teratomas) (93). Testicular seminomas and yolk sac tumors also express *GATA6* (93). The presence of GATA4 and/or GATA6 in these cells has suggested a role for these factors in early human germ cell differentiation. *GATA6* is also expressed in normal human testis, at least during fetal development. GATA6 in fetal human testis is most abundant in Sertoli and Leydig cells (92), where it may have overlapping roles with GATA4 if one transposes the comparable rodent data to humans (38).

Several mutations in either the *GATA4* or *FOG2/ZFPM2* genes have been identified in human cases of differences of sexual development (DSD) (96–100). While most of the reported *GATA4* and *FOG2/ZFPM2* variants appear to have a benign impact on XY DSDs (98), at least three FOG2/ZFPM2 (p.S402R; p.R260Q; p.R260Q/M544I) (97), and two *GATA4* missense variants (p.G221R; p.W228C) (96, 98), are considered to be pathogenic with reduced transcriptional activities on the human *AMH* promoter, a known GATA4 target. The fact that most of the reported variants are heterozygous in nature suggests that correct functional dosage of these transcription factors is critical for typical XY sex differentiation in humans. In support of this, haploinsufficiency of either *Gata4* or *ZFPm2/Fog2* is also known to induce gonadal sex-reversal on specific genetic backgrounds in mice (43).

CONCLUSION

GATA factors are incontrovertibly essential regulators of developmental and functional processes in multiple organs systems. The mammalian testis is no exception where at least three GATA factors (1/4/6) play essential, and sometimes overlapping roles, in controlling the initiation of male gonad development, testis differentiation at the time of sex differentiation, and gene expression and function in both the fetal and mature testis (an illustrative overview in presented in **Figure 1**). Over the years, much of our knowledge of the mechanisms of GATA action in the testis have come from genetic manipulation of the different *Gata* genes in mice and the analysis of the *cis*-regulatory regions of many of its proposed target genes in cell lines. From this point forward, the challenge will be to fine-tune this knowledge base by ascertaining not only what testis genes are regulated by GATA factors but more so, by identifying the genes that are directly targeted by these factors. The recent development and applicability of new technologies has made this a reality as we are now capable of probing the role of individual *cis*-regulatory elements in suspected genes by genome editing. Therefore, understanding GATA function in the testis is far from complete, but rather has just begun.

AUTHOR CONTRIBUTIONS

RV and JT drafted and edited the final version of the manuscript. KM prepared the illustration and participated in the final editing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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AMH Regulation by Steroids in the Mammalian Testis: Underlying Mechanisms and Clinical Implications

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Anti-Müllerian hormone (AMH) is a distinctive biomarker of the immature Sertoli cell. AMH expression, triggered by specific transcription factors upon fetal Sertoli cells differentiation independently of gonadotropins or sex steroids, drives Müllerian duct regression in the male, preventing the development of the uterus and Fallopian tubes. AMH continues to be highly expressed by Sertoli until the onset of puberty, when it is downregulated to low adult levels. FSH increases testicular AMH output by promoting immature Sertoli cell proliferation and individual cell expression. AMH secretion also showcases a differential regulation exerted by intratesticular levels of androgens and estrogens. In the fetus and the newborn, Sertoli cells do not express the androgen receptor, and the high androgen concentrations do not affect AMH expression. Conversely, estrogens can stimulate AMH production because estrogen receptors are present in Sertoli cells and aromatase is stimulated by FSH. During childhood, sex steroids levels are very low and do not play a physiological role on AMH production. However, hyperestrogenic states upregulate AMH expression. During puberty, testosterone inhibition of AMH expression overrides stimulation by estrogens and FSH. The direct effects of sex steroids on AMH transcription are mediated by androgen receptor and estrogen receptor α action on *AMH* promoter sequences. A modest estrogen action is also mediated by the membrane G-coupled estrogen receptor GPER. The understanding of these complex regulatory mechanisms helps in the interpretation of serum AMH levels found in physiological or pathological conditions, which underscores the importance of serum AMH as a biomarker of intratesticular steroid concentrations.

Keywords: androgen insensitivity, androgen response element, estradiol, estrogen response element, dihydrotestosterone, ketoconazole, testotoxicosis, triptorelin

1 INTRODUCTION

Anti-Müllerian hormone (AMH) is a glycoprotein hormone (1) that belongs to the transforming growth factor beta (TGFβ) superfamily (2). It is produced essentially by Sertoli cells of the testis and granulosa cells of the ovary: after castration, AMH is no longer detected in serum (3, 4). AMH is synthesized as a homodimeric precursor consisting of two identical polypeptide chains, with a large N-terminal pro-region of 110-kDa and a small C-terminal mature domain of 25-kDa. AMH is subjected to post-translational proteolytic processing (5); the resulting N-terminal and C-terminal dimers remain associated in a non-covalent complex that is biologically active (6, 7). The pro-region is displaced from the non-covalent complex upon binding to an AMH receptor homodimer (8). AMH transduces its signal through a specific type II receptor, AMHR2, that is expressed at the cell surface in target organs (9) and interacts with the non-specific type I receptors ACVR1 (initially called ALK2), BMPR1A (ALK3) or BMPR1B (ALK6) (reviewed in (10). The most widely recognized action of AMH takes place during male fetal development, where it provokes the regression of the Müllerian ducts –the anlagen of the Fallopian tubes, the uterus and the upper part of the vagina (reviewed in (11).

The human *AMH* gene is located on chromosome 19p13.3 (12) and consists of 5 exons spanning 2.8 kb (2). The 3' end of the 5th exon encodes the biologically active C-terminal domain of the protein. In the testis, the major transcription initiation site is located 10 bp upstream of the ATG codon (13). Most of the best characterized binding sites for transactivating factors with a relevant role in fetal life lie within the proximal 500 bp (**Figure 1**) (reviewed in (15), but other response elements for factors involved in AMH regulation in postnatal life are present in the distal promoter (16). Indeed, distal promoter sequences are necessary for the maintenance of AMH expression in the testis during postnatal life (17). While no canonical androgen response elements (AREs) have been described (18), a consensus sequence for estrogen receptor binding (ERE) is present at -1782 bp in the human *AMH* promoter (13, 19).

1.1 The Ontogeny of AMH Expression in the Testis

AMH is one of the earliest cell-specific proteins produced by Sertoli cells when the testis differentiates from the gonadal ridge

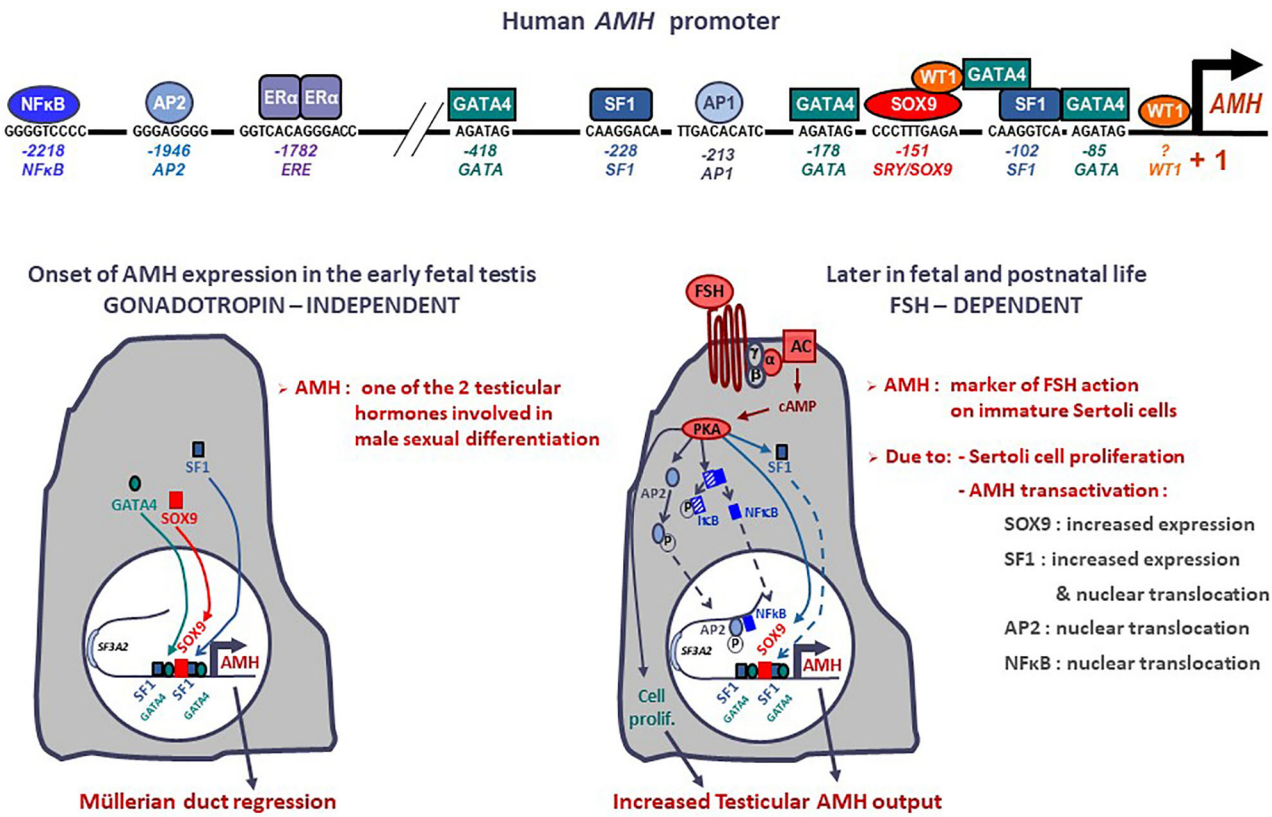

FIGURE 1 | Onset and upregulation of *AMH* expression. Transcription factors SOX9, SF1, GATA4 and WT1 bind to specific response elements in the proximal *AMH* promoter and trigger *AMH* expression in early fetal life, independently of gonadotropin or steroid action, resulting in Müllerian duct regression. From the second trimester of gestation, FSH increases testicular AMH production by 2 different mechanisms: it promotes cell proliferation and upregulates *AMH* transcription *via* the transduction pathway involving the heterotrimeric (α/β/γ) G protein, adenylyl cyclase (AC), cyclic AMP and protein kinase A (PKA) resulting in increased expression and nuclear translocation of transcription factors SOX9, SF1, AP2 and NFκB. Modified with permission from Grinspon and Rey (14), © 2010 S. Karger AG.

in the XY embryo (20). AMH starts to be expressed at 12.5 days post-coitum in the mouse (21) and from the 8th week onwards in the human (22). Although its role in sex differentiation of the internal ducts takes place in the first phases of fetal development (see below), AMH continues to be produced by Sertoli cells at very high levels during the whole fetal life and, postnatally, until the onset of puberty (**Figure 2**). During pubertal development, Sertoli cells progressively express less AMH (25), and the directional secretion switches from the basal to the adluminal compartment (26). Consequently, AMH concentration in serum is high during childhood but decreases during puberty, when it increases in seminal plasma [reviewed in ref (27)]. The decrease in AMH expression coincides with the establishment of the blood-testis barrier and the onset of germ cell meiosis (**Figure 3**) (28–30), which are androgen-dependent processes. During adulthood, serum AMH is approximately ten- to twentyfold lower as compared to the prepubertal period in males (24), but still twofold higher than in females (31).

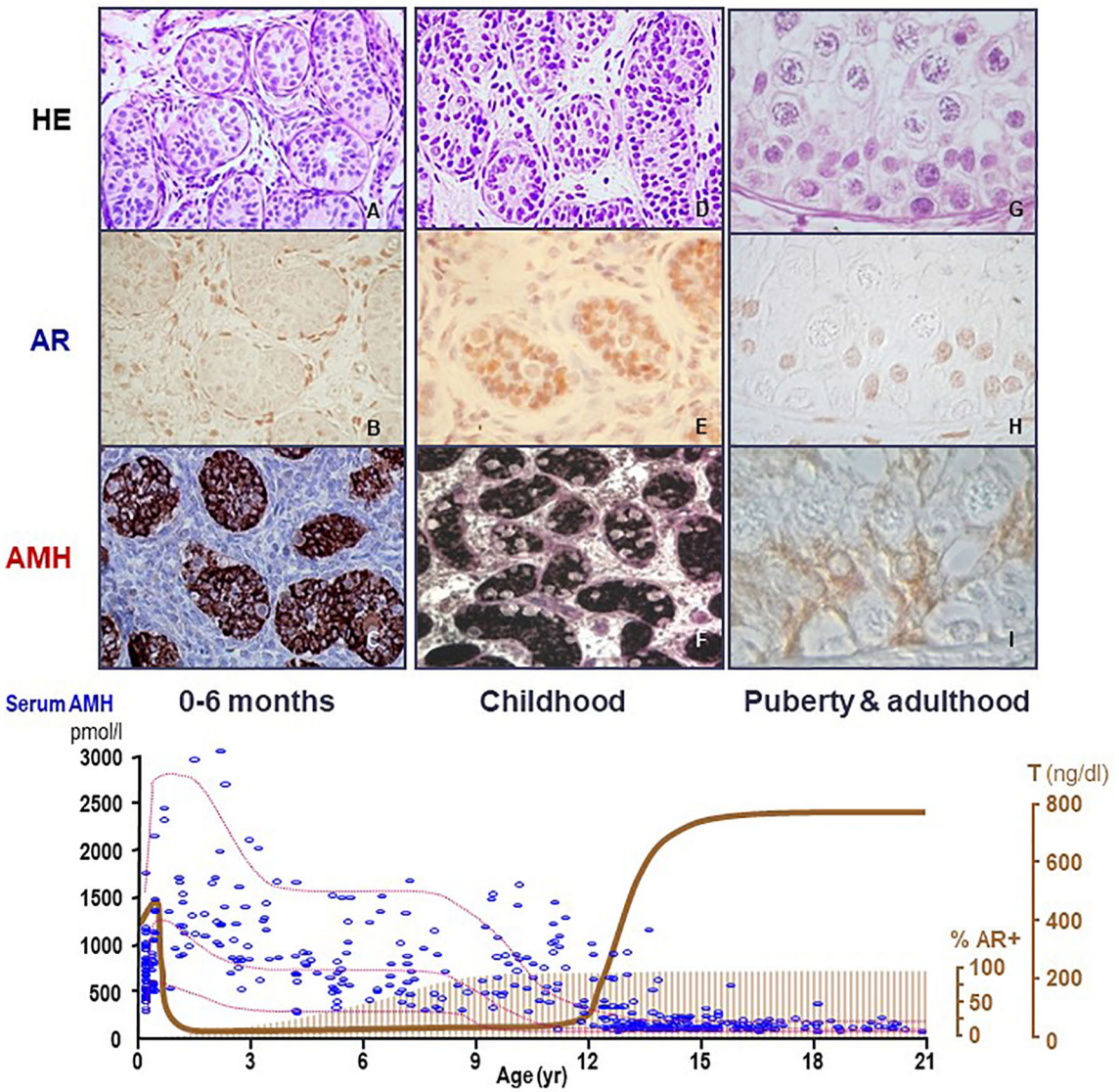

FIGURE 2 | Relationship between AMH production, androgen receptor (AR) expression and androgen levels in the human testis. During the postnatal period of activation of the hypothalamic-pituitary-testicular axis (0-6 months, first column, **A–C**), testosterone (T) levels are high (bottom graph, serum hormone levels); however, Sertoli cells do not show maturational changes because they do not express the AR (upper graph, second line, immunohistochemistry). Therefore, AMH expression is high (upper graph, third line, AMH immunohistochemistry) and germ cells do not enter meiosis (upper graph, first line, HE: hematoxylin-eosin staining). During the "quiescent" period of the gonadal axis (childhood, second column, **D–F**), androgen synthesis is negligible, which explains why Sertoli cells remain immature even though they have started to express the AR. In puberty and adulthood (third column, **G–I**), T increases, inducing Sertoli cell maturation, reflected in the decline of AMH expression and the onset of adult spermatogenesis. Bottom graph shows % AR+: percentage of Sertoli cells expressing the AR. AMH (pmol/l) and T (ng/dl): schematic representation of AMH and T serum levels from birth to adulthood in males.

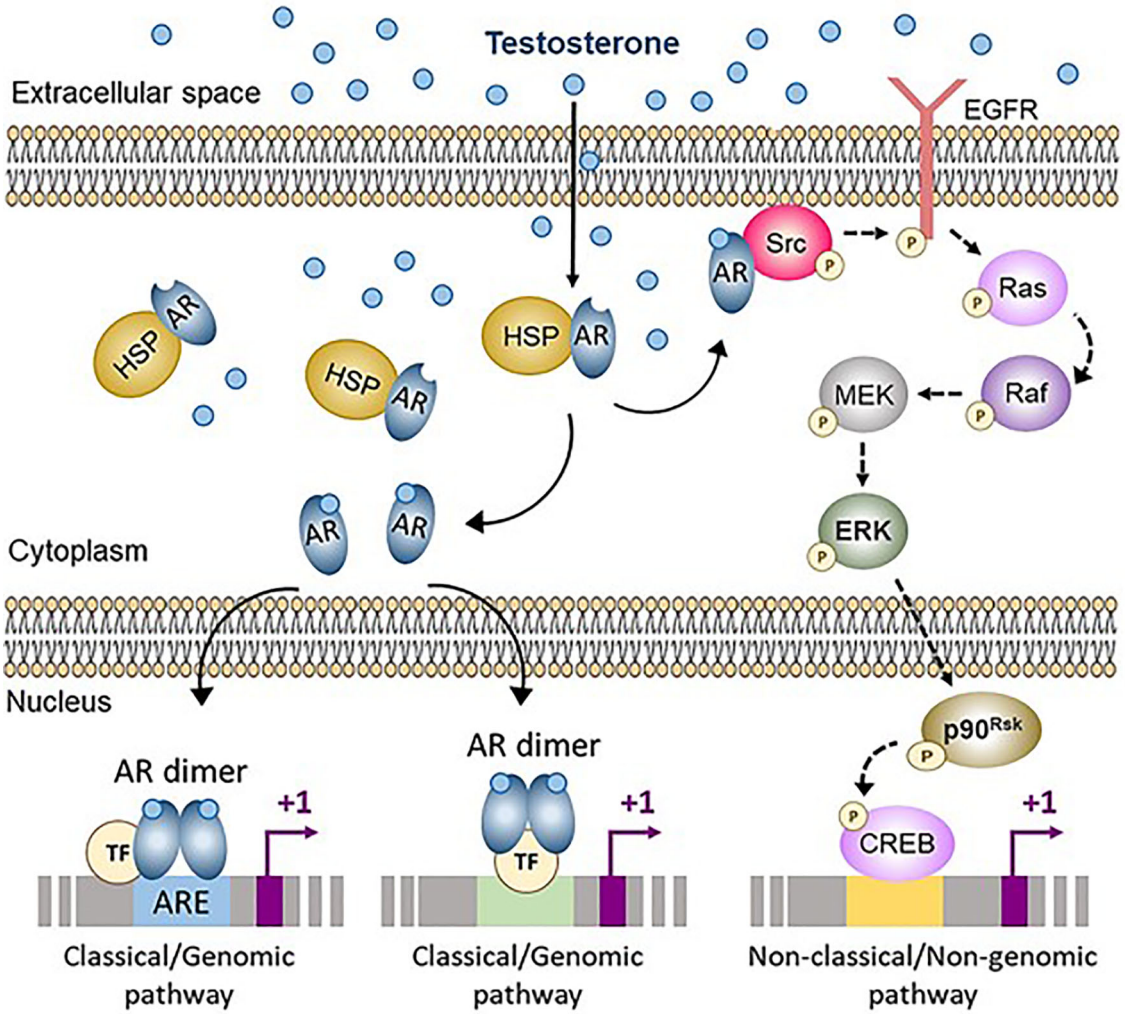

FIGURE 3 | Classical (genomic) and non-classical (non-genomic) androgen signaling in Sertoli cells. In cells not exposed to androgens, the androgen receptor (AR) is bound to heat-shock proteins (HSP) in the cytoplasm. Testosterone and other androgens are steroids that easily cross the cell membrane and bind to the AR, that is released from HSP. The ligand-bound AR can either translocate to the nucleus and interact with androgen response elements (ARE) or with other transcription factors (TF), thus activating the classical/genomic pathway, or migrate to the inner side of the cell membrane and interact with Src, thus activating the non-classical/non-genomic pathway. CREB, cAMP response element binding protein; EGFR, Epidermal growth factor receptor; ERK, Extracellular signal-regulated kinase; MEK, Mitogen-activated protein kinase; Src, Steroid receptor coactivator. Reproduced with permission from Edelsztein and Rey (18)

1.2 Physiological Role of AMH in Male Development

In mammals, the earliest stages of intrauterine development are characterized by the existence of a sexual undifferentiated period where no differences can be observed, except at the chromosomal level, between XX and XY embryos. The gonadal ridges and the precursors of the external genitalia are identical in both sexes, and there are two sets of internal ducts: Wolffian ducts that give rise to the male internal genitalia, and the Müllerian ducts that differentiate into the female internal genitalia. The pioneering work by Alfred Jost (32) demonstrated the existence of a Mullerian inhibiting activity, specific to the fetal testis, explaining why the female internal organs do not develop in the male fetus. In the absence of AMH action, e.g. in individuals with mutations of the genes coding for AMH or its specific receptor AMHR2, the Fallopian tubes and the uterus are present [reviewed in ref (15)]. The absence of AMH action is also evidenced in 46,XY disorders of sex development (DSD) due to complete gonadal dysgenesis [reviewed in ref (33)]. Interestingly, the window of action of AMH on Müllerian ducts is limited to the earliest stages of intrauterine life (34); this explains why the development of internal genitalia is not disrupted by ovarian AMH production that starts when Müllerian ducts are already insensitive to its action (35). The fact that AMH continues to be secreted by the testis after the critical window of Müllerian duct regression and that it is

produced by the ovaries at clearly detectable levels suggest that AMH may have other physiological roles. Nonetheless, individuals with *AMH* or *AMHR2* null mutations resulting in complete loss of AMH expression or action do not show other evident phenotypes [reviewed in ref (11)].

1.3 AMH as a Biomarker in Physiological and Pathological States

Independently of its physiological role(s), AMH is a proven biomarker of the immature Sertoli cell population in normal and pathological conditions [reviewed in ref (36–39)]. Serum AMH reflects the mass of immature Sertoli cells, i.e. of functional testicular tissue, from fetal life to adulthood. In the fetus (22), newborn and child (40), detectable AMH in serum indicates the existence of testes, which is particularly useful for the differential diagnosis between bilateral cryptorchidism and anorchidism in boys with nonpalpable gonads. In patients with DSD, serum AMH levels are commensurate with the amount of testicular tissue, except for those with a Persistent Müllerian duct syndrome due to *AMH* mutations (41). Undetectable AMH is indicative of no testicular development in 46,XY girls with complete gonadal dysgenesis. Circulating levels are below the normal male range in patients with 46,XY DSD due to partial gonadal dysgenesis –where both Sertoli and Leydig cell development is impaired– but normal or high in 46,XY DSD due to isolated steroidogenic defects –where only Leydig cell function is affected– or to androgen insensitivity or 5α-reductase deficiency –where both Leydig and Sertoli cell development is preserved (33). In patients with 46,XX DSD, serum AMH above the female range indicates the existence of functional testicular tissue suggesting the diagnosis of ovotesticular or testicular DSD, in contrast with other forms of XX virilization, such as aromatase deficiency, congenital adrenal hyperplasia of androgen-secreting tumors, which present with serum AMH in the female range (42). In normally virilized boys, low serum AMH is seen in patients with primary (24, 43, 44) or central hypogonadism (45). Conversely, high AMH is suggestive of Sertoli cell tumors (46), excessive signaling downstream the FSH receptor pathway like in McCune-Albright syndrome (47), or hyperestrogenism (19). During pubertal development, the decline in serum AMH levels is an early sign of Sertoli cell maturation (48), while an excessive decrease to undetectable levels is typical of Klinefelter syndrome (49). Conversely, the persistence of elevated AMH levels despite an increase in testosterone production is indicative of androgen insensitivity (50).

2 STEROID-INDEPENDENT REGULATION OF TESTICULAR AMH EXPRESSION

2.1 AMH Expression During Early Fetal Development

The onset of AMH expression in the fetal testis is independent of gonadotrophin or steroid regulation (**Figure 1**). SOX9, upregulated by SRY, triggers *AMH* expression by binding to a specific element on the proximal *AMH* promoter (51). Subsequently SF1 (51–53), GATA4 (54) and WT1 (55) cooperate to further upregulate *AMH* transcription whereas DAX1 represses the transcriptional cooperation between SF1 and GATA4 (56).

As mentioned, although its action takes place in the early stages of fetal sex differentiation, AMH continues to be produced by Sertoli cells throughout life (**Figure 2**). The proximal *AMH* promoter plays a major role in the initiation of fetal expression, when AMH induces the regression of Müllerian ducts, but it proves insufficient to maintain AMH expression thereafter, when sequences farthest from the transcription start site are required (17).

2.2 AMH Regulation by FSH in Late Fetal and Postnatal Life

From the second half of gestation, FSH increases testicular AMH output through two different mechanisms (**Figure 1**): it induces Sertoli cell proliferation and upregulates AMH expression at the individual cell level (16, 30). The continuous effect of high FSH levels on Sertoli cells for almost one year (the last 6 months of fetal life plus the 3-6 months of postnatal life induces a progressive increase in serum AMH (57), which peaks at 5-6 months of age (58). This explains why serum AMH levels are low, in coincidence with small testicular size, in boys with congenital central (hypogonadotrophic) hypogonadism (45, 59) and increase after FSH treatment (60, 61). The underlying molecular mechanisms include the classical cyclic AMP-dependent pathway triggered by the FSH receptor coupled to the Gαs protein (16), which activates SOX9 (62) and SF1 (62) acting on the proximal *AMH* promoter, but also NFκB and AP2, which bind to response elements lying approximately 2 kb upstream of the *AMH* start site (62). These molecular mechanisms explain the elevated AMH production observed in boys with McCune-Albright syndrome carrying a gain-of-function somatic mutation in the *GNAS1* gene encoding the Gαs protein (47, 63).

2.3 Lack of Androgen-Mediated Regulation of Testicular AMH Expression in Fetal and Early Postnatal Life

The fetal period and the stage of postnatal activation of the hypothalamic-pituitary-testicular axis occurring during the first 3 to 6 months after birth, usually referred to as "mini-puberty", are characterized by the coexistence of high testosterone levels and AMH expression in the testes (**Figure 2**). The continuous exposure to high androgen levels for approximately one year neither affects AMH expression nor induces histologic maturational changes in Sertoli cells or spermatogenic onset. Immunohistochemical studies have clarified why: Sertoli cells do not express the AR and, therefore, they are physiologically insensitive to androgens until the end of the first year of life in humans (**Figure 2**). Subsequently during infancy and childhood, AR expression increases progressively in Sertoli cells until full expression is observed by the age of approximately 8 years (64–66). An equivalent ontogeny can be observed in rodents (30, 67). Interestingly, boys with high intratesticular androgen

concentration due to precocious puberty show normal serum AMH during the first year of life but declining levels thereafter, when the AR starts to be expressed in Sertoli cells (68).

3 REGULATION OF TESTICULAR AMH PRODUCTION BY ANDROGENS

3.1 Downregulation of Testicular AMH Expression at Puberty

Testosterone is a well-known inducer of Sertoli cell maturation during puberty (69). During childhood, the pituitary-gonadal axis is mostly quiescent: LH levels are very low or undetectable and typical Leydig cells are absent in the testis interstitial tissue. Therefore, although Sertoli cells already express the androgen receptor (AR), they are not exposed to androgens (**Figure 2**). When the axis reactivates at the beginning of pubertal development, LH induces the differentiation of Leydig cells that start producing testosterone. Androgen concentration increases rapidly within the testis, even though this is not reflected in the circulating levels until more advanced pubertal stages. Intratesticular testosterone triggers a number of morphologic and functional changes in Sertoli cells [reviewed in ref (18)]. One distinctive change is the decrease in AMH production that occurs as one of the first clinical signs of pubertal development in boys (48), as well as in numerous other mammals (3, 25, 70, 71), in coincidence with the onset of germ cell meiosis (50). Results obtained in experimental mouse models support these observations (30). In the naturally occurring *Tfm* mice, carrying a functionally impaired AR (30), and in mice with an artificially mutated *Ar* gene (72), AMH expression does not wain at the expected age of puberty.

3.1.1 Molecular Mechanisms

Androgen signaling in target cells typically occurs through the AR engaging either the classical (or genomic) or the non-classical (or non-genomic) pathways (**Figure 3**) (73). The classical pathway involves the intracellular AR acting as a transcription factor. In the inactive state, the AR is bound to cytoplasmic heat-shock proteins. When testosterone or the more potent and non-aromatizable androgen dihydrotestosterone (DHT) binds to the AR, a conformational change leads to the release of the AR from the heat-shock proteins, AR phosphorylation, homodimer formation, nuclear translocation and interaction with androgen response elements (ARE), specific DNA sequences in the regulatory regions of target genes (18). Typical ARE are palindromic: in humans, the consensus sequence is 5'-AGAACAnnnTGTTCT-3' (74). The androgen-bound AR can also regulate target gene expression in the absence of ARE, by interacting with trans-activating factors that bind to their specific binding sequences (75). Androgen signaling through the genomic pathway requires 30-45 minutes to induce transcriptional regulation (76).

Non-classical pathways induce cellular changes more rapidly, within seconds to minutes (**Figure 3**). Androgen action leads the AR to localize near the plasma membrane, where it induces Src. This tyrosine kinase phosphorylates the epidermal growth factor receptor, which triggers the MAP kinase cascade ultimately resulting in the phosphorylation of specific transcription factors (73). Alternatively, the Zn^{2+} transporter ZIP9 has been proposed as a membrane AR that induces the phosphorylation of Erk1/2 and the transcription factors CREB and ATF1 (77).

Traditionally, maturation changes induced by androgens were believed to result from upregulation of target genes. However, high-throughput techniques have revealed similar amounts of up-regulated and down-regulated genes in response to androgen action in the maturing testis (78). However, the pathways underlying androgen-mediated downregulation have been barely explored. Using the mouse prepubertal Sertoli cell line SMAT1 (79), we have recently clarified the molecular mechanisms involved in AMH downregulation during puberty (80). The decrease in AMH production is a direct effect of androgens on Sertoli cells, not involving negative feedback on pituitary gonadotrophins or down-regulation of activating transcription factors. Indeed, FSH and testosterone have opposing effects on AMH expression at puberty, with the inhibitory effect of androgens largely exceeding the stimulatory effect of FSH. In SMAT1 cells, both testosterone and the nonaromatizable androgen DHT decrease the activity of a 3-kb human *AMH* promoter in the presence, but not in the absence, of the AR (**Figures 4A, B**), and this inhibition is prevented by the antiandrogen bicalutamide. Using human *AMH* promoters of different lengths in luciferase assays, we demonstrated that androgen-mediated downregulation involved the promoter sequences lying within the proximal 430 bp (**Figure 4A**). No canonical ARE can be found in the *AMH* promoter, and intact SF1 response elements are required for the negative regulation by androgens to occur (**Figure 4B**). This inhibitory effect on *AMH* expression could be mediated by a direct interaction between the ligand-bound AR and the SF1 elements, i.e. blockage by competition, or alternatively due to a protein-protein interaction between the ligand-bound AR and promoter-bound SF1, i.e. blockage by interaction. In any case, the AR prevents SF1 from upregulating *AMH* promoter activity (**Figures 4C, D**).

3.1.2 Clinical Implications

Clinical studies have shown that AMH decline is not merely associated with chronological age but rather with the maturation status of the testes. Indeed, a decrease in serum AMH is an early sign of an increase in intratesticular testosterone concentration reflecting the activation of LH pulses occurring with pubertal onset. The decrease in serum AMH occurs already in Tanner 2 stage of puberty in boys, that is earlier than the increase in serum testosterone, taking place in Tanner 3 stage (24, 48, 81, 82). Furthermore, AMH is low for age in boys aged 2 to 8 years with precocious puberty, independently of gonadotrophin levels, and recovers prepubertal levels when testosterone production is effectively curtailed by treatment (**Figures 5A, B**) (48, 68). Serum AMH can be used as a marker of effective treatment. In fact, for Sertoli cells to resume their prepubertal status and increase AMH production, intratesticular androgen levels should remain continuously low for at least 6 months. If adherence to treatment is erratic and intratesticular

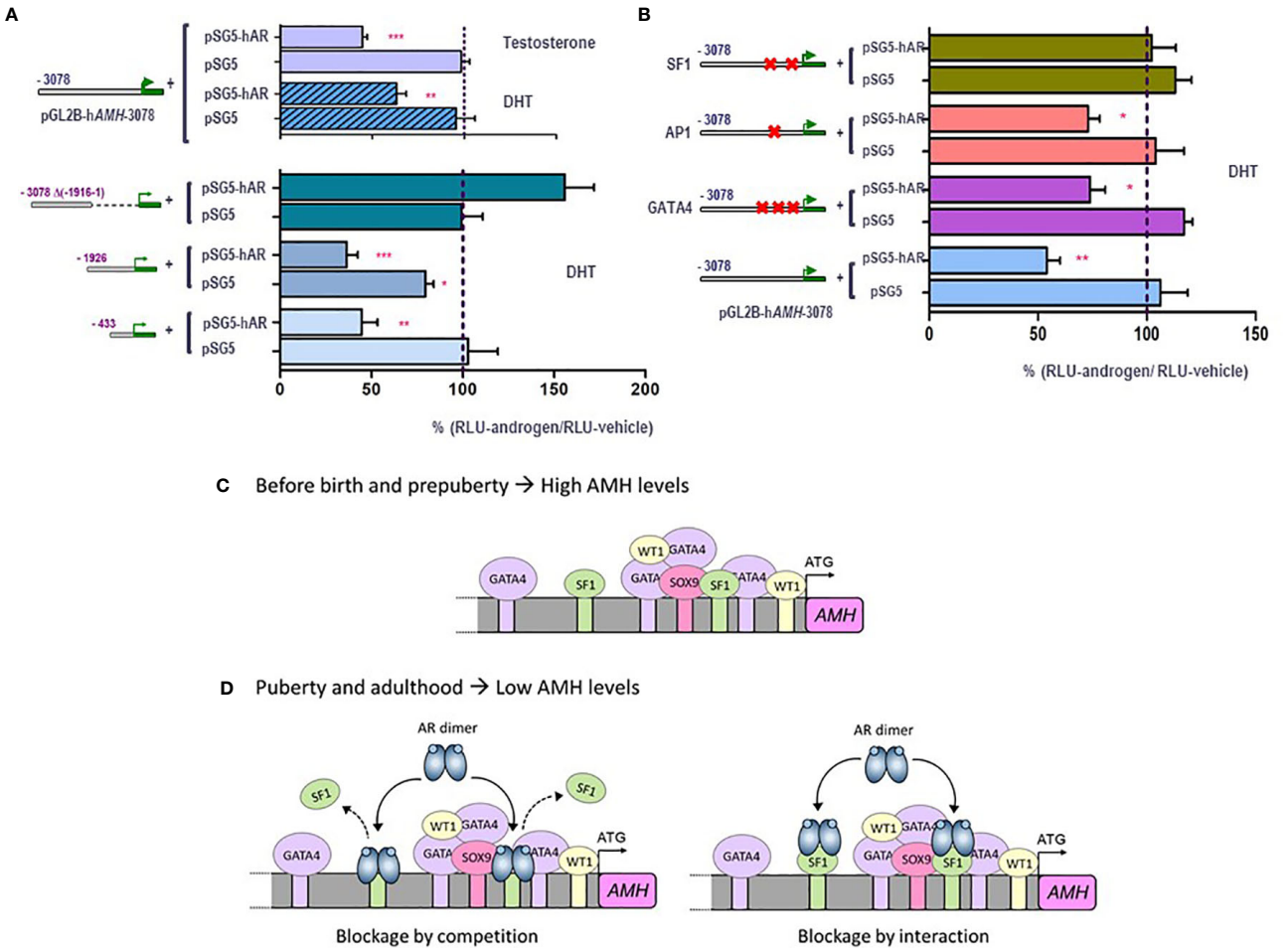

FIGURE 4 | Molecular mechanisms involved in androgen-induced downregulation of *AMH* expression in Sertoli cells. A and B: luciferase assays in SMAT1 Sertoli cells transfected with a luciferase plasmid under control of the human *AMH* promoter (pGL2B-hAMH) of different lengths and co-transfected with an expression plasmid of the androgen receptor (pSG5-AR) or pSG5 devoid of the AR. Cells were exposed to testosterone or DHT 10^{-7} M, or vehicle, and results were expressed as relative luciferase units (RLU) comparing cells exposed to androgens and those exposed to vehicle (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). A 100% level indicates the basal *AMH* promoter activity. Testosterone and DHT induce an inhibition of *AMH* promoter activity when the AR is present but not in its absence, when 433 to 3076 bp of the promoter are present but not when the proximal 1916-bp sequences are lacking **(A)**. The inhibition induced by androgen persists even if the binding sites for AP1 or GATA4 are mutated, but not when SF1 sites are mutated **(B)**. **(C, D)** schematic of androgen regulation of AMH expression. In fetal and postnatal periods before the onset of puberty, the lack of androgen action allows the *AMH* promoter activity induced by SOX9, SF1 and GATA4, resulting in high AMH production. During puberty and adulthood, the steroid-bound AR represses *AMH* promoter activity by competition or by interaction with SF1. Modified with permission from Edelsztein et al. (80) © 2018 The Authors and Edelsztein and Rey (18) © 2019 The Authors.

testosterone concentration shows intermittent variations, serum AMH never increases to prepubertal levels (**Figure 5C**) (48).

On the other hand, testicular AMH production remains high in adolescents and adults with androgen insensitivity due to mutations in the *AR* gene (50, 83–85). The increase in AMH expression has been ascribed to SOX9 upregulation in Sertoli cells (86).

4 REGULATION OF TESTICULAR AMH PRODUCTION BY ESTROGENS

Like for androgens, the testis is both a source and a target organ of estrogens, synthesized from androgens by the cytochrome P450 enzyme aromatase encoded *CYP19A1*. Aromatase expression is present in the postnatal testis (64) and is stimulated by FSH action on Sertoli cells (87). Estrogen signaling involves 3 different receptors. The canonical nuclear estrogen receptors α (ERα) and β (ERβ) act through their binding capacity on DNA sequences known as estrogen response elements (ERE). ERα and ERβ show a 97% identity in their DNA-binding domains, while their ligand-binding domains have an identity of 60%. Both ERs bind to estrogens with a similar affinity (88). The membrane ER, named GPR30, GPER1 or simply GPER, is a seven-transmembrane domain, G protein-coupled receptor, predominantly present in the endoplasmic reticulum, that mediates rapid cellular responses involving second messengers, ion channels and kinase activities (89).

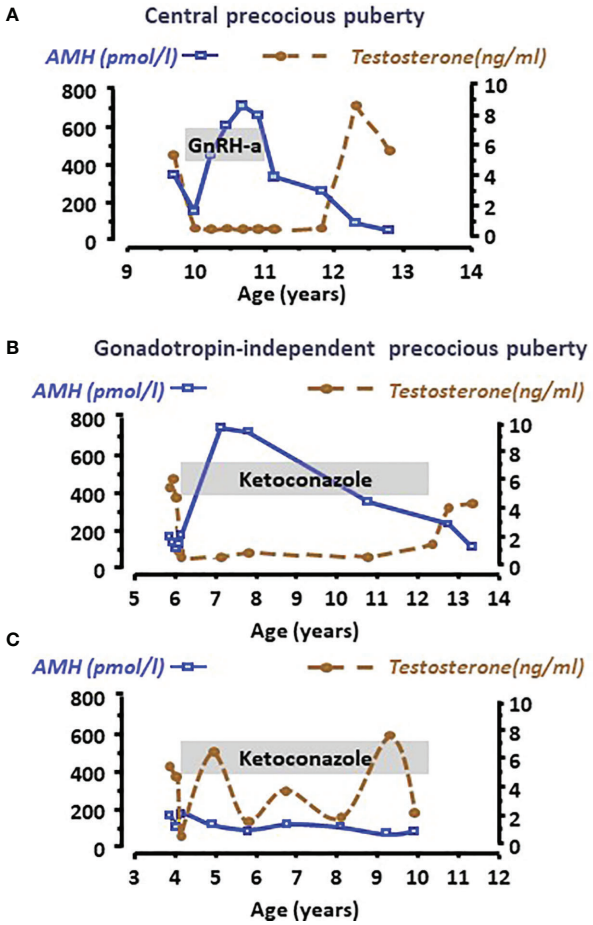

FIGURE 5 | Serum AMH as a biomarker of intratesticular androgen concentration in boys with central precocious puberty **(A)** or with gonadotropin-independent precocious puberty **(B, C)**. Serum AMH is low at diagnosis, indicating the inhibitory effect of high androgen concentration reflected in high serum testosterone levels in all cases. When testosterone production is effectively curtailed by the adequate treatment, such as a GnRH analogue in the patient with central precocious puberty **(A)** or ketoconazole in the patient with gonadotropin-independent precocious puberty **(B)**, Sertoli cells recover their immature status and increase AMH production until treatment is discontinued. Conversely, when adherence to treatment is erratic **(C)**, intratesticular and serum testosterone concentration does not remain at prepubertal levels and Sertoli cells do not revert their pubertal status, which is reflected in low serum AMH. Modified with permission from Rey et al. (48) © 1993 The Endocrine Society.

The 3 ERs are expressed in the testis, but there are differences in the timing and cellular types according to species (90). Sertoli cells of the prepubertal and pubertal human testis express ERα (19) and ERβ (19, 64). In rodents, ERα expression predominates in the prepubertal testis while ERβ is more abundant in the adult (91). GPER has been identified in Sertoli cells from the onset of puberty (92).

4.1 Upregulation of Testicular AMH Expression in Postnatal Life

At the onset of puberty, testicular AMH expression increases concomitantly with FSH and estradiol (E2) levels in normal boys (93). This simultaneous elevation in the 3 hormones is also observed in patients with androgen insensitivity, in whom the inhibitory effect of androgens on AMH is disrupted. Patients with androgen insensitivity show increased aromatization of androgens to estrogens, resulting in the development of breasts. As already discussed, AMH upregulation can be explained by a direct action of FSH on Sertoli cell proliferation and on AMH transcription. However, AMH is also elevated in boys with Peutz-Jeghers syndrome (46), in whom Sertoli cell proliferations produce high estrogen levels leading to suppressed FSH (46). Interestingly, the human *AMH* promoter contains a half ERE (**Figure 1**) (13), and E2 regulates AMH expression in the ovary (94).

We have recently tested the hypothesis that E2 upregulates AMH expression in the prepubertal and pubertal testis, using experimental mouse models. Treatment of 4-day-old mice with ICI 182780, an antagonist of ERα and ERβ, resulted in significant decrease in serum AMH (**Figure 6A**), indicating that abolishing ER signaling results in a decreased testicular AMH production. Nuclear ERs (91) and GPER (95) are involved in Sertoli cell proliferation, which could in part explain the increase in AMH output by the testes exposed to high estrogen levels.

4.1.1 Molecular Mechanisms

The existence of an ERE on the *AMH* promoter prompted us to test whether estrogens could have a direct effect on AMH expression at the individual Sertoli cell level. Luciferase assays clearly indicated that the activity of a 3-kb human *AMH* promoter was increased after exposure to E2 of SMAT1 Sertoli cells transfected with any of the 3 ERs. However, to elicit a significant response ERβ-transfected SMAT1 needed to be incubated with E2 10^{-7} M (19), a concentration 100-fold higher than that observed in the testes of adolescents (96), while ERα- and GPER-transfected SMAT1 cells showed a maximal response to E2 at the physiological concentration of 10^{-9} M (19). These observations suggest that ERα and GPER, but not ERβ, are involved in the upregulation of AMH expression by E2. Further support was provided by experiments showing that PPT, a potent and selective ERα agonist, increased *AMH* promoter activity while MPP, a specific ERα antagonist, inhibited the *AMH* promoter activity (**Figure 6B**). The effect induced by E2 *via* GPER was more modest. Its specificity was confirmed when SMAT1 cells were exposed to the potent and selective GPER agonist G1 or to the selective GPER antagonist G-15 (**Figure 6C**).

The upregulation induced by E2 in SMAT1 cells on the 3-kb *AMH* promoter was also observed when a 1926-bp promoter with an intact ERE present at position -1782 was transfected, but no estrogenic activity was detected when using *AMH* promoter constructs consisting of the proximal 433 bp or of sequences lying between -1926 and -2590 or between -1926 and -3078, which are devoid of the half-ERE (**Figure 6D**) (19). Furthermore, the response to E2 was abolished by a mutation introduced in the half-ERE at -1782 by site-directed mutagenesis. A direct interaction between ERα and the half-ERE was confirmed in electro mobility shift assays. Complex formation with ERα was observed when ERE wild-type probes, but not with mutant ERE

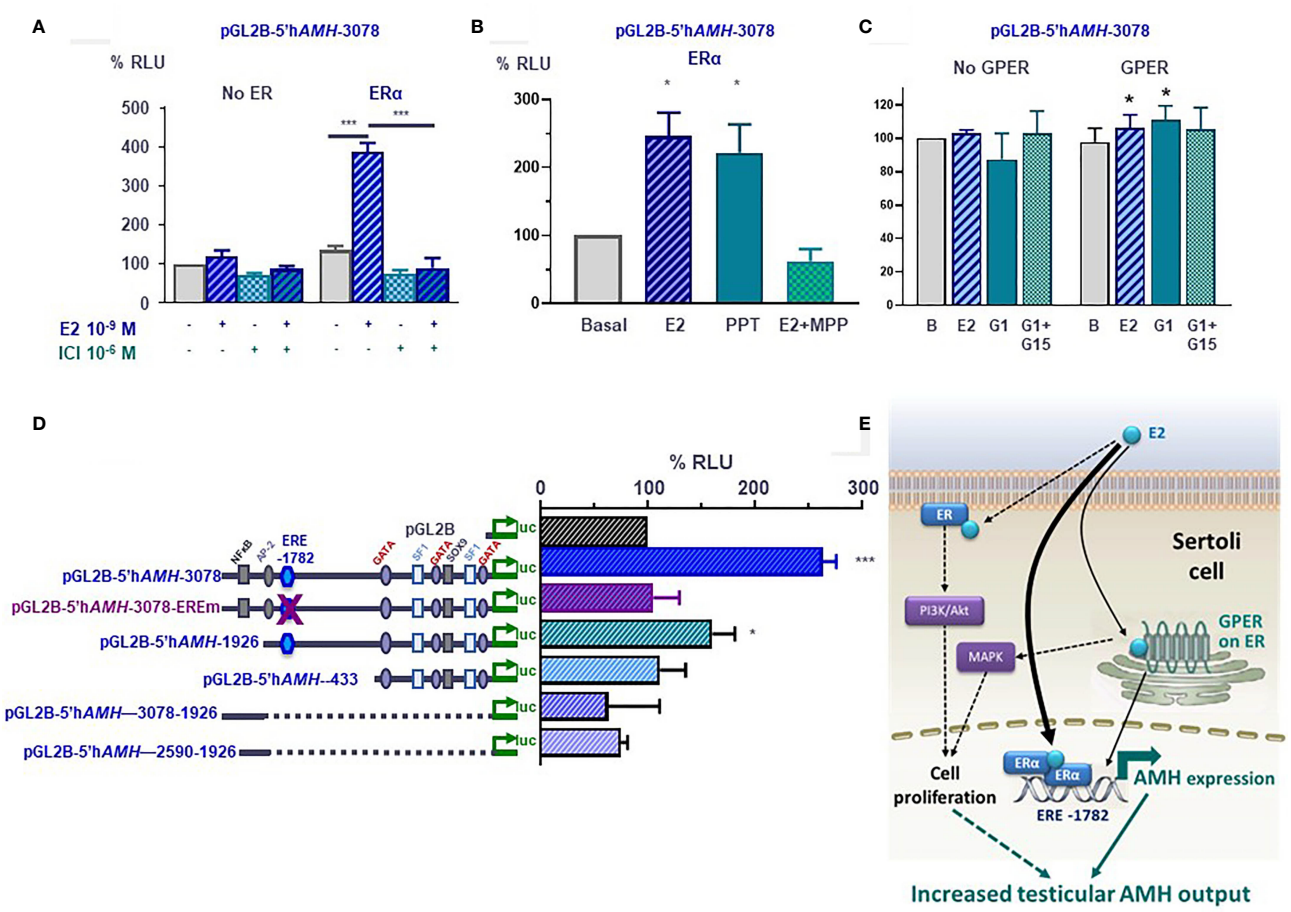

FIGURE 6 | Molecular mechanisms involved in estrogen-induced upregulation of *AMH* expression in Sertoli cells. **(A–D)** Luciferase assays in SMAT1 Sertoli cells transfected with a luciferase plasmid under control of the human *AMH* promoter (pGL2B-hAMH) of different lengths and co-transfected with an expression plasmid of the estrogen receptor alfa (ERα) or the G protein-coupled estrogen receptor (GPER) or the control plasmids devoid of the estrogen receptor sequences. Cells were exposed to estradiol (E2), the ERα/β antagonist ICI 182780, the ERα-specific agonist PPT or antagonist MPP, or the GPER-specific agonist G1 or antagonist G15. Results were expressed as relative luciferase units (RLU) comparing cells exposed to estrogens, agonists and/or antagonists (* $p<0.05$, *** $p<0.001$). A 100% level indicates the basal *AMH* promoter activity. E2 induce an upregulation of *AMH* promoter activity when the ERα or the GPER is present but not in their absence **(A–C)**, and when the estrogen response element (ERE) at -1782 of the promoter is conserved **(D)**. **(E)** Proposed model for E2 regulation of *AMH* expression in Sertoli cells. E2 upregulates AMH transcription *via* ERα binding to the ERE. GPER also upregulates *AMH* expression more modestly. The increased AMH expression results in a higher testicular AMH production. Another potential mechanism is the increase in Sertoli cell proliferation induced by membrane-bound ERα signaling through the PI3K/Akt pathway, and/or GPER *via* MAPK signaling. Reproduced with permission from Valeri et al. (19)© 2020 The Authors.

probes, were used. Altogether, these results indicate that E2 at physiological concentrations increases the activity of the *AMH* promoter through ERα binding to the half-ERE site present at position -1782 (**Figure 6E**). The intracellular pathway involved in the more modest *AMH* promoter upregulation induced through the GPER still needs to be elucidated.

4.1.2 Clinical Implications

The cellular and molecular mechanisms involving ERα and, more modestly, GPER in E2 induction of the activity of the *AMH* promoter (19) and of Sertoli cell proliferation (91) provide biological plausibility to explain the increase in serum AMH levels observed in patients with androgen insensitivity (50, 84) or with Peutz-Jeghers syndrome (46). It could also explain why serum AMH is low despite elevated circulating E2 in estrogen insensitivity caused by defective ERα function (97).

5 CONCLUDING REMARKS

AMH production by Sertoli cells reflects the differential regulation exerted by intratesticular levels of androgens and estrogens in the prepubertal testis, with specific variations according to the developmental stage. In the fetus and the newborn, the high intratesticular androgen concentrations do not regulate AMH expression because the AR is not yet expressed in Sertoli cells, whereas intratesticular estradiol can moderately increase AMH production since ERs are present in

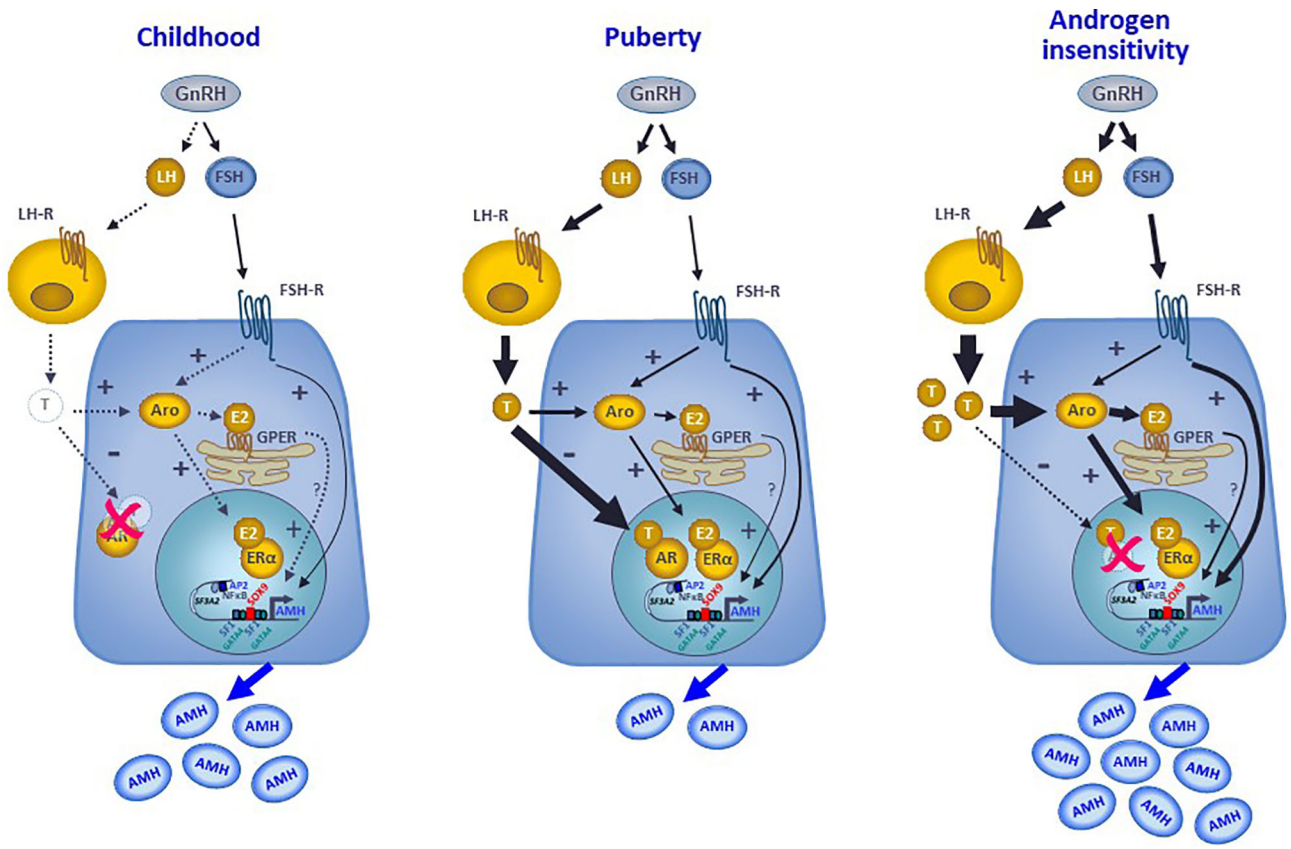

FIGURE 7 | Interaction between androgens, estrogens and FSH on the regulation of AMH expression in Sertoli cells. During childhood, the hypothalamic-pituitary-gonadal axis is quiescent, and the extremely low steroid levels do not exert any physiological regulation on AMH production that is mostly hormone-independent. At puberty, the reactivation of the GnRH neuron and the gonadotropes result in higher LH and FSH levels. LH induces testosterone secretion by testicular Leydig cells. FSH acts on its receptor in the Sertoli cell membrane, resulting in a direct upregulation of AMH expression, through the cyclic AMP-PKA pathway involving transcription factors SOX9, SF1, AP2 and NFκB, and in an indirect upregulation of AMH by inducing aromatase expression. Aromatase converts androgens into estrogens, which can upregulate AMH directly by binding to the nuclear ERα or indirectly acting through the GPER expressed in the membrane of the endoplasmic reticulum. Nonetheless, the inhibitory effect of androgens overrides the stimulatory effect of FSH and estrogens on AMH expression, resulting in a decreased AMH secretion. In hyperestrogenic states with abrogated androgen action, such as the androgen insensitivity syndrome, the inhibitory effect of androgens does not exist, FSH and LH increase resulting in high testosterone that is converted to estradiol. Consequently, AMH production is substantially boosted.

Sertoli cells and aromatase activity resulting in androgen conversion to estrogens is stimulated by FSH. During childhood in humans, or the quiescent period of the hypothalamic-pituitary-gonadal axis described in most mammals, the extremely low steroid levels do not exert any physiological regulation on AMH production (**Figure 7**). However, precocious androgen synthesis results in AMH downregulation since the AR is expressed in Sertoli cells from the second year of life. On the contrary, testis-borne hyperestrogenic states provoke an increase in AMH expression (**Figure 7**). Finally, during puberty the inhibitory effect of intratesticular testosterone levels overcome the stimulatory effects of estrogens and FSH (**Figure 7**). Apart from the indirect regulation of testicular function that they exert through their feedback on gonadotropins, both androgens and estrogens have a direct effect on *AMH* gene transcription. These direct effects are mediated by the classical mechanism involving nuclear AR and ER receptor activity on target gene promoters and, more modestly, by estrogen action through the membrane GPER (**Figure 7**). The comprehension of these complex regulatory mechanisms helps in the interpretation of serum AMH levels found in physiological or pathological conditions, highlighting the capacity of serum AMH as a biomarker of intratesticular steroid concentrations, which are not always reflected in serum levels.

AUTHOR CONTRIBUTIONS

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

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Conflict of Interest: Until 2020, RR received royalties for the development of an AMH ELISA kit and honoraria for technology services using the AMH ELISA.

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REVIEW
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Anti-Müllerian Hormone Signal Transduction involved in Müllerian Duct Regression

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Over seventy years ago it was proposed that the fetal testis produces a hormone distinct from testosterone that is required for complete male sexual development. At the time the hormone had not yet been identified but was invoked by Alfred Jost to explain why the Müllerian duct, which develops into the female reproductive tract, regresses in the male fetus. That hormone, anti-Müllerian hormone (AMH), and its specific receptor, AMHR2, have now been extensively characterized and belong to the transforming growth factor-β families of protein ligands and receptors involved in growth and differentiation. Much is now known about the downstream events set in motion after AMH engages AMHR2 at the surface of specific Müllerian duct cells and initiates a cascade of molecular interactions that ultimately terminate in the nucleus as activated transcription factors. The signals generated by the AMH signaling pathway are then integrated with signals coming from other pathways and culminate in a complex gene regulatory program that redirects cellular functions and fates and leads to Müllerian duct regression.

Keywords: anti-müllerian hormone, transforming growth factor-β, bone morphogenetic protein, Müllerian duct regression, signal transduction

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INTRODUCTION

Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS), is a member of the transforming growth factor-β (TGF-β) family expressed in Sertoli cells of the fetal and postnatal testis and granulosa cells of the postnatal ovary and has important roles in male and female reproductive development (1). In the male vertebrate embryo, AMH is responsible for the regression of the Müllerian duct (MD), the anlagen of the uterus, Fallopian tubes, and upper part of the vagina (2). Testosterone is produced later in male sexual development and is responsible for differentiation of the epididymis, vas deferens, and seminal vesicles from the Wolffian duct. However, sexual development of the male is not complete unless AMH causes regression of the MD. In the adult male, AMH has a role in Sertoli and Leydig cell differentiation and function (3, 4). In females, AMH inhibits primordial follicle recruitment and the responsiveness of growing follicles to follicle-stimulating hormone (5). In addition, AMH has been reported to have roles within the nervous system (6, 7) and the hypothalamic-pituitary-gonadal axis (8, 9).

Over 33 TGF-β ligands have been identified. AMH is closer in lineage and function to a group of TGF-β family members, which includes the bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and activins. These proteins have important roles in embryonic

patterning and morphogenesis, as well as more specialized roles within organs, such as the control of gonadal function and the regulation of bone and muscle mass (10). Another branch of the family, the TGF-βs, appeared later in evolution with the vertebrates and regulate more newly acquired processes including injury repair, cellular proliferation, adhesion, and immunity (11). TGF-β ligands mediate their effects by assembling a transmembrane receptor complex of type I and type II components, both of which contain intracellular serine/threonine kinase domains. The close juxtaposition of the two receptors results in the phosphorylation and activation of the type I receptor kinase by the constitutively active kinase domain of the type II receptor (12–14). Once activated, the type I receptor phosphorylates a receptor-regulated Smad (R-Smad), which interacts with the Co-mediator Smad4 to form a complex. This complex then moves to the nucleus where it associates with other transcription factors and binds to a Smad-binding element (SBE) in the promoter or enhancers of target genes (15). Five type II receptors (TGFR2, ActR2, ActR2B, BMPR2, and AMHR2), seven type I receptors (ALK1-7), and five R-Smads (1-3, 5, and 8) have been identified. There are two Smad pathways: TGF-β, activins and some GDFs stimulate the phosphorylation of Smad2 and Smad3, whereas BMPs and some GDFs stimulate the phosphorylation of Smad1, 5, and 8. Due to the limited number of type I and II receptors, each receptor interacts with multiple ligands, and many ligands interact with multiple receptors. Uniquely for the family, AMH and its type II receptor AMHR2 are mutually specific, as indicated by the identical phenotypes of *Amh* and *Amhr2* null mice (16). However, AMH does share the type I receptors, ALK2, ALK3, and ALK6, and the Smads 1, 5, and 8 with the BMP pathway (17).

Regression of the MD induced by AMH in the male fetus, proceeds *via* a paracrine mechanism, since it is the surrounding mesenchymal cells that express AMHR2 (18, 19). Thus, AMH signaling either activates a paracrine factor leading to apoptosis of the epithelium, or alternatively, represses a survival signal required for epithelial cell growth (20). Breakdown of the basement membrane (21–23), proteolysis (20), epithelial cell migration (24), and apoptosis and epithelial to mesenchymal cell transformation (25), have been shown to play a role in regression. This review will cover what is now known about the canonical AMH signaling pathway (**Figure 1**) and place it in context with other signaling pathways that are required for MD regression.

THE CANONICAL AMH SIGNALING PATHWAY

AMH

The human *AMH* gene is composed of five exons, maps to chromosome 19 p13.3 (26), and encodes a protein of 560 amino

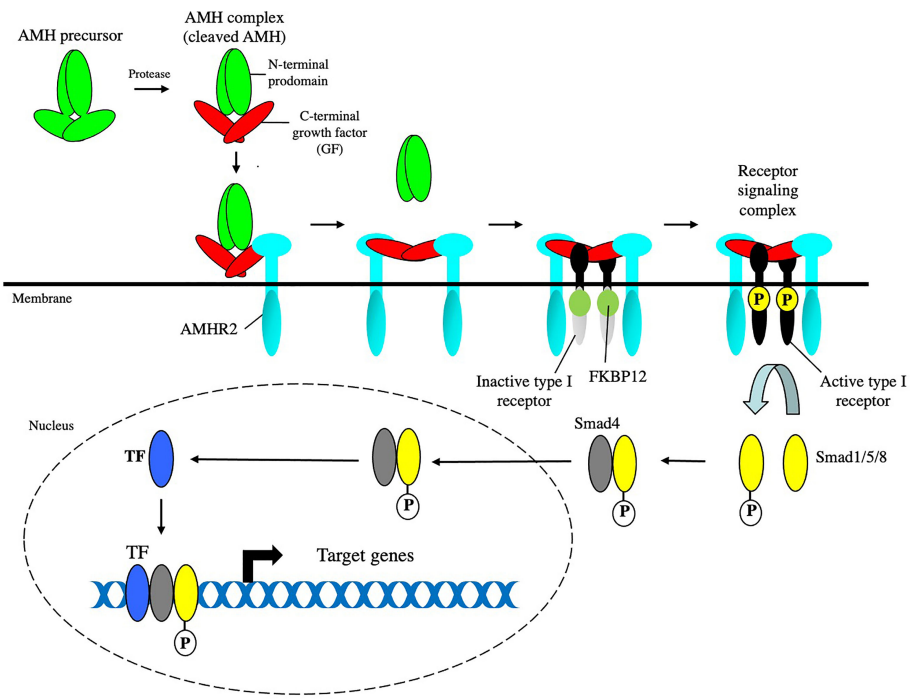

FIGURE 1 | The canonical AMH signaling pathway. AMH assembles a receptor signaling complex composed of type I and type II receptors. The kinase domain of AMHR2 is constitutively active and phosphorylates and activates the kinase domain of the type I receptor (ALK2 or 3), which phosphorylates Smad1, 5, and 8. The phosphorylated Smads form a heterotrimer with Smad4, which translocates to the nucleus, and binds to other transcription factors (TF). The Smad/TF complex binds to promoter or enhancer regions of target genes to regulate transcription.

acids containing a 24 amino acid signal sequence. The bovine testicular (27) and human recombinant (28) proteins are synthesized as homodimeric precursors, containing an N-terminal prodomain and a smaller C-terminal mature domain. The C-terminal domain, herein referred to as the growth factor (GF), contains a cysteine knot motif that is characteristic of all TGF-β family members, and has binding sites for AMHR2 and the type I receptor (29) (**Figure 2A**) (29, 30). Cleavage of the precursor at monobasic sites between the two domains is required for binding to AMHR2, but after cleavage the prodomain and C-terminal homodimers remain associated in a noncovalent complex (28). The prodomain has been shown to play an important role in the folding of the GF and the secretion of the AMH precursor and noncovalent complex (31).

Only ~5% of AMH secreted from bovine testis and Chinese hamster ovary cells is cleaved at the monobasic sites, but complete processing can be achieved *in vitro* with plasmin (28). The kex2/subtilisin-like endoprotease PCSK5 may be responsible for cleavage *in vivo* (32). Variations in the level of proteolytic cleavage of AMH have been observed in body fluids during various developmental and metabolic conditions (33, 34). A higher level of cleavage was observed in the follicular fluid from females with polycystic ovary syndrome (PCOS), consistent with an autocrine role for AMH in the pathophysiology of PCOS in the follicle (35). However, while most of the cleaved AMH in the follicular fluid was competent for binding AMHR2, only a small fraction of the cleaved AMH in serum was found to be competent (35). These results indicate that AMH can be subjected to further structural changes after cleavage that prevent binding to AMHR2 and render it inactive.

AMH is expressed by Sertoli cells of the testes, starting eight weeks after fertilization in humans (36), and persisting at high levels until puberty, when they decrease to below detection in the adult (37). In the female, AMH is expressed in granulosa cells of the ovary, starting 23 weeks after fertilization with the appearance of primary follicles (38); the highest expression levels are in secondary and small antral follicles (39). AMH can be detected in the blood until the onset of menopause, when levels fall below detection, concomitant with the decrease in the number of developing follicles (40).

AMHR2

The human *AMHR2* gene is composed of 11 exons, maps to chromosome 12q13 (41), and encodes a 573 amino acid membrane protein containing a signal sequence, an N-terminal extracellular domain that binds AMH, a single transmembrane domain, and an intracellular domain with serine/threonine kinase activity. AMHR2 uses its transmembrane domain for insertion and orientation in the membrane, because its signal sequence is not functional (42). The extracellular domain (**Figure 2B**) has the structure of a three-finger toxin found in other TGF-β type II receptors (29), and four similar disulfide bridges. The intracellular domain has the structure of a two-domain kinase, as deduced from a molecular model (42). During biosynthesis of AMHR2, aberrant cleavage of the extracellular domain and the formation of promiscuous disulfide bonds can occur, leading to intracellular retention (43). In the absence of ligand, AMHR2 undergoes a high level of non-covalent homo-oligomerization at the plasma membrane (43), even higher than the levels observed for TGFR2 and BMPR2 (44).

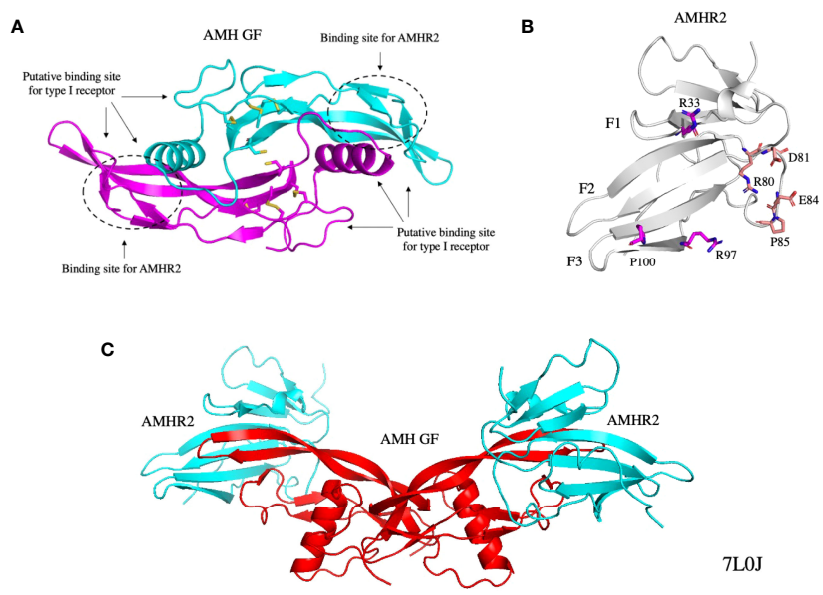

FIGURE 2 | Structures of the AMH GF **(A)**, AMHR2 ECD **(B)**, and the AMH GF bound to AMHR2 ECDs **(C)**. The binding sites of the type I receptor and AMHR2 on the AMH GF are indicated and some of the residues on the AMHR2 ECD that contact the AMH GF are shown as sticks. All structures were generated from PDB 7L0J (29). GF, growth factor; ECD, extracellular domain.

AMHR2 is expressed in the MDs of both sexes by mesenchymal cells surrounding the epithelium (18, 19) according to a cranio-caudal gradient (25), and disappears in the male after MD regression. AMHR2 is also expressed in the male and female gonads. In the testis, it is expressed from fetal life to puberty, whereas in the ovary, AMHR2 is expressed from fetal life to adulthood by granulosa cells of preantral and antral follicles (45). In humans, mutations in the genes coding for *AMH* or *AMHR2 cause* persistent Müllerian duct syndrome (PMDS), in which normal 46,XY males retain a uterus and fallopian tubes (46, 47). Many of these mutations lead to unstable and/or truncated proteins. Recently, loss of function heterozygous mutations in *AMH* and *AMHR2* were identified in a small subset of patients with congenital hypogonadotropic hypogonadism (48). Mutations in *AMH* and *AMHR2* produce a spectrum of phenotypes in a variety of vertebrate species (49).

Binding of the noncovalent AMH complex to AMHR2

An early question that arose prior to the identification of AMHR2, was whether the prodomain of AMH would prevent binding of the AMH GF to its receptor. Whereas the noncovalent complexes of some TGF-β ligands are latent and cannot bind their receptors, the noncovalent complexes of other ligands are biologically active, indicating that they can bind their receptors. Crystal structures of noncovalent complexes of TGF-β ligands (50–53) have provided insight into the way that prodomains interact with their GFs, revealing that the latency lasso, α2-helix, and β1-strand of the prodomain form proximal associations with the type II receptor binding site on the GF, while the α1- or α5-helix forms a proximal association with the type I receptor binding site (14). Still, it has not been determined whether these associations block access to receptors. The GFs of latent complexes are bound to their prodomains in a more confined state and either torsional force (TGF-β1 (50);) or proteolysis (GDF-8 (53);) is required for the GF to be released from their prodomains and allow receptor interactions. In contrast, non-latent complexes can bind their receptors while the prodomain is still bound to the GF. For example, the BMP-9 complex can bind to the type I receptor ALK1 (54) even though the α5-helix of the prodomain is associated with the type I receptor binding site in the noncovalent complex (51). The differences in how the latent TGF-β1 and non-latent BMP-9 complexes access their receptors are illuminated in **Figure 3**. The regions in the AMH prodomain that correspond to the β1-strand and α2-helix elements in TGF-β1 and BMP-9 are not well conserved (51), so structural verification will be required to determine whether the AMH prodomain contains these elements and if so, whether they interact with the type II receptor binding site on the AMH GF.

The AMH noncovalent complex is biologically active (28, 55) and can bind to AMHR2 (56). However, when the AMHR2 is on a surface (as a fusion protein with IgG) or on the surface of cells, the prodomain is displaced after binding to AMHR2 (56). The

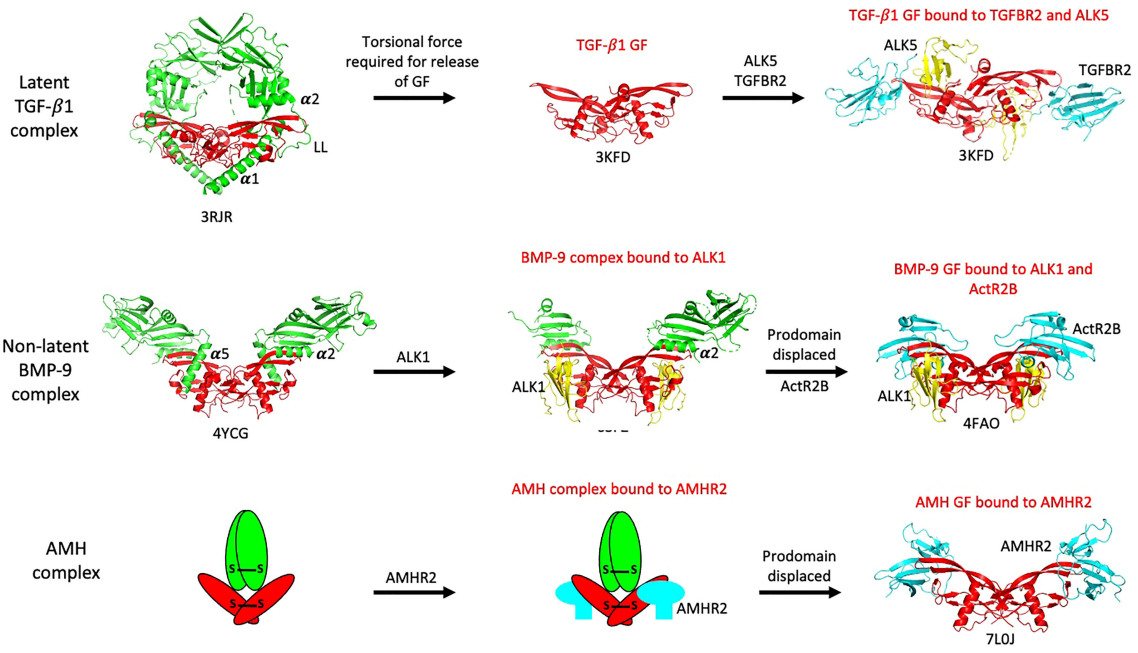

FIGURE 3 | Summary of how various TGF-β family ligands access their receptors. The latent TGF-β1 complex requires torsional force to liberate its GF and allow access to receptors. The non-latent BMP-9 complex can bind to its type I receptor, ALK1, without inducing prodomain displacement, while the AMH complex can bind to AMHR2 in solution without inducing prodomain displacement. Prodomains are shown in green, GFs in red, type I receptor ECDs in yellow, and type II receptor ECDs in cyan. The PDB file names are shown beneath each structure. The α1-, α2-, and α5-helices and latency lasso (LL) in the prodomains are labeled; these elements are located in close proximity to receptor binding sites on the GFs.

prodomain of the BMP-7 complex has been shown to be displaced by the type II receptor BMPR2 (57), while the prodomain of the BMP-9 complex has been shown to be displaced by the type I receptor ALK1 (54, 58). When ELISA assays were used to measure the dependence of prodomain displacement on AMH concentration, it was found that prodomain displacement only occurred at low concentrations of the AMH complex, indicating the AMH complex can bind to surface captured AMHR2 in two different states, with one being susceptible to dissociation (59). Subsequently it was shown that when the AMH complex is bivalently bound by two AMHR2 molecules, the dissociation constant for the AMH complex is increased by 1000-fold, compared to the unbound state. Monovalent binding to one AMHR2 molecule had a minimal effect on the dissociation constant, indicating that the receptor binding site in the AMH complex is fully accessible to AMHR2. Furthermore, displacement did not occur when the AMHR2 was in solution, indicating that displacement of the prodomain is caused by a conformational change in the growth factor induced by bivalent binding to AMHR2 on a surface (59). These results are summarized in **Figure 4**. In **Figure 3**, the non-latent complexes of BMP-9 and AMH are compared.

Interactions of the AMH GF with AMHR2

Due to the limited number of type II receptors, many TGF-β receptors bind multiple ligands. In contrast, AMH and AMHR2 have a monogamous relationship (16, 41). The crystal structure of AMH bound to AMHR2 has provided insights into the reasons for this specificity (29) (**Figure 2C**). The binding sites for AMHR2 on the GF are shown in **Figure 2A**; some of the residues in contact with the GF on AMHR2 are shown in **Figure 2B**. For a detailed description of the interactions between AMH and AMHR2, readers are referred to the chapter in this edition by Thomas B. Thompson and co-authors. Previous mutational studies have also provided insight into how AMH interacts with AMHR2 (31, 42, 60). Of note, the Q496H mutation in the pre-helix loop of the GF may disrupt binding of the type I receptor (31).

Binding of Type I receptors to the AMH/AMHR2 complex

After the AMH complex binds bivalently to AMHR2, the GF undergoes a conformational change which leads to the displacement of the prodomain as described above. Presumably, it is at this stage that the low affinity type I receptors are recruited. Several observations support this notion. It has been proposed that binding of activin by two ActR2 chains immobilize it in a type I receptor binding-competent orientation (61, 62). BMP-7 requires a cooperative interaction with ActR2 to bind ALK2, which does not rely on receptor-receptor contacts (63). Furthermore, it has been shown that the BMP-2 homodimer (64) and a BMP-2/6 heterodimer (65) must have two functional type II binding sites to activate their receptors and stimulate downstream signaling on cells. Together, these observations suggest that the conformational change induced in the AMH GF upon bivalent binding to AMHR2 is required for the recruitment of the type I receptor. However, without further experimentation, it cannot be ruled out that recruitment of the type I receptor also involves a cooperative interaction with AMHR2, perhaps mediated by their cytoplasmic domains.

To date, there have been few reports in the literature of direct binding of any type I receptor to the AMH/AMHR2 complex. In

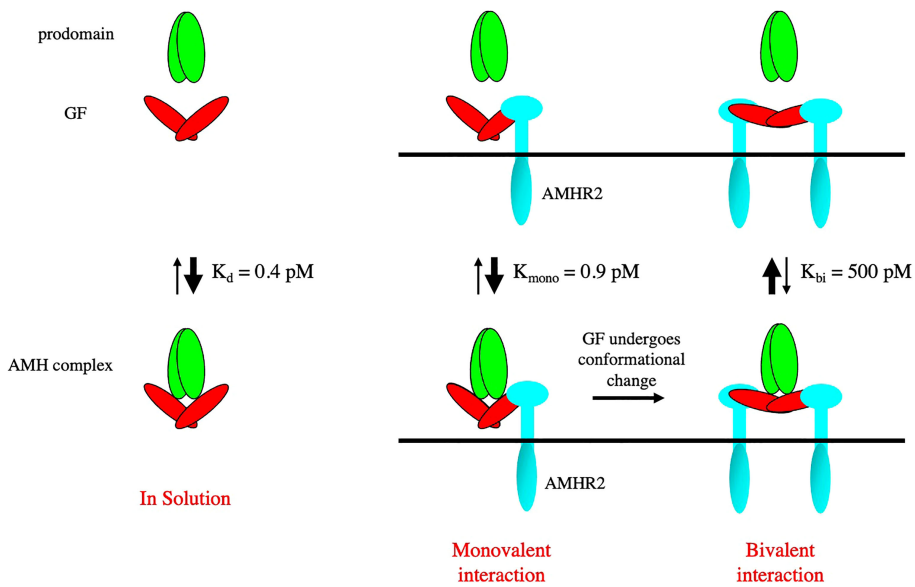

FIGURE 4 | A bivalent interaction of AMHR2 with the AMH noncovalent complex leads to a conformational change in the GF and displacement of the prodomain. The dissociation constants of the AMH complex bound monovalently and bivalently to AMHR2 are shown. Bivalent binding causes a 1000-fold increase in the K_d of the complex leading to rapid dissociation.

one study, co-immunoprecipitation assays indicated that ALK-6 was the only type I receptor that could interact with AMHR2 in cells in a ligand dependent manner (66), although subsequent analyses suggested that ALK-6 acts as a negative regulator of intracellular signaling (67). Evidence supporting ALK2 as a type I receptor has come from experiments which measure downstream signaling in cells facilitated by different type I receptors. Assays using a luciferase reporter indicated that ALK2, but not ALK3 could enhance the AMH signaling response (68), while dominant negative variants of ALK2 could attenuate the AMH induced activation of a reporter gene (68, 69). In a Sertoli cell line, AMH signaling was attenuated by a dominant negative variant of ALK3 and by small interfering RNAs (67). ALK2 could compensate for the absence of ALK3 and act in synergy with ALK3 at high concentrations of AMH. It has been reported that in certain cases, ALK2 and ALK3 can bind to the same BMP-2 or BMP-6 homodimer (70). A ligand independent association of ALK2 with AMHR2 was observed in COS cells transfected with the two receptors (71).

Targeted gene disruption experiments in mice have provided the most compelling evidence for the involvement of ALK2 and ALK3 in the regression of the MD mediated by AMH. Because mice with mutations in *Alk2* and *Alk3* are not viable, conditional knockouts were generated using the *cre/loxP* system. A Cre recombinase expression cassette was targeted into the *Amhr2* locus in order to drive expression of the Cre recombinase in the mesenchymal cells of the MD (72). The *Amhr2-cre* mice were then crossed with mice carrying conditional null alleles of *Alk2* or *Alk3* (i.e. LoxP sites were inserted on both sides of the genes making them susceptible to removal by the Cre recombinase in the cells where it is expressed). Conditional inactivation of *Alk3* was found to block MD regression in ~55% of the males (73), whereas conditional inactivation of *Alk2* did not block MD regression in any of the males. When both *Alk2* and *Alk3* were conditionally inactivated, MD regression was blocked in 100% of the males. These results indicated that *Alk3* is the primary type I receptor for AMH induced MD regression, but *Alk2* in the absence of *Alk3* can transduce the AMH signal (73). Mice with mutations in *Alk6* are viable and males do not have retained MDs, indicating that *Alk6* is not essential for AMH induced regression of the MD (69, 74).

The overall structures of the type I and II receptors are depicted in the schematic diagram shown in **Figure 5A**. All type I and II receptors share sequence and structural homology

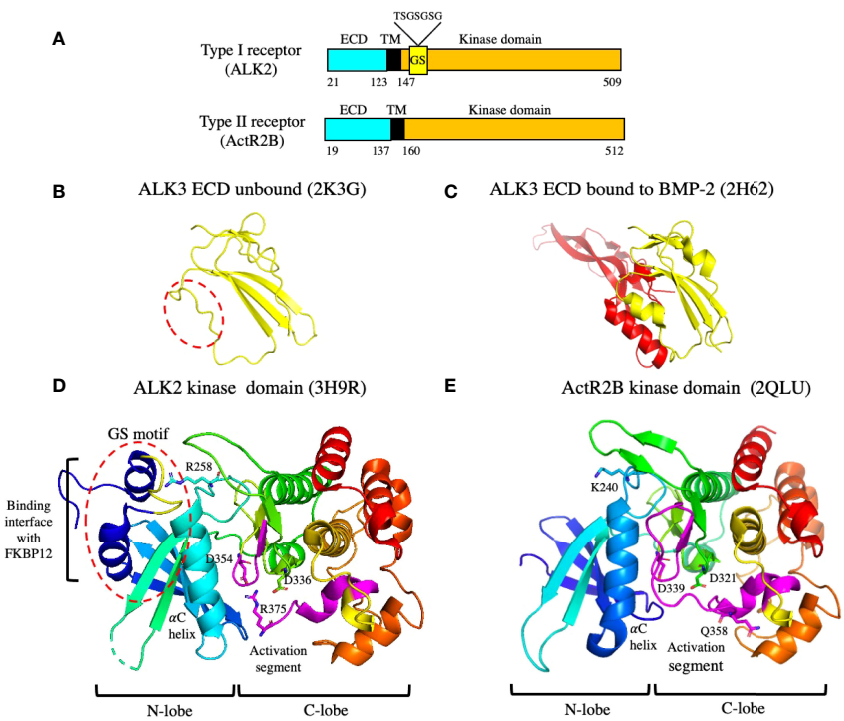

FIGURE 5 | Organization and structures of the type I and type II receptors. **(A)** A schematic diagram showing the organization of the type I and II receptors. The type I receptor contains a GS motif that is phosphorylated by the type II receptor on serine residues, leading to its activation. The amino acid sequence of the GS loop containing the serine residues that are phosphorylated is shown above the GS motif. The numbering corresponds to amino acid residues in UniProtKB accession numbers Q04771 (human ALK2) and Q13705 (human ActR2B). ECD: extracellular domain; TM: transmembrane. **(B, C)** Structures of the ALK3 ECD in solution **(B)** and bound to the BMP-2 ECD **(C)**. The dashed red oval shows that the alpha helix in ALK3 involved in binding BMP-2 is not present in the unbound state. **(D, E)** Structures of an inactive type I receptor kinase domain (ALK2) **(D)** and an active type II receptor kinase domain (ActR2B) **(E)**. The GS motif in ALK2 is indicated by the red dashed oval; it interacts with FKBP12 as shown. The GS motif is composed of two alpha helices connected by a loop (yellow) which contain the serine residues that are phosphorylated by the type II receptor. ALK2 contains a salt bridge between residue R375 and residues D336 and D354. Both aspartate residues are required in all kinases for Mg-ATP binding and catalysis. PDB files used to generate the structures are indicated.

in both their ECD and kinase domains, indicating that they arose from a common ancestor (14). **Figure 3** shows how type I receptors bind to the TGF-β1and BMP-9 GFs and reveals that their binding modes are somewhat different. In the case of TGF-β1, the type I and II receptor ECDs make contact (75), while in the case of BMP-9, they do not (76). Following TGFR2 binding to TGF-β3 or TGF-β1, the type I receptor binds tightly to the TGF-β/TGFR2 composite surface (75, 77). This may explain why with the TGF-βs, one pair of bound type I and II receptors can signal independent of the other pair (78), while in the case of BMPs, two type II receptors are required for signaling (64, 65). Thus, the TGF-βs use a cooperative model for assembling the signaling complex, whereas several BMPs, activin, and AMH appear to follow an allosteric model, in which bivalent binding to the type II receptor results in a conformational change in the GF and the exposure of the binding site for the type I receptor. In the latter case, cooperative effects could also be involved in the recruitment of the type I receptor.

The ALK3 ECD undergoes a conformational change after binding to ligand. The structures of ALK3 ECD unbound (79) and bound to BMP-2 (80) are shown in **Figures 5B, C**, respectively, revealing that the α-helix in ALK3 is not present in the unbound state. This α-helix makes important contacts with the ligand, indicating that ALK3 uses an induced fit mechanism to adapt to the binding interface in BMP-2. The flexibility of ALK3 may explain how it can bind to so many different ligands including AMH.

Activation of Type I receptors

The structures of the kinase domains of the type I receptor ALK2 (81) and the type II receptor ActR2B (82) are shown in **Figures 5D, E**. The structures look similar, with both kinases containing an N-lobe, consisting mostly of a five stranded β-sheet, and a C-lobe that is mostly α-helical. However, the ALK2 kinase is in an inactive conformation, whereas the ActR2B kinase is in an active conformation. One major difference is the presence of the GS motif in the type I receptor located just inside the membrane (**Figure 5A**). The GS motif is indicated by the red dashed circle on the ALK2 structure in **Figure 5D** and is composed of two α-helices connected by a loop (colored in yellow). This loop contains several serine residues which are phosphorylated by the type II receptor kinase resulting in its activation. One of the α-helices in the GS motif interacts with the inhibitory protein FKBP12, which has been shown to bind to and stabilize the inactive conformations of several type I receptors (83, 84). In this inactive conformation of ALK2, certain elements critical for catalytic activity are not optimally positioned (81). The GS loop is protected from phosphorylation by residue R258 which buries the GS loop in the kinase N-lobe between the αC helix and β4 strand; the correct positioning of the αC helix is critical for kinase activation. In addition, the inactive conformation is stabilized by the formation of salt bridges between residue R375 in the activation segment and residues D336 (in the catalytic HRD motif) and D354 (in the DFG motif). Both aspartate residues are required in all kinases for Mg-ATP binding and catalysis. These salt bridges are not formed in the active type II kinase domain of ActR2 (**Figure 5E**).

Bringing the inactive type I receptor close to the active type II receptor through interactions of their ECDs with ligand, allows phosphorylation of the serine residues in the GS loop. Phosphorylation of the GS loop in ALK5 results in much higher specificity of the type I receptor kinase for the C-terminal serine residues of Smad2 (see below) and prevents binding to FKBP12 (85). Exactly how the type II kinase phosphorylates the serine residues in the GS loop is not known, since there are no structural studies to date that provide insight into the organization of the intracellular domains in an active receptor complex containing two type I and two type II receptors. Recently, it has been reported that ALK2 and BMPR2 form a heterodimeric complex *via* their C-terminal lobes that is essential for ligand induced receptor signaling (86).

Phosphorylation of Smads

All Smads share a similar structural organization with an amino-terminal Mad-homology 1 (MH1) domain, a central proline-rich linker, and a carboxy-terminal MH2 domain (**Figure 6A**) (15). The two groups of R-Smads, Smad2 and 3 and Smad1, 5, and 8, share 84-90% identity within the groups (Smad2 and 3 compared or Smad1, 5, and 8 compared), and 56-60% identity between groups (Smad2 and 3 compared to Smad1, 5, and 8). The MH1 domain contains a β-hairpin structure that mediates the binding of Smads to DNA, while the MH2 domain mediates the association with Smad4 and subsequently with other transcription factors after nuclear translocation. Smad activation is dependent on the type I receptors that phosphorylate a common Ser-X- Ser motif present at the carboxyl terminus of the MH2 of R-Smads (13). The TGF-β and activin-specific receptors phosphorylate Smad2 and 3, whereas the BMP-specific receptors phosphorylate Smad1, 5, and 8. AMH has been shown to induce phosphorylation of Smad1, 5, and 8 in all of its target organs (66, 68, 69), as well as induce an interaction with Smad4 and nuclear translocation (66). The *Amhr2-cre* mice have also been used to conditionally knockout the *Smad1, 5, and 8* genes in the mesenchymal cells of the MDs in mice. The results indicated that *Smad5* is the major R-Smad used in AMH-induced MD regression, but *Smad1* and *Smad8* are both capable of replacing *Smad5* (73).

At present there are no structural studies that inform explicitly on how the type I receptors interact with Smads. Anchor proteins that bind to phosphatidylinositol 3-phosphate and recruit Smads to the inner membrane and position them adjacent to activated type I receptors have been identified for Smad2/3 (SARA) (15) and for Smad1 (endofin) (87). For ALK5, it has been proposed that phosphorylation of the GS loop of the kinase domain by the type II receptor, switches the GS region from a binding site for the inhibitory protein FKBP12 into a recruitment site for the R-Smad substrate (85). Mutational studies have identified four residues within the β4-β5 loop of the type I receptor kinase domain that may determine Smad selectivity; an exchange of these residues between BMP and TGF-

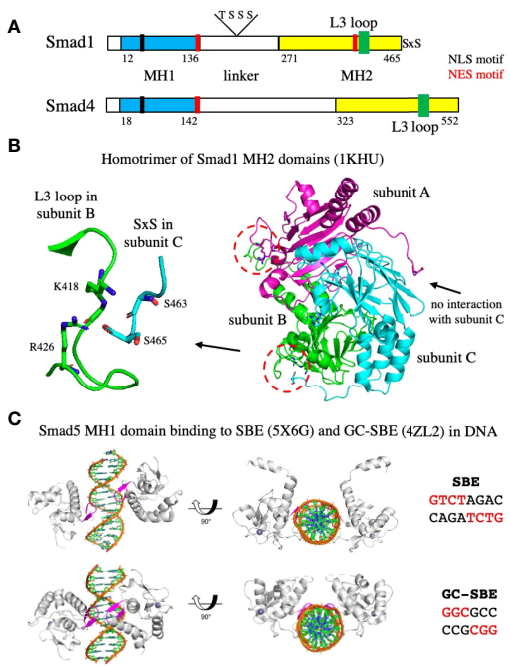

FIGURE 6 | Organization of Smads, homotrimerization of the MH2 domain, and binding of the MH1 domain to DNA. **(A)** A schematic diagram showing the organization of Smad1 and Smad4. Both Smads contain an MH1 domain, a central proline-rich linker, and a carboxy-terminal MH2 domain. Smad1 contains two serine residues at the C-terminus that are phosphorylated by the type I receptor kinase. NLS and NES motifs important in the export into and out of the nucleus, and threonine and serine residues phosphorylated by CDK8/9 (S206 and S214) or GSK-3 (T202 and S210) in the linker of Smad1 are shown. The numbering corresponds to amino acid residues in UniProtKB accession numbers Q15797 (human Smad1) and Q13485 (human Smad4). **(B)** Structure of the Smad1 MH2 domain homotrimer. The C-termini of subunits B and C interact with the L3 loops in subunits A and B, respectively, indicated by the red dashed circles. An enlargement of the interaction between the serine residues at the C-termini of subunit C with residues in the L3 loop of subunit B is shown on the left. The C-termini of subunit A does not interact with the L3 loop of subunit C. **(C)** Structures of the Smad5 MH1 domain bound to the SBE or GC-SBE elements in DNA. The β-hairpin responsible for DNA sequence recognition (shown in magenta) is embedded in the major groove of DNA. PDB files used to generate the structures are indicated.

β type I receptors results in their Smad selectivity being reversed (13). There are some leads as to how the type I receptor and Smad interact to allow phosphorylation, but nothing is conclusive. Ultimately, the carboxy-terminal SxS motif of the R-Smad must be engaged by the substrate pocket in the C-lobe of the kinase domain for phosphorylation to occur (13).

Phosphorylation of the C-terminal serine residues induces trimerization of the R-Smads. Biochemical analyses have indicated that Smad2 and Smad3 exist as monomers *in vivo* and form homoligomers upon phosphorylation of the SxS motif by ALK5 (88, 89). Oligomerization was found to occur in the absence of Smad4 and in the case of Smad2, the analysis showed that it was most likely a homotrimer. The crystal structure of the phosphorylated Smad2 MH2 domain revealed the formation of a homotrimer mediated by the C-terminal phosphoserine residues (89). Similar results were observed with Smad1. When the two serine residues at the C-terminus of Smad1 were mutated to aspartic acid to mimic the structural and electrostatic properties of phosphorylation, the modified Smad1 protein superactivated a Smad1/Smad4 dependent signaling response and underwent a monomer to trimer transition at high concentration (90). The X-ray structure of an unphosphorylated Smad1 MH2 domain revealed that it forms an asymmetric trimer (90), shown in **Figure 6B**. Two distinct structural arrangements of the C-terminal tails are apparent: The tails of subunits B and C interact with the L3 loops of subunits A and B, respectively, while the tail of subunit A rotates in a different direction, leaving the L3 loop of subunit C unoccupied. A blowup of the interaction between the tail of subunit C and the L3 loop of subunit B is shown on the left side of **Figure 6B**. The proximity of residue S465 at the C-terminus to residues K418 and R426 in the L3 loop, suggest that phosphorylation of S465 will generate electrostatic interactions between the phosphoryl group and the positively charged side chains of residues K418 and R426 that will strengthen subunit to subunit contact. Phosphorylation of S463 also introduces favorable electrostatic and H-bond interactions with residues in the L3 loop (90). Thus, phosphorylation of the C-terminal serine residues is likely to promote trimerization of Smad1.

The R-Smad homotrimers are subsequently converted to R-Smad/Smad4 heterotrimers. The absence of an interaction between the C-terminal tail of subunit A with the L3 loop of subunit C (**Figure 6B**) suggests that the homotrimeric interactions may be less than optimal, allowing preferential formation of the Smad1/Smad4 heterotrimer. Structural modeling of a 2 to 1 Smad1/Smad4 heterotrimer has indicated that the Smad4 subunit would be favorably tilted toward the Smad1 subunit (90). Activation of Smad2 in the presence of Smad4 prevented Smad2 homo-oligomerization, providing biochemical evidence that Smad2 can form heterotrimers with Smad4 (88). The crystal structures of the Smad2/Smad4 and Smad3/Smad4 complexes are heterotrimers, comprising two phosphorylated R-Smad subunits and one Smad4 subunit (91).

Nuclear translocation of Smads

After the formation of the R-Smad/Smad4 heterotrimer, it translocates to the nucleus to regulate target genes. Nuclear localization of Smads is controlled by two opposing signals: The nuclear localization signal (NLS) and the nuclear export signal (NES). The NLS motif is a single stretch of 5-6 positively charged amino acids that mediates the transport of proteins into the nucleus by docking with importin-α and -β at the cytoplasmic side of the nuclear pore (92). The NES motif is a short stretch of critically spaced hydrophobic residues, usually leucines (LXXLXXLXL), that conveys the protein out of the nucleus (93) and is mediated by exportin-1, also known as CMR1 (94). Smad1 contains an NLS motif in the MH1 domain (95) and two NES motifs, one in the MH1 domain (96) and one in the MH2 domain (95) (**Figure 6A**). Mutations in either NES motif convert Smad1 into an exclusively nuclear location. All motifs are required for optimal transcriptional activation by Smad1,

indicating that it is under constant nucleocytoplasmic shuttling, and that nuclear accumulation is the result of a change in the balance of NLS and NES opposing signals (95). At one point it was thought that Smad4 did not have an NLS motif and was brought into the nucleus through its interaction with the R-Smads. However, Smad4 was shown to contain an NLS motif at a similar location as Smad1, that requires additional basic residues on the C-terminal side up to 86 amino acids away to confer nuclear localization (97). Smad4 contains an NES signal which overlaps with the NES2 motif in Smad 1, that is required for optimal transcriptional activation and is inactivated by TGF-β induced hetero-oligomerization with R-Smads (98). Conformational changes within MH1 and MH2 domains and associations between domains can either expose or mask the motifs and affect their functionality.

R-Smad–Smad4 complexes in the nucleus are further phosphorylated in the linker region by other kinases that induce interaction with additional transcription factors and subsequently with ubiquitin ligases (99) (**Figure 6A**). Phosphorylation of Smad1 by CDK8/9 at positions S206 and S214 creates docking sites for YAP, another transcriptional effector required for certain BMP actions (100). Phosphorylation at the CDK8/9 sites also trigger the subsequent phosphorylation at T202 and S210 by GSK-3, which regulates the duration of the activated pSmad1 signal by switching the docking site preference from YAP to the ubiquitin ligase Smurf1, resulting in its degradation (101). Thus, nuclear CDK8/9 participates in a cycle of Smad activation and disposal that is critical for optimal BMP signaling (100).

Binding of Smads to DNA

After translocation to the nucleus, the phosphorylated heterotrimeric Smad complexes are selectively recruited to target genes. The MH1 domains of Smad2/3-Smad4 complexes mostly bind to the GTCTAGAC site (referred to as the Smad binding element, SBE) (102). In contrast, the best-defined Smad1/5-binding site is GGCGCC (referred to as the GC-SBE) (103) and is present in the enhancers of BMP-responsive genes, such as the ID and VENTX genes (104–106). Crystal structures have been solved for the MH1 domains of Smad1 (107), Smad3 (102), and Smad4 (108) bound to the SBE site. Two MH1 domains are bound to the motif, with each MH1 domain binding on opposite sides of the DNA to a half site of the SBE (GTCT) (102). In all three MH1 domains, an 11-residue β-hairpin, responsible for DNA sequence recognition, is embedded in the major groove of DNA (99), and the amino acids that make contacts with the bases in the DNA are conserved. The structures of the Smad5 MH1 domain bound to the SBE and the GC-SBE have also been determined (109) (shown in **Figure 6C**). The Smad5 MH1 domain contacts the SBE and the GC-SBE with the same β-hairpin (shown in magenta) and the same conserved amino acids. However, due to the shorter GC-SBE motif, the two MH1 domains are closer together than on the SBE motif. In addition, binding of the Smad5 MH1 domain induces different DNA conformations in the SBE and GC-SBE sequences (109).

The affinity of Smads for DNA is weak (K_d = 0.1 μM) (102), which may explain why multiple SBEs are needed to get transcriptional activation in cell culture. Although some MH1 domains do not show cooperative binding to palindromic SBEs, full length Smads would be expected to behave differently. As described above, MH2 domains undergo heterotrimerization, so MH1 domains as part of a trimeric Smad complex should be capable of binding with higher affinities than single MH1 domains to the SBE or GC-SBE sites, especially if multiple sites are present in the promoter or enhancer (110). Higher-affinity interactions should also be promoted by the recruitment of additional transcription factors that bind to the MH2 domains and bind to different DNA sequences. The initiation of Smad dependent gene transcription involves the assembly of a preinitiation complex containing general transcription factors and RNA polymerase II, and its correct positioning at the transcription start site of a target gene. This complex is then regulated by Smads and their associated transcription factors bound at proximal promoter regions or at more distal enhancers (103). The cooperating transcription factors can be either activators or repressors. An important transcription factor that interacts with Smad1/5 is RUNX2, which mediate the activation of genes involved in osteoblast formation, such as osteocalcin, osteopontin, collagen alpha-1(X) chain, and Smad6 (103). Smad1 has also been shown to interact with HoxC8, Nkx3-2, YY1, and GATA factors 4, 5, and 6 (110), as well the myeloid lineage regulator CEBPA and the erythroid regulators GATA factors 1 and 2 (111). Recently a crystal structure of the Smad2 MH2 homotrimer in complex with a domain of the transcriptional activator CBP was reported, showing that CBP forms an amphiphilic helix on the hydrophobic surface of Smad2 (112).

NETWORK OF SIGNALING PATHWAYS INVOLVED IN MÜLLERIAN DUCT REGRESSION

The signaling pathways that have been implicated in MD regression are depicted in the schematic diagram shown in **Figure 7**. Below, each of these pathways are discussed (going from left to right) with a goal of showing how they are integrated to induce regression. Much of this is based on the work of Richard R. Behringer and his colleagues at the University of Texas (113).

The Wnt7A pathway is necessary for expression of AMHR2

Wnt7a is expressed in the MD epithelium and is required for MD regression during male sexual differentiation. Male mice lacking Wnt7a have retained MDs due to the absence of AMHR2 (114). Wnt7a belongs to the Wnt family of ligands which play an important role in the development and differentiation of many cell types during development and adult homeostasis. In the canonical Wnt pathway, Wnts signal by binding to seven-pass transmembrane receptors, Frizzled (Fz), which results in the inhibition of the serine/threonine kinase, GSK-3, mediated by Disheveled (Dvl). This leads to the accumulation β-catenin and translocation to the nucleus where it associates with TCF/LEF

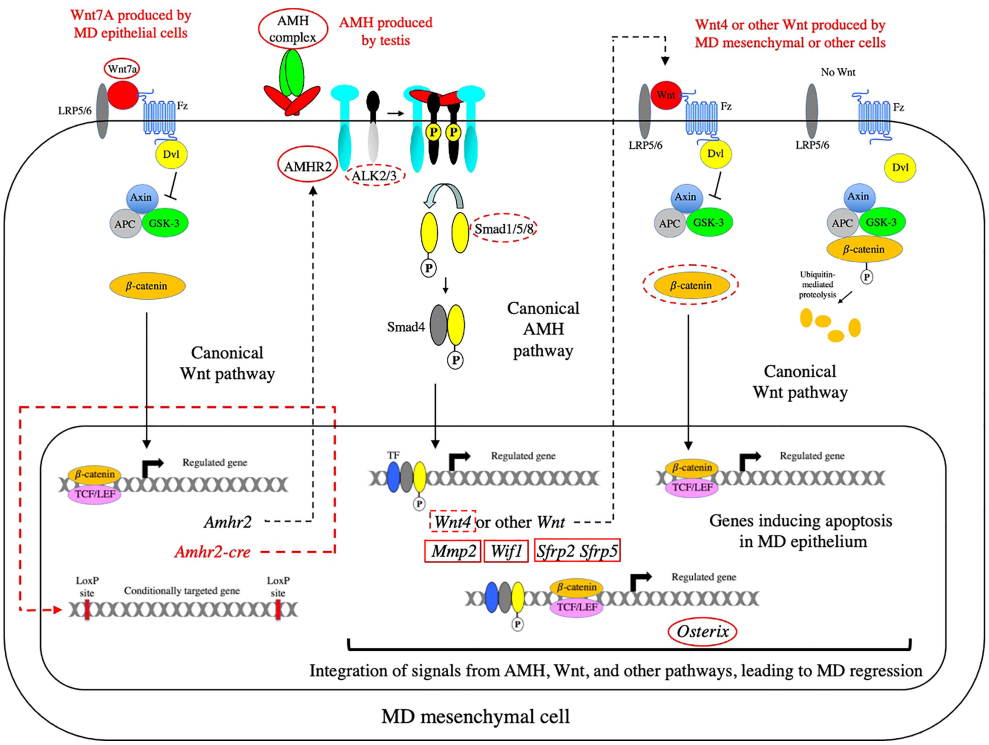

FIGURE 7 | The network of signaling pathways required for MD regression. The AMH canonical signaling pathway is shown in relation to the upstream and downstream Wnt canonical pathways that are required for MD regression. The canonical Wnt pathway on the right of the diagram shows how catenin is degraded in the absence of a Wnt signal. In the absence of Wnt signaling, TCF factors associate with Groucho family proteins to repress transcription (not shown). When Wnt binds to its receptors (Fz and LRP5/6), Disheveled (Dvl) is recruited and activated, resulting in the inhibition of the serine/threonine kinase, GSK-3, and leading to the accumulation of β-catenin and its translocation to the nucleus. In the nucleus, β-catenin displaces Groucho to bind TCF/LEF and activate transcription. Genes or proteins that affect MD regression as null mutations or conditional knockouts are indicated with red solid or dashed ovals, respectively. Genes that do not affect MD regression as null mutations or conditional knockouts are indicated with red solid or dashed rectangles, respectively. β-catenin is not circled in the Wnt7a pathway shown on the left because the conditional knockout of β-catenin did not affect expression of AMHR2. Since both *Amhr2* and the *Cre-recombinase* (under the control of the *Amhr2* promoter) would be induced at the same time, there is almost certainly a time delay between establishment of a competent AMH signaling pathway and the inactivation of β-catenin in the Wnt7a pathway required for the induction of AMHR2. Fz, Frizzled; LRP5/6, LDL-receptor-related protein 5/6; APC, adenomatous polyposis coli protein.

and activates transcription (115) (See **Figure 7**). Wnt signaling can also be mediated by two other pathways: The planar cell polarity pathway which regulates the cytoskeleton, or the calcium pathway which regulates calcium inside the cell (115). Wnt7a has been shown to signal through the canonical pathway in a number of oncological models (116, 117) and in synapse formation (118). There is evidence that Wnt7a induction of AMHR2 is also mediated by the canonical pathway. A luciferase reporter gene driven by the *AMHR2* promoter was activated by β-catenin and the activation was dependent on TCF4 binding sites in the AMHR2 promoter (119). Both *Wnt7a* (114) and β-catenin (120) are required for female MD differentiation indicating that β-catenin is a downstream effector of Wnt7a for this function.

Genes induced *via* the AMH canonical pathway

Several genes have been identified that are induced *via* the AMH canonical pathway. *Wnt4* is expressed in a sexually dimorphic pattern during the period of MD regression. *Wnt4* starts to be expressed at E13.5 in the MD mesenchyme of male mice, but not female mice, and persists in males throughout the period of regression (121). *Wnt4* expression was absent in *Amhr2*-null males, but present in the MD mesenchyme of females ectopically expressing human AMH, indicating that AMH signaling is both required and sufficient for *Wnt4* expression at the onset of MD regression (121). A conditional knockout of Wnt4 using *Amhr2-cre* mice showed that MD regression occurred normally in the absence of *Wnt4*, indicating that it is not required for MD regression.

Expression of a matrix metalloproteinase, Mmp2, is highly upregulated in the MD mesenchyme of male, but not female mice at day E13 (20). In *Amh*-null males, the male-specific activation of Mmp2 was not observed. MD regression was partially blocked in rat urogenital organ culture by protease inhibitors and antisense oligonucleotides to Mmp2, while exogenous Mmp2 triggered apoptosis in the MDs of female urogenital ridges (20). A role for Mmp2 in regression would be

consistent with previous studies showing a correlation between MD regression and degradation of the extracellular matrix (21–23). However, a knockout of *Mmp2* in male mice did not affect MD regression, indicating that it is not required for MD regression. Humans with defects in MMP2 display a severe impairment of bone and cartilage development, suggesting that Mmp2 may act redundantly with other Mmp genes in the mouse.

Wnt inhibitory factor 1 (WIF1) is a secreted frizzled-related protein that blocks binding of Wnts to receptors. *In situ* hybridization revealed a pattern of expression of *Wif1* in male mesenchyme similar to *AMHR2*, that was absent in urogenital ridges from females and *Amhr2*-null males (122). Exposure to exogenous AMH induced *Wif1* expression in the MD mesenchyme of female urogenital ridges, while knockdown of *Wif1* expression in male urogenital ridges by *Wif1*-specific siRNAs resulted in MD retention, consistent with WIF1 playing a role in MD regression (122). However, a knockout of *Wif1* in male mice did not affect MD regression, indicating that it is not required for MD regression, or that there is another similar gene that can replace its function. Two members of a different family of Wnt inhibitors, secreted frizzled-related proteins (SFRPs), are also expressed in a sexually dimorphic pattern (123). SFRP2 and SFRP5 proteins are expressed in the mesenchymal cells of the male MD, but not in mice with *Wnt7a* null mutations which lack AMHR2. Mice containing null mutations in both *Sfrp2* and *Sfrp5* exhibit normal MD regression.

β-catenin is required for MD regression

With several Wnts and Wnt antagonists being induced by AMH in the MD mesenchyme and Wnt7a having an essential role in MD regression, the question was raised whether the Wnt canonical pathway played an essential role in regression. An early insight into this matter was the observation that AMH signaling induces the accumulation of β-catenin in the cytoplasm and the transcription factor, LEF1, in the nucleus of male MD mesenchyme cells (25). Cytoplasmic accumulation of β-catenin was also observed in MD mesenchymal cells of female rat urogenital ridges exposed to AMH, but not in untreated female urogenital ridges. Subsequently, it was shown that conditional inactivation of β-catenin in the MD mesenchyme using *Amhr2-cre* mice, caused MD retention in males, indicating a requirement of β-catenin for MD regression (121). The accumulation of β-catenin in the cytoplasm was significantly reduced and LEF1 upregulation was not observed in the MD mesenchymal cells of the conditional β-catenin mutant males at E14.5, confirming that β-catenin had been specifically inactivated in these cells. However, at E13.5, both *AMHR2* and *Wnt4* were expressed in the MD mesenchyme of the conditional β-catenin mutant males, indicating that AMH signaling was activated in the MD mesenchymal cells in the absence of β-catenin function.

The perturbations observed after the conditional inactivation of β-catenin and the finding that AMH is required for the cytoplasmic accumulation of β-catenin in male MD mesenchymal cells (25) indicate that there is a Wnt/β-catenin pathway required for MD regression which is downstream of the AMH and Wnt7a signaling pathways (121). Although *Wnt4* would be a likely candidate for this Wnt/β-catenin pathway, the conditional knockout of *Wnt4* did not cause MD retention, indicating that either more complete recombination might be required by the Cre-recombinase, perhaps because *Wnt4* encodes a secreted protein, or another Wnt mediates this Wnt/β-catenin pathway (121). Apoptosis of MD epithelial cells requires this downstream Wnt/β-catenin pathway. When β-catenin was inactivated in males, the level of cleaved caspase-3 positive in the MD epithelium was significantly reduced, indicating that β-catenin is required in MD mesenchymal cells to induce apoptosis in MD epithelial cells (121).

Integration of AMH, Wnt, and other pathways

The expression of *Osterix (Osx)*, a C2H2-type zinc transcription factor, in male MD mesenchymal cells, is regulated by both of the AMH and Wnt canonical pathways (124). *Osx* was identified using transcriptional profiling methods and shown to have a male specific pattern of expression in MD mesenchymal cells. *Osx* expression was absent in *Amhr2*-null males, but present in the MD mesenchyme of females ectopically expressing human AMH, indicating that AMH signaling is both required and sufficient for *Osx* expression at the onset of MD regression. In mice with β-catenin conditionally inactivated, there was a significant decrease in the level of *Osx* transcripts in the MD mesenchyme, indicating that *Osx* is also regulated by the Wnt/β-catenin pathway. Furthermore, MD regression was delayed in *Osx*-null male mice, demonstrating that *Osx* regulates MD regression (124). In **Figure 7**, the activation of *Osx* is shown by activated Smad and β-catenin/TCF complexes binding individually to promoter/enhancer sequences, but it has been shown that BMP can induce an interaction between Smad1 with β-catenin and TCF4 to activate the c-myc promoter (125). Also, Smad3 (126) and Smad4 (127) have been shown to associate with β-catenin and TCF/LEF1 to activate the Xenopus twin promoter. So formally, it is possible that the Smad and β-catenin/TCF complexes associate to activate *Osx* transcription.

In hindsight, it is not surprising to find that Wnt and AMH pathways are integrated to bring about regression of the MD, since previous studies have established that Wnt and BMP pathways interact during other biological processes. This interaction can occur in the cytoplasm as effectors of one pathway are modified by effectors from the other pathway, and in the nucleus where the Smad and TCF/LEF transcription factors converge to regulate transcription. During embryonic patterning in *Xenopus*, dorsal-ventral or anterior-posterior cell fates are controlled by gradients of BMPs or Wnts, respectively (128); integration may occur at the level of Smad1 phosphorylation by GSK-3 (101). Both pathways participate in the development (129) and regeneration (111) of the hematopoietic system. Chromatin immunoprecipitation studies have shown that Smad1 and TCF7L2 occupy sites close to other transcriptional regulators in the promoters of hematopoietic genes (111). It is likely that other signaling pathways are involved in MD regression. Overactivation of the hedgehog

pathway has been shown to interfere with MD regression, indicating that the AMH and hedgehog signaling pathways probably intersect in places (130).

Smad independent pathways

Activated receptors of the TGF-β family also signal through non-Smad signaling pathways. This includes the mitogen-activated protein kinase (MAPK) pathways, which comprise the extracellular signal-regulated kinase (ERK), c-Jun amino terminal kinase (JNK), and p38 MAPK families. In addition, the activated receptors can signal *via* IκB kinase (IKK), phosphatidylinositol-3 kinase (PI3K) and Akt, and Rho family GTPases (131, 132). The non-Smad pathways are, in general, activated by phosphorylation of tyrosine residues on either type I or II receptors, and the recruitment of proteins that bind to phosphotyrosine. Tyrosine phosphorylation can result from autophosphorylation or cross phosphorylation of type I and II receptors, or phosphorylation by other tyrosine kinases (131). Although the kinases of TGF-β type I and II receptors mostly phosphorylate serine and threonine residues, they are also capable of phosphorylating tyrosine residues (133). At present, there are only a limited number of reports that AMHR2 can signal *via* Smad independent pathways. AMH was reported to inhibit the androgen dependent growth of a prostate cancer line *via* an NFκB-dependent, but Smad-independent mechanism (134). In another study, blocking AMH signaling in two lung cancer cell lines resulted in lower levels of phosphorylated (and activated) Akt; lower levels of the phosphorylated Akt activator, PDK1, were also observed (135).

Non-Smad signaling pathways are associated with TGF-β receptors that reside in caveolar compartments of the cell membrane (a special type of lipid raft), similar to receptor tyrosine kinases, whereas Smad-mediated signaling pathways are associated with TGF-β receptors that reside in clathrin-dependent endosomal compartments (132). Localization to caveolae can lead to restricted lateral movement and an enrichment of receptors (136). It should also be noted that, in addition to transcriptional regulation, R-Smads have a role in chromatin remodeling required for transcriptional activation and in the processing of microRNAs involved in RNA silencing (15).

CONCLUDING REMARKS

The goal of this review article was to illuminate how AMH interacts with its type II receptor to activate a canonical pathway resulting in the induction of several genes and how the AMH canonical pathway is integrated with a Wnt pathway required for MD regression. However, details in some areas have been limited in the interest of space and comprehension. For more information, the reader is referred to the following reviews (14, 99, 132, 137). One topic that was not covered is how the AMH signals can be distinguished from BMP signals, an issue that is receiving considerable attention. As noted in a recent review article by Nickel and Mueller (138), "It seems illogical that on the one hand Nature has diversified growth factors of this family to more than 30 known members, but at the same time restricted the signaling outcome of all ligands to initiate intracellular signaling pathways in just two different flavors: Smad1/5/8 or Smad2/3". Part of the answer may lie in understanding how differences in the binding affinities and kinetics of specific ligand-receptor interactions affect the assembly of receptor signaling complexes, and whether distinct receptor signaling complexes can be assembled because of these differences that have distinct signaling properties (138).

AUTHOR CONTRIBUTIONS

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

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Molecular Mechanisms of AMH Signaling

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Anti-Müllerian Hormone (AMH) is a secreted glycoprotein hormone with critical roles in reproductive development and regulation. Its chemical and mechanistic similarities to members of the Transforming Growth Factor β (TGF-β) family have led to its placement within this signaling family. As a member of the TGF-β family, AMH exists as a noncovalent complex of a large N-terminal prodomain and smaller C-terminal mature signaling domain. To produce a signal, the mature domain will bind to the extracellular domains of two type I and two type II receptors which results in an intracellular SMAD signal. Interestingly, as will be discussed in this review, AMH possesses several unique characteristics which set it apart from other ligands within the TGF-β family. In particular, AMH has a dedicated type II receptor, Anti-Müllerian Hormone Receptor Type II (AMHR2), making this interaction intriguing mechanistically as well as therapeutically. Further, the prodomain of AMH has remained largely uncharacterized, despite being the largest prodomain within the family. Recent advancements in the field have provided valuable insight into the molecular mechanisms of AMH signaling, however there are still many areas of AMH signaling not understood. Herein, we will discuss what is known about the biochemistry of AMH and AMHR2, focusing on recent advances in understanding the unique characteristics of AMH signaling and the molecular mechanisms of receptor engagement.

Keywords: anti-müllerian hormone (AMH), anti-müllerian hormone receptor type II (AMHR2), transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), activin, persistent müllerian duct syndrome (PMDS), cell signaling, prodomain

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INTRODUCTION

Anti-Müllerian Hormone (AMH), also known as Müllerian Inhibiting Substance (MIS), was first described by Alfred Jost in 1946 as a secreted testicular factor which drove the regression of the Müllerian ducts in the male fetus (1). Importantly, dysregulation of this mechanism presents clinically as Persistent Müllerian Duct Syndrome (PMDS), in which loss of function mutations in AMH or its signaling receptor, Anti-Müllerian Hormone Receptor Type II (AMHR2), lead to persistence of Müllerian duct derivatives – uterus, fallopian tubes, cervix, and upper vagina – in males (2, 3). In women, AMH is a negative regulator of folliculogenesis and dysregulation of the signaling pathway has been implicated in two leading causes of female infertility: Polycystic Ovary Syndrome (PCOS) and Primary Ovarian Insufficiency (POI) (4). Since its initial description, more recent characterization of this hormone has provided foundational insights into our current understanding of the structure and function of AMH and its signaling pathway.

AMH is a glycoprotein hormone (5) which shares structural and mechanistic homology with signaling proteins of the Transforming Growth Factor β (TGF-β) family (6). This family consists of over 30 secreted signaling ligands that have essential functions for many processes regulating cell homeostasis and human development, including reproductive development (7). These ligands are synthesized from a precursor consisting of a large N-terminal prodomain and smaller C-terminal mature signaling domain (**Figure 1A**). Folding, dimerization, and secretion are regulated by prodomains, which are cleaved from the smaller signaling domain and, in most cases, remain noncovalently associated (**Figure 1B**) (8). Ligands signal by binding to the extracellular domain (ECD) of two type I and two type II serine/threonine receptor kinases. This complex brings the intracellular kinase domain (ICD) of the constitutively active type II receptor in close enough proximity to phosphorylate the GS domain of the of the type I receptor ICD, relieving inhibition and activating Smad transcription factors (9) (**Figure 1C**). Signaling within the TGF-β family is limited to specific combinations of the seven type I receptors, Activin-like kinases 1-7 (ALK1-7), and five type II receptors, ActRIIA, ActRIIB, BMPR2, TβR2, and AMHR2 (7). It has been shown that AMH will mainly signal using ALK2 (10, 11) or ALK3 (12–14), type I receptors used by the bone morphogenetic protein (BMP) branch of TGF-B ligands, and activation of BMP R-Smads 1, 5, and 9 as well as activation of BMP reporter genes (15, 16). The other BMP type I receptor, ALK6, has a stimulatory or inhibitory effect depending on the tissue type (17, 18). AMHR2 is unique within the TGF-β family as it is the only receptor specific for a single ligand (19). In this review, we will summarize the current biochemical understanding of AMH as a TGF-β ligand from secretion to signal, with a focus on recent efforts to characterize the binding of AMH to AMHR2 and the looming gaps the field must overcome in order to better understand this important biological pathway.

PROCESSING AND REGULATION OF AMH & AMHR2

AMH was first identified as a TGF-β ligand by sequence similarity of its C-terminal mature signaling domain with Activins and TGF-βs (20) and the proteolytic processing of this domain (6). The full open reading frame of human AMH (UniProtKB P03971-1) consists of a signal sequence (SS) (residues 1-24), prodomain (residues 25-451), and mature domain (residues 452-560) (**Figure 1A**) (6). Human AMH is processed canonically; mammalian proprotein convertases (PCs), such as furin, will cleave the proprotein downstream of an a R-X-X-R motif at amino acid position 448-451 to generate the 109 amino acid mature domain (21–24). Similar to other family members, PC cleavage separates the N-terminal prodomain from the C-terminal mature domain, which allows for assembly into a noncovalent complex (**Figure 1B**) (6, 25–27). Only the cleaved, processed, dimer form can properly bind its receptors and induce downstream signaling (28), but evidence of mixed circulating species of AMH suggests a regulatory role of this processing (29). Both the processed and unprocessed species are found in the serum and follicular fluid (25, 30) in varying ratios depending on age (27), sex (27), and disease state (31, 32). Interestingly, alternative cleavage products resulting from serine proteinase activity within the prodomain (**Figure 1A**) have been described during purification (6, 25, 33–35), however their biological relevance remains unknown.

The processing of AMHR2 (UniProtKB Q16671-1) is less characterized than its ligand. While there has been robust study of type II receptor regulation within the TGF-β family *via* mechanisms of internalization (36–40), homo- and heteromeric complex formation (41–43), and glycosylation (44, 45), for AMHR2, the understanding of regulation is currently limited to biosynthetic processing and surface presentation alone (46, 47). Unlike other type II receptors, it has been suggested that functional presentation of AMHR2 at the membrane is negatively regulated by cleavage or by disulfide-linked oligomerization of the extracellular domain, leading to increased retention within the ER (46). Additionally, those functional receptors that are presented appear to organize in clusters of homo-oligomers, resulting in a lack of lateral mobility (46). In mammals, receptor splice variants have been identified that result in the deletion of either amino acids 377-471 (Amhr2Δ9/10) within the kinase domain, or 17-77 (Amhr2Δ2) within the extracellular domain (47–49) (**Figure 1A**). Although their mRNA expression level in the testes and brain is 5% or less of the normal receptor, the existence of these variants raises interesting questions about their regulatory function in the AMH signaling pathway (47). Thus, continued investigation of the functional consequences of these or other splice variants is necessary to understand their potential impact on signaling. Lastly, Unlike other TGF-β ligands, investigations into mechanisms of extracellular antagonism of AMH have not been definitive (50–52). Nevertheless, regulation of ligands by protein antagonists represents a significant feature of TGF-β ligands, and the lack of known AMH-binding proteins is either a missing piece of the known mechanism or an interesting aberration from other family ligands.

THE ROLE OF THE AMH PRODOMAIN

It is widely accepted that the prodomains of TGF-β ligands are required for proper folding and dimerization of the mature signaling ligand (53–55). While most prodomains are similar in size, an indication of secondary structure elements, there are exceptions. For example, GDF15 maintains the smallest prodomain of 18.5 kDa whereas AMH has evolved the largest of the prodomains at 45 kDa. Furthermore, unlike the ligands which typically have a conserved patter of cystines, the prodomains exhibit significant differences in the number and placement of cysteines, indicating structural divergence (7). For TGF-β1-3 the prodomains from two different chains are joined by a disulfide bond (56). The intermolecular disulfide bond increases the affinity of the prodomains for the mature ligand thorough avidity effects. Similarly, AMH also exhibits an

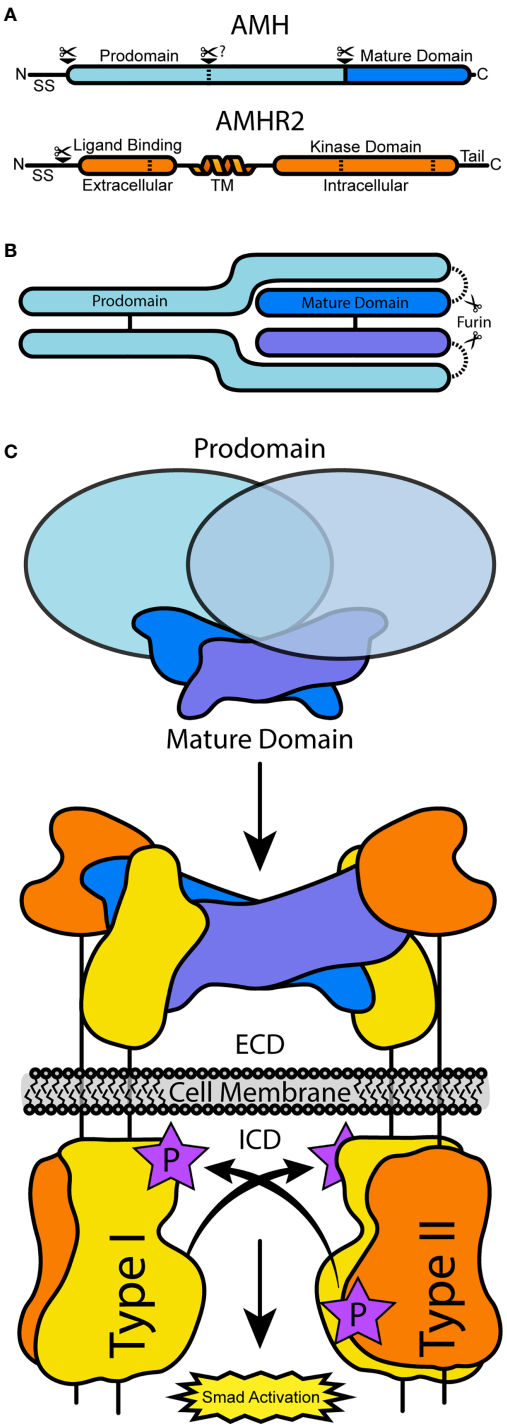

FIGURE 1 | A schematic of AMH and AMHR2 processing and receptor assembly. **(A)** The full translated sequences of AMH and AMHR2 undergo processing to cleave the signal sequence. In AMH, PCs will cleave at the solid bar, separating the prodomain and mature domain, while alternative processing may occur at the dashed bar. In AMHR2, dashed bars represent alternative splicing sites. **(B)** Assembled AMH pro-complex, which may or may not be cleaved. **(C)** AMH-driven receptor assembly at the cell surface, resulting from AMH binding AMHR2 and prodomain dissociation. Type I receptors are activated and in turn activate BMP R-Smads.

interchain disulfide bond which likely increases its affinity for the mature ligand (6).

For some ligands, such as the TGF-βs, GDF8, and GDF11 the prodomain maintains the ligand in a latent state, and activation occurs thorough proteolysis (57–59) of the prodomain or an integrin-mediated stretching mechanism (56), both of which liberate the bound ligand from the prodomain. For AMH, BMPs, and other activin ligands, the prodomain does not render the ligand latent and the ligand is either thought to signal in the presence of the prodomain or that the prodomain is readily displaced by binding the signaling receptors. For AMH, the prodomain has been shown to allosterically regulate AMH binding to AMHR2 without inhibiting signal (28, 60). This mechanism is similar to the non-latent BMP7 pro-complex, however unlike AMH, the BMP7 prodomain has a weakly competitive interaction with the BMP type II receptors and unchanged type I receptor interactions (61). Further parallels might be drawn from the crystal structure of the BMP9 pro-complex bound by ALK1, which shows that the type I receptor can associate without displacing the prodomain (62), but this remains untested for AMH. Unlike most BMPs, the AMH prodomain has a 10-100 fold higher affinity for the mature ligand (K_d = 0.4 pM) (60). Despite this high-affinity interaction, bivalent binding to AMHR2 presented on a surface is able to disrupt interactions and attenuate binding 1000-fold (60). Displacement appears to be dependent on the avidity as neither monovalent binding nor soluble receptor are able to induce prodomain displacement. Thus, while certain ligands have high affinity for their prodomain and confer latency, many BMP ligands have lower affinity for their prodomains and are more readily displaced by receptor binding (8, 58). AMH appears somewhat unique in that it maintains a very high affinity for the ligand, but the prodomain can also be displaced by cell surface receptors. The high affinity of the prodomain of AMH suggests that the prodomain is likely to play an important role in either protecting AMH or facilitating signaling.

As mentioned, the prodomain seems to be an additional and principal factor of regulation within the signaling pathway. The prodomain is required for proper folding, homo- or heterodimerization, and secretion (7, 63–65), and the presence of PMDS mutations within the prodomain support this mechanism for AMH (2). In the serum, there is no unbound mature AMH ligand (25), suggesting a role for the prodomain in shuttling the mature domain to nearby and distant targets. The endocrine character of AMH is a robust research area, as we have yet to fully comprehend the breadth of extragonadal signaling targets (4, 18, 66, 67). For other ligands, the pro-complex also functions as a shield from extracellular antagonists (23, 33, 64, 68, 69). The interface between BMP antagonist Crossveinless 2 and BMP2 is analogous to the interface between mature BMP9 and the BMP9 prodomain (7), so the large AMH prodomain might function to protect AMH from interactions with a milieu of extracellular matrix (ECM) components. On the other hand, prodomains seem to be important for targeting the mature ligand to the cell surface through interactions with heparin (8), fibrillin (70), and other components of the ECM (7). However, unlike many BMPs, AMH does not have

large positively-charged patches of amino acids which would limit its interactions with heparin; it instead has a significant hydrophobic character (71). We do know, however, that the prodomain is necessary for activity in tissue-based assays (72) but dispensable for cell-based assays (47, 52, 73). This suggests that the prodomain likely does not play a major role in the signaling mechanism but might play a larger role in the availability of the ligand by mediating ECM interactions or conferring protection from degradation or antagonism.

The prodomain itself may also be subject to regulatory mechanisms common to the TGF-β family. In this vein, the previously mentioned alternate cleavage sites of AMH (6, 74) (**Figure 1A**) might have some bearing on the activity of the noncovalent complex. Conformational changes (7), alternative cleavage (57, 75, 76), or other uncharacterized modifications (77) have been shown to prime the noncovalent complex for receptor interactions. Furthermore, there are 2 N-linked glycosylation and several possible O-linked glycosylation sites predicted within the AMH prodomain (78) comprising 13.5% of the complex mass (71). Differential modification of glycosylation may impact protein-protein interactions or cleavage, as observed in drosophila with the ortholog of BMP7, Gbb (76). Largely, we lack understanding of the regulatory role of the AMH prodomain beyond its absolute necessity for secretion and activity in the body. Whether the AMH prodomain, which is the family's largest and most divergent, has additional function beyond increasing the availability of the AMH signaling ligand is not known. While recent advances in modeling using AlphaFold can help to visualize structure and domain architecture of the AMH prodomain (79), the lack of structural definition of this region and low homology cause difficulty in effectively modeling the AMH prodomain (62, 63, 80–82). As such, structural and biochemical characterization of the prodomain structure and its interfacing interactions with the mature ligand and receptors will help ascertain its function.

STRUCTURAL DEFINITION OF AMH AND AMHR2

The TGF-β family is part of the cystine knot growth factor (CKGF) superfamily (7) which have a conserved fold and sequence. The overall shape can be described as an opposite-facing left and right hands in a Vulcan salute joined at the palm (**Figure 2A**). This creates a concave pocket between the wrist helix of one chain and the fingertips of the opposing chain, to which the type I receptor is recruited, and a convex surface on a single chain at the "knuckle" region, to which the type II receptor binds for Activins and BMPs (84). The extracellular ligand-binding domain of the type II receptors adopt a three-finger toxin fold, which has also classically been described with a hand-like morphology, consisting of three anti-parallel beta strand fingers and a central palm region (**Figure 2B**). These features are also conserved for AMH and AMHR2 as shown in the recently solved structure of the extracellular complex (85).

In general, ligands have evolved two central binding modes to interact specifically with their type II receptors (**Figure 2C**). Ligands of the Activin and BMP class bind at the convex, knuckle surface of the ligand fingers, while the TGF-β class ligands bind the fingertips (84). For AMH, the binding mode was unknown until the recently-solved crystal structure of AMH bound to AMHR2 (PDB:7L0J) (85). This structure provided a critical piece for understanding ligand-receptor interactions and disease-causing mutations (**Figures 2D–E**), revealing that while similar to the general binding mode of BMP and Activins, AMH utilizes a modified mode of type II receptor binding (85).

The receptor binding interaction of AMH and AMHR2 is unique within the family. While TGF-β class ligands bind TβR2 using finger 1 of the receptor and the fingertips of the ligand, Activin and BMP class ligands bind ActRIIA and ActRIIB at the palm of the receptor and the knuckles of the ligand (86–91) (**Figure 2C**). Like Activins and BMPs, AMH binds AMHR2 using the palm of the receptor and the knuckles of the ligand, however this interface is shifted towards the fingertips by about 7.5 Å. Additionally, fingers 1 and 3 of AMHR2 wrap around the ligand making unique contacts with sections of AMH not observed with Activins and BMPs, especially within fingers 3 and 4 of AMH and the connecting loop (85). While the mature ligand is similar to each of the other three classes (the root mean square deviation of the Cα positions is below 2 Å for BMP2, GDF11, and TGF-β1) the AMH ligand adopts a flat character of the fingers akin to TGF-βs.

The structure of AMH bound to AMHR2 highlighted structural differences in each that are likely responsible for specificity. Of note, AMH has a truncated finger 1/2 loop relative to other ligands that facilitates the wrap-around mechanism of AMHR2 (85). More significant variance is observed on the receptor side with differences between AMHR2 and other type II receptors undoubtedly contributing to specificity. Most notably is the conformation of finger 1 of AMHR2 which is extended compared to other receptors and forms a favorable interaction with AMH. While the number of disulfide bonds are similar, a shift of one cysteine (Cys60) results in unique structural character of AMHR2. The altered location of the disulfide bond brings together the finger 2/3 loop and finger 3 to create unique surface for AMH binding (85). These distinct conformational features of AMHR2 promote the selectivity of AMH binding and signaling.

Where we still lack critical information is in our understanding of the nature of interactions between AMH and its type I receptors. The affinity of AMH for its type II receptor has been shown to resemble TGF-βs or Activins, while affinity for the type I receptor, though not yet directly tested, is assumed to follow the same low affinity archetype as the above ligand classes (28, 73, 85). What is known is that AMH lacks two conserved tryptophan residues present in the type I receptor binding site of BMPs. Importantly, these residues have been shown to be necessary for proper signaling in members of the BMP class (92). In fact, the entire type I binding interface is dissimilar to that of BMPs (86, 93, 94) and contains more polar and charged residues, yet AMH will signal using the same type I receptors – ALK2, ALK3, ALK6 – as BMPs. It will be interesting to determine how AMH

accommodates for its binding and specificity of type I receptors with these differences. It is possible that type I receptor binding is shifted relative to BMPs and might even potentially interact with the type II receptor in a cooperative mechanism similar to TGF-β, however, this has yet to be explored.

DISCUSSION

Recent studies have revealed a wealth of information about the molecular mechanisms of AMH signaling, but the field has a long way to go towards a full understanding of the intricacies of this

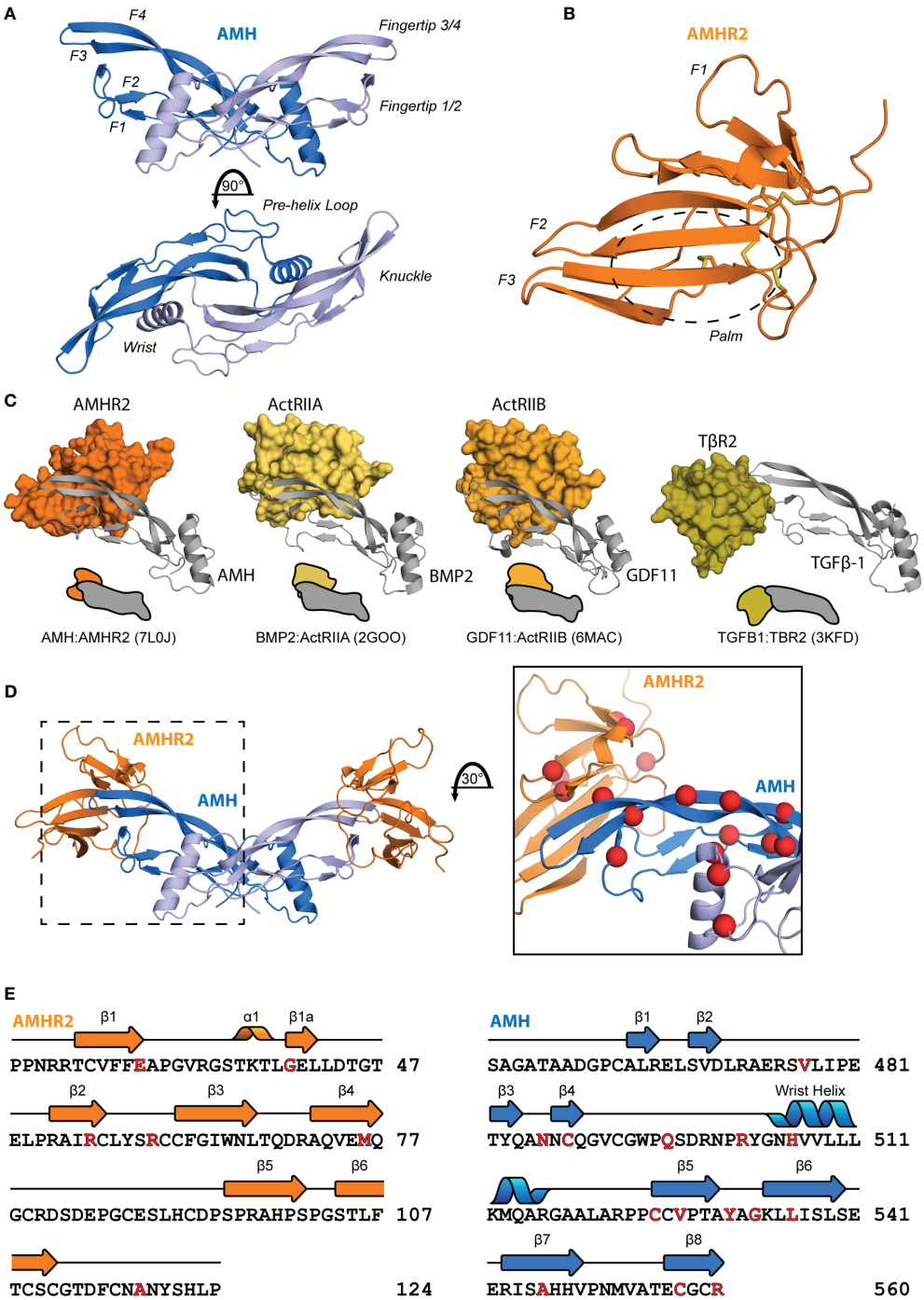

FIGURE 2 | Structural features of AMH and AMHR2. Diagram of the structure of mature AMH **(A)** and AMHR2 ECD **(B)**. **(C)** Comparison of the binding modes of each ligand class to a type II receptor. **(D)** Mapping of PMDS mutations (83), indicated by red spheres, to the binary complex. **(E)** Sequence of AMH and AMHR2 annotated with secondary structure features and highlighted in red with the above PMDS mutations.

unique pathway. The biochemistry of AMH is certainly less characterized than its TGF-β family counterparts. Knowledge of these structures and their interactions can help explain the expanding genetic information linked to human diseases, such as PMDS and PCOS. For example, several mutations have been identified in the prodomain, however, we lack the structural information needed to better understand how these mutations impact AMH function.

The interaction between mature AMH and the extracellular domain of AMHR2 is perhaps the most actionable piece of recent data characterizing AMH. The crystal structure demonstrates unique features that set AMH apart from other ligand classes, as well as an atomic-level explanation for PMDS mutants which map to the interface (**Figures 2D–E**). This story is, of course, incomplete without a structure of mature AMH bound by a type I receptor. While the mutual exclusivity of the AMH and AMHR2 interaction is an interesting feature within the TGF-β family, equally interesting is how the intracellular kinase domain of AMHR2 can employ type I receptors shared with BMPs yet propagate an AMH-specific signal. Intracellular interactions remain something of a black box for the fields of both TGF-β and AMH biology.

Looking ahead, further structural studies of the AMH ligand and receptor are warranted; these studies must be supported by stronger assay development. Most importantly, the field should address discrepancies between *in vitro* and *in vivo* studies, especially concerning the prodomain. It has been known for some time that the AMH prodomain is required for biological function. This feature is reflected in tissue-based assays but not in cell-based assays where the mature ligand will suffice. Better care should be taken to include the prodomain, when possible, to better replicate the biological context of AMH and allay concerns about differences between these two assay systems. Additionally, the ability to distinguish between the transcriptional outcome of AMH and BMP signaling would be a great and powerful tool for probing the mechanisms of the signaling pathway at every level. Research into this area might also help to answer a major question of AMH signal in general: is there a signaling cascade unique to AMH, or does AMH modulate a pre-existing BMP signal to generate unique outcomes? Although the research areas in need of attention are difficult, fresh data and new techniques have done wonders to answer critical questions and spark novel hypotheses about how this pathway truly functions.

AUTHOR CONTRIBUTIONS

JH and KH developed the concept. JH wrote the manuscript. KH and TT edited and revised the manuscript for important intellectual content. All authors read and approved the submitted version.

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REVIEW
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One Tool for Many Jobs: Divergent and Conserved Actions of Androgen Signaling in Male Internal Reproductive Tract and External Genitalia

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In the 1940s, Alfred Jost demonstrated the necessity of testicular secretions, particularly androgens, for male internal and external genitalia differentiation. Since then, our knowledge of androgen impacts on differentiation of the male internal (Wolffian duct) and external genitalia (penis) has been drastically expanded upon. Between these two morphologically and functionally distinct organs, divergent signals facilitate the establishment of tissue-specific identities. Conversely, conserved actions of androgen signaling are present in both tissues and are largely responsible for the growth and expansion of the organs. In this review we synthesize the existing knowledge of the cell type-specific, organ specific, and conserved signaling mechanisms of androgens. Mechanistic studies on androgen signaling in the Wolffian duct and male external genitalia have largely been conducted in mouse model organisms. Therefore, the majority of the review is focused on mouse model studies.

Keywords: androgen, androgen receptor (AR), external genitalia (ExG), wolffian duct, penis, masculinization

INTRODUCTION

Androgen signaling pathway is essential for development of male organs across vertebrate animals (1). This pathway is responsible for the growth and differentiation of the prostate, penis, epididymis, vas deferens and seminal vesicle during embryonic development for normal structural development and function. Since the 1940s, we have known that the loss of testis-derived androgens disrupts male reproductive development. XY embryos without the testes develop into female sexual characteristics due to the absence of male-specific reproductive structures (2, 3).

Due to the dependency on androgen signaling, male reproductive structures are particularly sensitive to disrupted fetal development and adulthood dysfunction (4, 5). The incidence rate of children born as intersex, a condition where patients contain a combination of male and female reproductive organs, is relatively common, with incidence ranging from 0.37-1.7% cases per year (6). The intersex spectrum includes defects of the penis (e.g. hypospadias and micropenis) and defects in the reproductive tract (e.g. cryptorchidism). Often times the androgen-related congenital

defects of the external genitalia and Wolffian duct lead to compromised reproductive functions and infertility.

In this review, we discuss the diverse and conserved androgen actions in two critical reproductive organs, the Wolffian duct-derived tissues and the male external genitalia, specifically the penis, during embryonic development. We review the literature with the goal of understanding the pleiotropic nature of AR. To do this, we tease apart the cell populations of each organ. Then we investigate the androgen signaling programs that are specific to each cell population. By reviewing the literature in this way, we can begin to discern the molecular determinants that specify the distinct androgen signaling programs in each cell population. To further understand the general functions of AR, we discuss common androgen signaling events in both the Wolffian duct and male external genitalia. Because our knowledge about androgen signaling in male sexual differentiation derive largely from mouse studies, the majority of the following sections focus on mouse models. Reviewing both the internal and external reproductive tract provides a roadmap for future studies and allows us to investigate the many diverse and conserved action of androgen signaling.

MECHANISMS OF ANDROGEN SIGNALING PATHWAY

Androgen signaling pathway is mediated by the androgen receptor (AR), which mainly acts as a ligand-activated transcriptional factor (7). Loss of function mutations of AR in mice lead to feminization of internal reproductive tract organs and external genitalia even though the fetal production of androgens is unaffected (8). Human XY individuals with androgen insensitivity syndrome caused by disruptions in the androgen signaling cascade develop phenotypically female internal and external genitalia (9). Conversely, XX patients with congenital adrenal hyperplasia, a disorder characterized by excessive production of androgens in adrenal glands, develop masculinized external genitalia (10). Thus, androgens are the driving force for male sexual differentiation.

Androgens are first produced by the Leydig cells in the fetal testis, in which express the key steroidogenic enzymes for androgen production Cytochrome P450 17a1 (*Cyp17a1*) and 3β-Hydroxysteroid (3β-HSD) (11). These two enzymes and other enzymes in the cascade of steroidogenesis convert cholesterol to androgens (11, 12). The testis-derived androgens then enter the blood circulation and reach the target organs. Once androgen reaches an organ that expresses AR, it passively diffuses through the cell membrane and either bind directly to AR in the cytosol, or be converted into a more potent androgen, dihydrotestosterone, by the 5-α reductase (SRD5a1 and SRD5a2) enzymes (13). Binding of testosterone or dihydrotestosterone to AR triggers the release of AR from molecular chaperones and translocation of AR to the nuclei, where it regulates target gene expression.

In addition to the classic genomic action, binding of androgens to AR in the cytoplasm initiate signal transduction pathways to modulate cellular proliferation and migration, which is also known as non-genomic actions of the AR (14). When the DNA-binding domain, which transmit the genomic action of AR, is removed from AR, male mice display a complete androgen insensitive phenotype with feminized external genitalia and loss of internal reproductive tracts (Wolffian duct and prostate) (15). These results underscore the genomic actions of the AR are indispensable for normal male sexual differentiation. Another study generated transgenic mice expressing mutant *Ar* with only genomic or non-genomic actions in the absence of endogenous AR (16). They found that seminal vesicle and epididymis are degenerated in both mutant mice, suggesting both genomic and non-genomic actions are required for normal development of Wolffian duct-derived tissues. On the other hand, main actions of AR in external genitalia are mediated through the genomic actions based on the observation that mice with a mutated non-genomic region of *Ar* develop normal penises (17).

HETEROGENEITY OF ANDROGEN ACTIONS IN THE WOLFFIAN DUCT

Ontology of Wolffian Duct-Derived Organs

Wolffian ducts are paired embryonic structures that give rise to male internal reproductive tract organs including the epididymis, vas deference and seminal vesicles (18). In both XY and XX mice embryos, the Wolffian duct derives from the intermediate mesoderm at around embryonic day 8.5 (E8.5) and elongates craniocaudally till it reaches the cloaca by E9.5 (19). The Wolffian duct is surrounded by its mesenchyme, which expresses AR first on E12.5 in both sexes (20, 21). In XX embryos, where ovaries do not produce androgens, AR action does not take place, leading to degeneration of Wolffian ducts (18). In addition to the lack of AR action, Wolffian duct degeneration in XX embryos requires COUP-TFII (Chicken ovalbumin upstream promoter transcription factor II or NR2F2), an orphan nuclear receptor specifically expressed in the Wolffian duct mesenchyme (22). On the other hand, in XY embryos, fetal testes produce androgens starting from E12.5 (23). Under the influence of testis-derived androgens, the Wolffian ducts are stabilized and then undergo regionalization and differentiation into the epididymis, vas deferens and seminal vesicle in a craniocaudal fashion.

Mesenchymal AR Actions Are Critical for Wolffian Ducts Differentiation

Although the AR is expressed in both the epithelium and mesenchyme during Wolffian duct differentiation, AR action in the mesenchyme appears to govern fetal Wolffian duct development. Ablation of AR in the Wolffian duct epithelium does not affect Wolffian duct maintenance or morphogenesis (20). The specific role of AR in Wolffian duct mesenchyme has not been determined directly by mesenchyme-specific AR knockout model; however, classic tissue recombination studies imply that the mesenchymal AR actions govern androgen-

induced epithelial morphogenesis. For example, the epithelium of upper Wolffian ducts (future epididymis) develops seminal vesicle-like structures when combined with lower Wolffian duct mesenchyme (prospective seminal vesicle) (24). The urogenital sinus is another androgen target tissue, which undergoes prostatic morphogenesis upon androgen actions but develops vagina-like morphology in the absence of androgen. When wild-type mesenchyme is grown adjacent to either wild-type or Tfm (testicular feminized mice that lacks functional AR) epithelium, the urogenital sinus still undergoes prostatic morphogenesis. In contrast, when urogenital sinus epithelium is recombined with the mesenchyme from Tfm embryos, the epithelium develops vagina-like structures (25). These results demonstrate that mesenchymal AR actions dictate Wolffian duct differentiation.

How Do Mesenchymal AR Actions Induce the Stabilization of Wolffian Ducts?

One possible signal downstream of mesenchymal AR actions in promoting Wolffian duct survival is epidermal growth factor (EGF), which regulates a wide range of cellular events including cell survival and proliferation (26). *Egf* expression is elevated in the whole mesonephric tissue during fetal male reproductive tract differentiation and flutamide (AR antagonist) exposure during sexual differentiation decreased *Egf* in male mice embryos (27). Receptors for EGF or EGFR is an integral membrane tyrosine kinase that is activated upon binding of multiple ligands including EGF. Expression of EGFR is also induced by testosterone and inhibition of EGFR using an anti-EGFR antibody blocks Wolffian duct growth in cultured murine mesonephros (28). Global *Egfr* knockout in mice causes genetic background dependent placental abnormalities and embryonic lethality before E11.5, preventing analysis of Wolffian duct development in male embryos (29). *Egf* knockout mice have normal phenotype (30), suggesting other ligands for EGFR might compensate for the loss of *Egf* in Wolffian ducts.

To promote Wolffian duct stabilization, mesenchymal AR actions need to antagonize inhibitory effects of another mesenchymal transcriptional factor, COUP-TFII. COUP-TFII is an orphan nuclear receptor expressed in the mesenchymal compartment of many developing organs (31, 32), including the mesonephros in XX and XY embryos (33). In XX embryos, the absence of *Coup-tfII* increased expression of two mesenchymal growth factors *Fgf7* and *Fgf10*, which activated their receptor FGFR2 in Wolffian duct epithelium to promote Wolffian duct survival in XX embryos in the absence of AR actions (22). These results suggest that under normal conditions, COUP-TFII action in the Wolffian duct mesenchyme is to inhibit Wolffian duct survival by suppressing FGF signaling. In XY embryos, to promote the stabilization of Wolffian ducts, the mesenchymal AR is speculated to induce the EGFR-mediated signaling pathway in Wolffian duct stabilization, therefore, providing another survival signal different from *Fgfs* that are suppressed by COUP-TFII. Genome-wide binding of COUP-TFII in the XX and XY mesonephros reveals that COUP-TFII binding motif is different from established AR binding motifs, providing another evidence that these two mesenchymal transcriptional factors target differential genes in regulating Wolffian duct survival (34).

How Do Mesenchymal AR Actions Induce Region-Specific Gene Expression and Differentiation in Wolffian Ducts at the Fetal Development?

AR actions in the mesenchyme drive the differentiation of the Wolffian duct into three morphologically and functionally distinct organs: the epididymis, vas deferens and seminal vesicle during fetal development from rostral (i.e. anterior) to caudal (i.e. posterior) regions (**Figure 1**). Regionalization of the Wolffian duct along the rostral-caudal axis is regulated by region-specific expressed HOX genes in the epididymis (*Hoxa9* (36), *Hoxd9* (36) and *Hoxa10* (37), vas deference (*Hoxa10* (37)

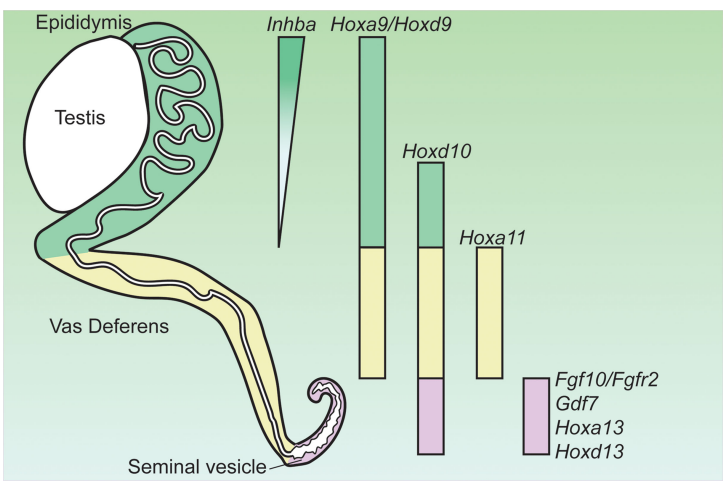

FIGURE 1 | Expression of genes critical for the regionalization of the Wolffian duct and potentially involved in region-specific androgen actions. Hox, homeobox; Inhba, inhibin β A; Gdf7, growth differentiation factor 7; Fgf10, fibroblast growth factor 10; Fgfr2, fibroblast growth factor receptor 2. Adapted from (35).

and *Hoxa11* (38)), and the seminal vesicle (*Hoxa13* and *Hoxd13*) (39) (**Figure 1**). Deletion of these region-specific Hox genes can often cause homeotic transformations. For example, *Hoxa10* mutant males have anterior transformation of the cauda epididymis and the proximal vas deferens (37, 40). The vas deferens in either individual *Hoxa11* knockout or *Hoxa11* and *Hoxd11* (a paralog to *Hoxa11*) double males (38, 41) resemble an epididymis. Seminal vesicles become hypoplastic in both *Hoxd13*$^{-/-}$ and *Hoxa13* $^{+/-}$; *Hoxd13*$^{-/-}$ compound mutant mice (42). In addition to *Hox9-13*, additional region-specific *Hox* transcripts, such as *Hoxc4*, *Hoxc6*, *Hoxc9* have been identified in the epididymis by comparing gene transcriptional profiles of the epididymis, vas deferens and efferent duct at E14.5, E16.5, E18.5 and P1 (43). Although roles of *Hox* genes in the regionalization of the Wolffian duct is well recognized, it is still unknown how these *Hox* transcription factors interact with AR actions and determine region-specific AR actions.

Mesenchymal AR actions induce region-specific paracrine mesenchymal growth factors for regulating epithelial morphogenesis in Wolffian duct differentiation. In the rostral (anterior) region, AR action promotes epididymal coiling which relies on the action of inhibin β A (*Inhba*) (44). *Inhba* is a critical component of inhibins and activins (members of TGFβ superfamily ligand) and is highly expressed in the mesenchyme of the anterior Wolffian duct before the initiation of epididymal morphogenesis (**Figure 1**) (44). Androgen partially increased *Inhba* expression after E13.5 based on *ex vivo* organ culture studies (44). Male embryos lacking *Inhba* fail to develop epididymal coiling due to a dramatic decrease in epithelial proliferation even if AR expression is intact (44).

In addition to INHBA, mesenchyme-derived WNT ligands or WNT signaling activators may also play a role in epididymal morphogenesis. The WNT signaling pathway is an evolutionarily conserved pathway that regulates organogenesis (45). The WNT signaling pathway includes the extracellular WNT ligands, which stimulate intracellular signal transduction cascades to regulate gene expression and cellular differentiation in target cells (46). Two intracellular pathways in the target cells mediate WNT signaling: β-catenin (CTNNB1)-dependent or -independent pathways (46). The WNT/β-catenin signal also depends on the Wnt signaling activators, R-spondins (RSPOs), which elicit their functions by binding to the WNT co-receptors (*Lgr4-6*, the leucine-rich repeat-containing G-protein-coupled receptors) and preventing the destabilization of the Wnt receptors. As a result, RSPOs act in concert with WNTs to promote the activation of the β-catenin-mediated intracellular signaling (46). Wolffian duct epithelium displays high WNT/β-catenin activity during epididymal coiling (47). When β-catenin is deleted specifically in the Wolffian duct epithelium before the region-specific patterning, the epididymis fails to coil at birth with significant reduction in proliferation and increases in cell death (47, 48). The defective morphogenesis (cystic formation) in the epididymal region is also observed when *Pkd1* (a receptor for mediating WNTs actions) is deleted in the epithelium (49, 50). The WNT receptor LGR4 is specifically expressed in the cranial region of fetal Wolffian ducts and postnatal epididymides (51, 52). Inactivation of *Lgr4* caused the cystic formation in the epididymis (51). Given the critical roles of WNT/β-catenin activities in the epithelium and AR actions in the mesenchyme in epididymal morphogenesis, mesenchymal AR might induce WNT ligands or activator as paracrine signals to regulate epididymal morphogenesis.

In the caudal region, another secreted ligand of TGFβ superfamily GDF7 is required for seminal vesicle growth, morphogenesis, and epithelial differentiation (**Figure 1**) (53). *Gdf7* is expressed in the seminal vesicle mesenchyme but its expression in other regions of Wolffian duct is not reported (53). *Gdf7*$^{-/-}$ males develop normal testis, epididymis, vas deferens and prostate; however, seminal vesicles are dramatically smaller and lack epithelial folding with decreased number of basal cells (53). In addition to *Gdf7*, AR actions in caudal Wolffian duct development are mediated by FGF10/FGFR2 signaling (54, 55). *Fgf10* (fibroblast growth factor 10) is a member in a gene family of generally extracellular signaling peptides, which are key regulators in organ development. The mesenchyme of the caudal Wolffian ducts expressed high *Fgf10*, which is increased by androgen treatment (**Figure 1**) (56). Global knockout of *Fgf10* led to the absence of seminal vesicle and distal vas deferens despite of normal testicular development (55). FGF10 has a high specificity for FGFR2, which is the major FGF receptor in the Wolffian duct epithelium (54). Wolffian duct-specific deletion of *Fgfr2* led to the degeneration of the caudal Wolffian ducts (54). FGF7 was also considered as a key mesenchymal paracrine growth factor for seminal vesicle development in *ex vivo* culture condition (57, 58). However, *Fgf7* knockout males does not display any reproductive phenotype (MGI: 95521), suggesting that *Fgf7* is dispensable in AR actions in promoting Wolffian duct development *in vivo*. These observations demonstrate that mesenchymal AR actions regulate region-specific morphogenesis *via* paracrine growth factors.

How Are Mesenchymal AR Actions Affected by Epithelium-Derived Signals?

The crosstalk between the mesenchyme and epithelium during organogenesis is never a one-way street. The Wolffian duct epithelium-derived signal also has reciprocal inductive effects on the mesenchyme and can potentially influence mesenchymal AR actions. Wolffian duct epithelium specifically synthesizes a paracrine factor WNT9B, a member of the WNT ligands (45). *Wnt9b*$^{-/-}$ male embryos fail to form Wolffian duct derived organs at birth despite of normal testis development (59). The loss of the Wolffian duct-derived organ is also observed in male embryos that lack the direct upstream regulator of *Wnt9b, HNF1B* (60). In the *Hnf1β*$^{-/-}$ mice, the Wolffian duct are still partially present on E14.5 during sexual differentiation of Wolffian ducts. These observations suggest that Wolffian ducts in *Hnf1β*$^{-/-}$ *or* *Wnt9b*$^{-/-}$ male embryos could be in process of degeneration on E14.5 when androgen actions are supposed to promote Wolffian duct stabilization. These observations indicate that signals derived from Wolffian duct epithelium are required for mesenchymal actions in stabilizing Wolffian ducts.

HETEROGENEITY OF ANDROGEN SIGNALING IN THE EXTERNAL GENITALIA

Androgen signaling is absolutely essential for sex differentiation of the external genitalia, making it susceptible to disruptions of androgen signaling as a result of genetic mutations or exposure to anti-androgenic chemicals. Defects of external genitalia are some of the most common birth defects in the world (61). Hypospadias, which is where the urethra exits not at the tip but along the shaft of the penis, is one of the most common birth defects of penis (61). Even though the requirement of androgen signaling in penis development has been known since the 1940's, the causes for more than 70% of hypospadias cases remain unknown (62). One possible explanation for the unknown nature of hypospadias, is that penis formation is a product of hormone action and local cell-cell interactions. The intertwined actions of these pathways make the penis susceptible to genetic mutations and environmental exposure (62, 63). Researchers are only beginning to understand how all the cell types of the penis coordinate under the control of androgen to form a normal penis.

Establishment of the Unique Androgen Responsive Programs in Penis Cell Populations

Penis development can be divided into two major phases, androgen-independent and androgen-dependent development (**Figure 2**) (66). The androgen-independent phase involves the transformation of the primitive cloaca into the genital tubercle, the precursor of the penis. At the end of embryonic development, the genital tubercle morphs into the penis that consists of the distal dorsal glans, distal ventral glans, proximal glans, prepuce, corporal bodies, and urethra epithelium (67). The distal dorsal glans, distal ventral glans, and urethral epithelium have distinct cellular origins. It is not elucidated whether the proximal glans, prepuce, and corporal bodies come from diverse cell origins, or a derived population of mesenchymal cells. It is the diverse origins of the cells and their cell states from the androgen-independent phase that imparts diverse androgen responsiveness during the androgen-dependent phase.

During the androgen-independent phase of fetal development, the cloaca arises between the hindlimbs of the embryo of both sexes (**Figure 2**). The cloaca is an ancestral structure present in most vertebrates where both the gastrointestinal and genitourinary tracts merge. Around mid-gestation or E10.5 in the mouse, the cloaca is a single chamber lined with epithelium, which is surrounded by mesenchymal cells. The mesenchymal cells contain two major populations that are associated with penis development, abdominal mesenchyme, and tailgut mesenchyme (**Figure 2**). The abdominal mesenchyme eventually differentiates into the distal dorsal glanular mesenchyme and the tailgut mesenchyme differentiates into the ventral glanular mesenchyme. The abdominal mesenchyme is specifically marked with the expression of *Alx4* gene while the tailgut mesenchyme is enriched for *Six1* and *Six2* genes (68, 69). Mutations of *Alx4* in mice lead to severe defects of the distal dorsal glans (68). Cultured genitalia with the abdominal mesenchyme removed, develop with an absent dorsal glans, displaying the importance of these progenitor cells in normal penis development (68). Mutations in the *Six1* and *Six2* genes cause defects in the ventral glans and urethra closure defects (69). *Six2* expressing cells at E13.5 and E14.5 comprise the distal ventral glans by E17.5 (64). These observations of *Alx4* and *Six2* expressing cells reveal that before the penis is formed, the distal dorsal glanular and distal ventral glanular mesenchyme have already established their unique identities in the cloaca.

Once the cloaca is separated into the gastrointestinal and genitourinary tracts around E13.5, the urethra epithelium appears along the ventral aspect of the genital tubercle. At this same time in development, two preputial buds form on either side of the established genital tubercle and the proximal glanular mesenchyme becomes present at the base of the genital tubercle (65). It is not clear if the preputial buds and the proximal glanular mesenchyme are derived from the abdominal or tailgut mesenchyme or if they originate from other sources.

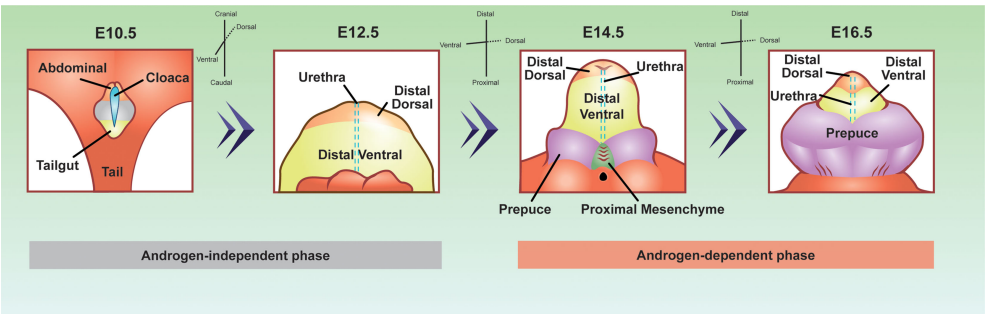

FIGURE 2 | *Developmental origins of the penis cell populations in the embryonic mouse penis.* The colors represent conserved cell populations through time. Blue=cloacal/urethra epithelium, yellow=tailgut/distal ventral glanular mesenchyme, light orange=abdominal/distal dorsal glanular mesenchyme, purple=prepuce, green=proximal glanular mesenchyme, and grey= undefined penile cell population. The E10.5 depiction is looking at the lower half of the embryo, while the other time points are focused on the genitalia. E10.5 depiction was adapted and redrawn from (64). E12.5, 14.5, and 16.5 were adapted from whole mount, scanning electron microscope images from (65).

The above described androgen-independent phase ends around E13.5, when androgen production by the testis starts. Simultaneous with androgen production, AR protein is most present in the cytoplasm of the urethral epithelium and the surrounding mesenchyme (70). One day later on E14.5, AR protein become localized in the nucleus of the proximal glanular mesenchyme, distal dorsal glanular mesenchyme, distal ventral glanular mesenchyme, prepuce, and urethral epithelium (71). From E14.5 through the rest of embryonic development, AR protein steadily increase in all mesenchymal cells of the penis and remains present in portions of the urethra epithelium (48). Disruptions in androgen signaling by fetal exposure to anti-androgenic chemicals or genetic knockouts of *Ar* cause severe cases of hypospadias, underlining the importance of androgen signaling in penis development.

Androgen-Dependent Closure of the Proximal Urethra

The closure of the proximal urethra starts at E13.5 and E14.5 in the mouse embryo when the urethra is still an open sulcus at the base of the penis (72). By E15.5, the proximal portion of the urethra starts to close and form an internalized tube (72). To accomplish this event, extensive androgen-dependent communication occurs between the urethral epithelium and proximal glanular mesenchyme. Although the urethral epithelium expresses *Ar* mRNA and protein throughout development (67, 73), AR in the urethral epithelium is not required for urethral closure and proper penis formation (70, 74) (**Figure 3**). Conversely, inactivation of *Ar* in the surrounding mesenchyme causes severe cases of hypospadias, indicating the necessity of mesenchymal androgen signaling for urethra closure (70, 74–76).

The proximal glanular mesenchyme is a group of mesenchymal cells that surrounds the urethra. These mesenchymal cells are sites of strong androgen signaling, largely due to abundant expression of *Srd5a2* that convert testosterone to the more potent dihydrotestosterone (77). As a result, dihydrotestosterone is twice as concentrated in the proximal glanular mesenchyme compared to other areas of the penis (77). The abundance of androgens facilitates the expression of key androgen responsive genes in the proximal glanular mesenchyme, like the transcription factor *Mafb* and the AR co-chaperone *Fkbp5*, both essential for urethra closure (75, 78). The expression of *Mafb* is not only dependent on AR binding to the ARE, but also requires the co-regulator SP1 to bind at the *Mafb* enhancer element. Loss of SP1 binding motif in the *Mafb* enhancer element results in severe urethra closure defects (79) (**Figure 3**). The combination of the high concentration of androgens and unique presence of co-activator provide the proximal glanular mesenchyme with the unique ability to orchestrate urethra closure.

Two possible mechanisms of androgen-dependent urethra closure are proposed between the proximal glanular mesenchyme and the urethral epithelium. One is through mechanical forces that help push the urethra closed, and the other is cell-cell interactions through morphogens that instruct structural changes of the epithelium and mesenchyme. Androgen signaling in the proximal glanular mesenchyme induces contractility and expression of several muscle related genes such as myosin heavy chain 10 (*Myh10*), *Myh9*, and F-actins (80, 81). Myosin and F-actin provide the cells the capacity to contract and impart physical lateral forces on surrounding cell populations. Inactivation of *Myh9/10* in the proximal glanular mesenchyme in mice causes severe urethra closure defects arise due to the loss of capability to exert physical forces on the urethra (80) (**Figure 3**).

The contractility capability of the proximal glanular mesenchyme is not only induced directly by androgens, but also by androgen-dependent morphogen pathways from the urethra and proximal mesenchyme. The WNT signaling pathway, identified through the Gene Ontology analyses on the proximal mesenchyme, is specifically enriched in the proximal glanular mesenchyme of the penis (67). The main WNT ligands,

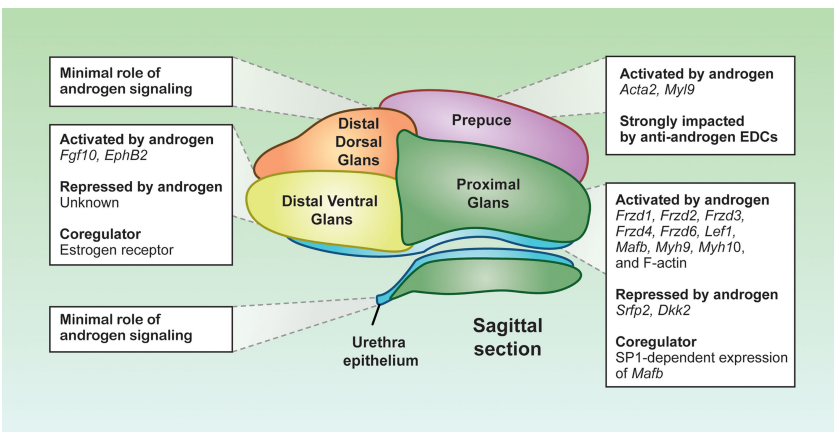

FIGURE 3 | *Distinct androgen responsiveness of the penile cell populations.* Representative sagittal section depiction of an E16.5 mouse penis. Each color represents a distinct cell population, displaying the localization of the cell populations in the penis. Boxes are the androgen-related programs for each subpopulation. Depiction was generated from sagittal sections of mouse penises generated in the Yao laboratory.

Wnt5a, Wnt6, Wnt7a, Wnt10a and *Wnt2*, are expressed throughout the penis, with *Wnt5a* being the main effector in the proximal glanular mesenchyme (82, 83). Removal of *Wnt5a* expression in the proximal glanular mesenchyme disrupts the contractility phenotypes of the proximal glanular mesenchymal cells and caused severe hypospadias-like phenotypes in the mice (84). However, *Wnt5a* expression does not seem to be controlled directly by androgens (84). Several of the WNT receptors and transcription factors, in contrast to *Wnt5a*, are induced by androgen signaling (85). The WNT receptor, FRZD6, and the WNT-related transcription factor such as LEF1 are significantly enriched in the male proximal mesenchyme, when compared to the female (85). Exposure of female embryos to androgen (methyltestosterone) increases expression of both FRZD6 and LEF1 proteins (86). Other WNT receptor gene expression, *Frzd1*, *Frzd2*, *Frzd3*, and *Frzd4*, are also significantly elevated in methyltestosterone exposed female (85). In addition, the WNT ligands and receptors, WNT inhibitors, *Dkk2* and *Sfrp2*, are higher in female genitalia compared to males, and the expression of these WNT inhibitors significantly lessen with androgen exposure (**Figure 3**) (74). These findings reveal that closure of the proximal urethra requires a complex WNT signaling crosstalk between the proximal glanular mesenchyme and urethral epithelium in an androgen-dependent fashion.

A Role for Estrogen Receptor Coordination With AR in Distal Urethra Closure

The closure of the urethra occurs in a proximal (base) to distal (tip) fashion along the ventral aspect of the penis (87). At E16.5 in the mouse embryo, the urethra is largely closed in the proximal portion, but remains open in the distal portion of the penis (65). The distal ventral glanular mesenchyme subsequently interacts with the urethra to form a tube and complete the closure of the urethra. Similar to the proximal glans, the distal ventral glans express myosin light chain 12a (*Myl12a*) and *Myl6* and is enriched with actin-cytoskeleton signaling pathway components, suggesting that it may exert similar mechanical forces on the distal urethra (67). The expression of these genes are also elevated in the male distal ventral glans compared to the female, an indication that they could be controlled by androgens.

Other than the androgen signaling, the distal ventral glans has the highest expression of estrogen receptor (ER) compared to the rest of the penis (67). Estrogen receptor and AR can be both antagonistic and agnostic to one another depending on hormone levels and tissue types (**Figure 3**) (88). For example, the estrogenic chemicals such as estradiol benzoate, diethylstilbesterol, and 17β-estradiol, cause only distal hypospadias (70, 89, 90). The degree of defects in urethra closure drastically differs among different anti-androgen exposure, which induces severe hypospadias where the urethra exits at the base of the penis (91). ER may be antagonizing AR at hormone response elements along the DNA within the distal ventral glans. Or estrogen-dependent signaling could result in gene expression that inhibits androgen-dependent signals (**Figure 3**). Although aberrant exposure to exogenous estrogens causes distal hypospadias, the ER signaling pathway appears to be essential for normal penis development in some capacity. Estrogen receptor α or *Esr1* knockout mice develop mild hypospadias in adulthood, similar to estrogen exposed mice (90). These data suggest that a properly tuned estrogen signaling in the penis is essential, where too little or too much estrogen is detrimental to penis development.

This proposed role of estrogen signaling in penis development is likely required to modulate appropriate morphogen expression. One such morphogen is fibroblast growth factor or FGF. In the fetal mouse penis, FGF10 ligand and its receptor FGFR2IIIβ are uniquely expressed in the distal ventral glans (92, 93). Global knockout of *Fgf10* causes severe hypospadias with no urethra closure occurring throughout the penis (92, 93). *Fgf10* is tightly linked to both androgen and estrogen signaling in many hormone responsive tissues (**Figure 3**). In the ventral prostate and the uterus, estrogen exposure and *Esr1* are essential for establishing appropriate *Fgf10* expression (94, 95). In the penis, *Fgf10* is induced with androgen supplementation and not much is known about it estrogen responsivity (93). The interactions between androgen and estrogen receptor signaling on gene expression remains to be elucidated in the distal ventral glans.

Androgen-Dependent Encapsulation of the Glans by the Prepuce

The prepuce in the male mouse embryo originates as two bilateral buds on the lateral sides of the glans at the onset of androgen-dependent development, followed by extensive outgrowth after androgen production in the fetal testis. Soon after the preputial buds form on the penis, the prepuce begins to express *Ar* (67). In the male embryo, the cells within the prepuce have a stretched, migratory phenotype with a clear directionality pointing toward the urethra (80). The prepuce starts to fuse along the midline of the penis at E15.5. Closely following the process of urethra closure, the prepuce eventually fuses to the tip of penis and fully encapsulates the glans and urethra.

Inactivation of the androgen signaling, either through global *Ar* knockout or exposure to environmental chemicals, result in severe aplasia of the prepuce and a lack of fusion along the ventral aspect of the penis (70, 91). The majority of hypospadias cases are accompanied with preputial defects (96, 97). When external genitalia are cultured without androgens, preputial cell migration does not occur (80). The migratory loss could be due to the diminished expression of smooth muscle actin (*Acta2*) and myosin light chain 9 *(Myl9*), which are both expressed in the developing prepuce (67) (**Figure 3**). The correlations between preputial fusion and urethra closure suggest a role of the prepuce in urethra closure.

Estrogen and Androgen-Dependent Differentiation of the Corporal Bodies

The corporal bodies of the penis are mesenchymal condensations that arise within the glans of the penis later in development around E17.5-E18.5 in mice (98). The corporal bodies are column condensations that run throughout the entire penis. In adult life, the corporal tissues are essential for erectile function.

Mice have three major corporal tissues, the corpus cavernosum, corpus cavernosum glandis, and corpus cavernosum urethrae (99). The corpus cavernosum is located in the internal portion of the penis and connects to the penis bone, which maintain extrusion of the penis from the prepuce during erection (98). Where the corpus cavernosum ends close to the proximal end (or base) of the penis, the external portion of the penis (glans) begins. Within the glans lies the corpus cavernosum glandis as a muscular ring around the outer edge of the penis. Toward the center of the glans is where the corpus urethrae located. The corpus urethrae helps maintain an open urethra during copulation (98). Development of the three corporal tissues are androgen-dependent in mice. In the *Tfm* mice, where AR is nonfunctional, the corporal tissues are drastically reduced (100). The corpus cavenosum glandis and corpus urethra express *Ar*, *Esr1*, and *Esr2*. *Esr1* knockout mice fail to develop the corpus cavernosum glandis, while the *Esr2* knockout mice fail to develop the corpus urethra (101). In humans, both corporal tissues express AR and ESR2 (102), suggesting a potential interplay between the androgen and estrogen signaling pathways.

Dorsal Mesenchyme of the Penis as an Androgen Insensitive Cell Population

In contrast to the tissues in the proximal and ventral penis, the distal dorsal glans seems to have weak androgen responsiveness and plays a minor role in the androgen-dependent processes of penis development. Unique to the male mice, a portion of the distal dorsal glans differentiates postnatally into a cartilaginous mating protuberance, or the male urogenital mating protuberance (MUMP) (99), which is not found in female mice nor in the human (99). In addition to the MUMP, the distal dorsal glans is involved in the overall growth and size of the penis. Genetic mutations of dorsal glans gene *Inhba* in mice caused the abnormally enlarged penis (103). The role of androgen signaling within the distal dorsal glans remains to be determined.

COMMONALITY OF AR- MEDIATED MALE SEXUAL DIFFERENTIATION OF WOLFFIAN DUCT AND EXTERNAL GENITALIA

Androgen Action Must Be Imposed Within a Short Developmental Period Known as Masculinization Programming Window

The action of AR must be imposed within a specific fetal programming window. For the Wolffian ducts, the masculinization programming window is E15.5–E17.5 in rat (104), presumably E14.5-16.5 in mouse given that mouse development is generally 1 day behind that of rat (86), and predicted to be approximately 8-12 weeks of gestation in humans (104). The critical window for the Wolffian duct overlaps considerably with the critical window for the penis, with timeframes ranging from E15.5-18.5 in the rat (104), E14.5-E17.5 in the mouse (105), and 8-12 weeks of gestation in the human (104) (**Figure 4**). Only within this programming window, disruption of androgen actions by AR antagonist flutamide results in partial or complete absence of Wolffian ducts by adulthood in rat. AR action-associated endpoints subside in the presence of flutamide, including cell proliferation, epididymal coiling, epithelial vimentin expression, and smooth muscle actin expression in the Wolffian duct inner stroma (106). Similarly, only within this window does impaired androgen action result in cryptorchidism and hypospadias (104). The most severe cases and highest incidence of hypospadias occurs with flutamide exposure between E15.5 and E16.5 (105). Flutamide exposures before E15.5 can result in severe hypospadias, but low incidence rates, while exposures after E16.5 result in both mild hypospadias cases and low incidence rates (70, 91). The same degree of hypospadias severity was shown to be true for AR knockouts at E14.5 and E17.5. The masculinization window also exists in the female embryos. For example, seminal vesicle formation in female rats is induced by androgen exposure during E15.5-17.5, the masculinization program window but not by exposure during E19.5-E21.5 (104). Female rats develop complete Wolffian ducts and penis only when they are exposed to androgens during this window. The window is tightly regulated, where exposure to androgens before or during the masculinization window does not advance or extend the timing of the critical window (107). The molecular determinants of opening/closing the programming window are not completely understood. It appears that the induction of AR protein in Wolffian duct and penile tissues (20) coincides with the opening of the window.

Androgen Action in the Mesenchyme Regulates Epithelial Morphogenesis

Extensive studies have demonstrated the essential role of AR action in the mesenchyme of Wolffian ducts and penis (**Figure 4**). Epithelial ablation of AR in the Wolffian duct or the penis do not affect maintenance and coiling of the Wolffian duct (20), nor urethra closure in the penis (74). The functional significance of the mesenchymal AR in penis development has been demonstrated in mesenchymal specific AR knockouts mice where severe urethral and penile defects were observed (74). Although the consequences of delating the mesenchymal AR has not yet been determined in Wolffian duct development *in vivo*, classic tissue recombinants studies provide evidence that the mesenchymal AR actions governs androgen-induced epithelial morphogenesis (25) (See the section *Mesenchymal AR actions are critical for Wolffian ducts differentiation*).

Androgen Actions Induce Epithelial Morphogenesis Through Both Growth Factors and Mechanical Forces

In response to androgens, the mesenchyme produces multiple mesenchymal growth factors (FGFs, EGF, and WNT) that mediate mesenchymal AR actions in controlling survival and differentiation of Wolffian ducts and penis (18, 74, 109). Aside from growth factor signaling, mesenchymal AR can also regulate epithelial morphogenesis by inducing expression myosin and actin related genes, which induce mechanical forces on the epithelium (108). During Wolffian duct morphogenesis, inner mesenchymal cells differentiate into smooth muscle on E16.5, the initiation of Wolffian duct coiling. The smooth muscle is known

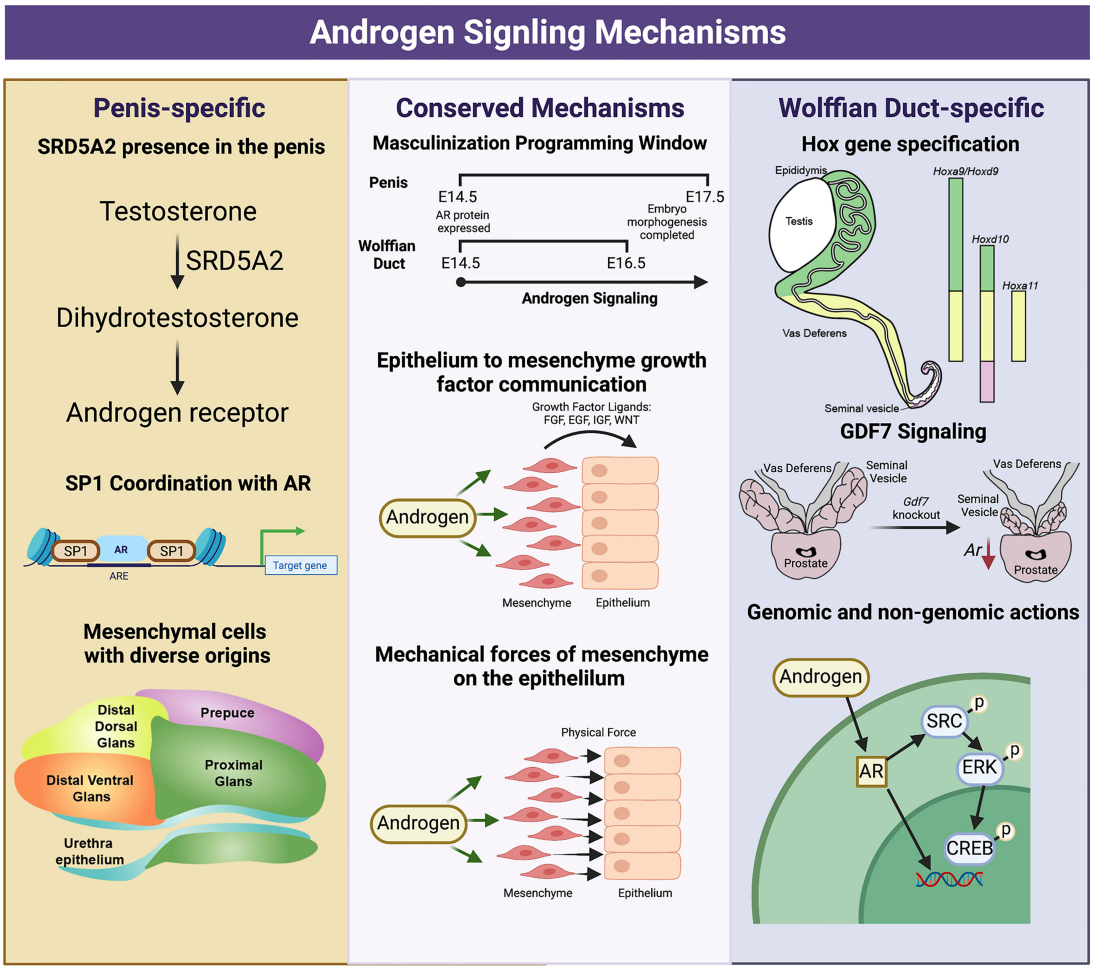

FIGURE 4 | *Divergent and conserved androgen signaling mechanisms in the fetal development of the Wolffian Duct and penis.* The Wolffian Duct and penis have conserved androgen signaling mechanisms that include a window of masculinization, growth factor signaling (EGF,FGF, and WNT) and mechanical forces from the mesenchyme to the epithelium. However, these two organs also possess divergent androgen signaling mechanisms. In the penis, *Srd5a2* is present and converts testosterone to dihydrotestosterone. AR and SP1 interact along the DNA to elicit both gene expression and chromatin modifications, and the mesenchymal cells of the penis have a more diverse community of mesenchymal cells from different development origins. In the Wolffian duct, mesenchyme in different regions expresses region-specific *Hox* genes that govern the regionalization of the Wolffian ducts. The differentiation of the cranial Wolffian duct to the seminal vesicle depends on *Gdf7*. Wolffian duct development requires both genomic and non-genomic androgen signaling while non-genomic androgen signaling is dispensable in the penis development. The figure summarizes data and reviews from (16, 18, 27, 28, 53, 108) on Wolffian ducts and (67, 77, 79, 80, 91, 93, 104, 106) on external genitalia. Figure Created with BioRender.com

to produce mechanical resistance. Blocking α-smooth muscle actin (aSMA), a marker of smooth muscle differentiation, significantly reduces tubule folding without affecting cell proliferation in the tubule or the length of the epididymal tubule (108). In the penis, androgen induced expression of myosin-related genes, *Myh10* and actomyosin contractility in the proximal mesenchyme. *In vivo* genetic ablation of both Myh10 and Myh9 in the bilateral or pharmacological inhibition of actomyosin contractility in *ex vivo* slice culture system induced defective urethral masculinization (80). These results suggest that mesenchyme-derived mechanical force is the other mechanism by which androgen induce epithelial morphogenesis.

SUMMARY

Although AR is a singular transcription factor, it can fill many roles throughout Wolffian duct and male external genitalia development. In both organs, plenty of research has identified androgen responsive programs, but there many questions about the molecular drivers of AR pleiotropy. Future studies that conduct cell type-specific investigations of AR co-regulator interactions, chromatin accessibility of AREs, and multi-organ androgen signaling conservation will bring us drastically closer to understanding the prevalence of androgen-related human birth defects.

AUTHOR CONTRIBUTIONS

CA, FZ, and H-CY were responsible for creating figures, writing content, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Testicular Sertoli Cell Hormones in Differences in Sex Development

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The Sertoli cells of the testes play an essential role during gonadal development, in addition to supporting subsequent germ cell survival and spermatogenesis. Anti-Müllerian hormone (AMH) is a member of the TGF-β superfamily, which is secreted by immature Sertoli cells from the 8th week of fetal gestation. Inhibin B is a glycoprotein, which is produced by the Sertoli cells from early in fetal development. In people with a Difference or Disorder of Sex Development (DSD), these hormones may be useful to determine the presence of testicular tissue and potential for spermatogenesis. However, fetal Sertoli cell development and function is often dysregulated in DSD conditions and altered production of Sertoli cell hormones may be detected throughout the life course in these individuals. As such this review will consider the role of AMH and inhibin B in individuals with DSD.

Keywords: AMH, inhibin B, testes, spermatogenesis, Sertoli cell

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INTRODUCTION

Gonadal development is a complex process, whereby the genital ridge is directed to develop into testes or ovaries. The testicular Sertoli cells play an essential role during gonadal development, in addition to supporting subsequent germ cell survival and spermatogenesis (1). Sertoli cells are responsible for the production of a variety of factors including hormones, binding proteins and signalling molecules that regulate testicular development and function throughout life. Anti-Müllerian hormone (AMH) and inhibin B are hormones that play a role in sex development during fetal life, as well as regulation of spermatogenesis in adulthood. Differences/Disorders of Sex Development (DSD) are a heterogeneous group of conditions with a wide range of aetiologies and clinical features. The presence of measurable AMH and inhibin B in infants with DSD suggests the presence of testicular tissue and indicates Sertoli cell function. However, in people with a DSD, fetal Sertoli cell development and function can be dysregulated. As both of these hormones can be useful clinically, this review will focus on the role of testicular AMH and inhibin B in individuals with DSD.

PATHWAYS OF TYPICAL SEX DEVELOPMENT AND TESTICULAR CELL DIFFERENTIATION

In human embryos, gonadal precursors are present from 32 days post conception and the gonads are bipotential until 6 weeks of gestation (2). As the testicular cords are established at 6-7 weeks post fertilisation, the Sertoli and interstitial cells (including Leydig-lineage cells) originate from common gonadal progenitors and subsequently differentiate (3, 4)

Sry, sex determination region on Y chromosome is largely responsible for testis differentiation. The Sry gene is expressed in pre-Sertoli cells at 7 weeks in the XY gonad and encodes a high mobility group (HMG) box transcription factor, which binds to specific target sequences in DNA, resulting in DNA bending (5). The expression of Sry is initiated by multiple transcription factors including GATA4/FOG2/NR5A11/WT1, resulting in induction of SOX9 expression. This is further augmented by the synergistic actions of Sry and NR5A1, leading to definitive Sertoli cell differentiation (6, 7).

Much of the data relating to sex differentiation in mammals is derived from mouse studies. However, studies using human fetal tissues provide support for these mechanisms also being important for sex development in humans. Mamsen et al. undertook gene expression analysis of key genes associated with gonadal development in 67 human first trimester fetuses obtained during elective termination of pregnancy. This study demonstrated that in the bipotential gonad, WT1 and NR5A1 were highly expressed, although concentrations of WT1 decreased over time. SOX9 gene expression increased to a peak at day 48. AMH was detected in Sertoli cells from 48 days. SRY expression peaked at 44 days post conception and then decreased to basal levels at day 60 (8). That said, SRY expression has also been identified in 46,XY gonads up to 18 weeks gestation in human embryonic and fetal tissue (9).

Once formed, Sertoli cells induce the development of fetal Leydig cells, *via* a *hedgehog* signalling pathway (10). At 8-9 weeks of development, the Leydig cells start to produce androgens and insulin-like factor 3 (INSL3) (11). Masculinisation of the indifferent external genitalia is induced by testosterone produced by the Leydig cells between weeks 9-20 of gestation (12, 13). Testicular descent is primarily under the control of the Leydig cells *via* the actions of INSL3 and testosterone. INSL3 regulates the first phase of testicular descent, acting *via* cyclic AMP with downstream effects *via* Wnt, β-catenin and BMP, causing the gubernaculum to swell, dilating the future inguinal canal and holding the testis close to the groin as the fetal abdomen enlarges between weeks 8-15 of gestation. The second stage of testis descent from the abdomen to the inguino-scrotal region occurs from approximately 25 weeks of gestation due to shortening of the gubernaculum cord and is dependent on the presence of sufficient exposure to androgen (14).

AMH – SECRETION AND REGULATION

AMH, previously known as Müllerian Inhibiting Substance (MIS), is a member of the TGF-β superfamily. It is a 140-kDa dimer glycoprotein, which is secreted by immature Sertoli cells from the 8th week of fetal gestation (15).

The AMH gene is located on chromosome 19 (16). Gonadotrophin-independent transcription is upregulated by SOX9, NR5A1, GATA4, WT1, AP-1 and AP-2. Late in fetal life and after birth, AMH transcription falls under the control of FSH *via* the adenyl-cyclase cyclic AMP (cAMP) pathway (17). Increased testicular AMH production in response to FSH activates protein kinase A (PKA)-mediated induction of SOX9, SF1, NFkB and AP-2, which bind to specific response elements on the AMH promoter (17, 18). FSH stimulation upregulates AMH transcription by phosphorylating the transcription factors binding to the promoter (19), an effect which is downregulated by testosterone (15).

Immunohistochemical labelling of testes has shown that the AR is expressed weakly in 2-15% of Sertoli cells from approximately the age of 5 months until the age of 4 years resulting in a physiological Sertoli cell androgen insensitivity during fetal and early postnatal life, which may protect the testes from premature Sertoli cell maturation (20). Expression progressively increases thereafter such that 90% of boys had high levels of AR expression from the age of 8 years (20). However, the AMH promoter does not have androgen response elements, and as such the androgen receptor must signal indirectly through SF-1 response elements (21).

INHIBIN B – SECRETION AND REGULATION

lnhibin B is a glycoprotein, secreted by Sertoli cells, which consists of α- and β-subunits. Most of its mechanism of action is *via* antagonism of activins on the activin type I and II receptors but there are some cells with specific inhibin-binding molecules, such as betaglycan (22). Recent genome wide association studies (GWAS) have demonstrated that LRRIQ1 and TSPAN19, two genes located on chromosome 12 may affect inhibin B production (23). Plasma inhibin B measurements reflect both Sertoli cell number and status of spermatogenesis (24). Inhibin B has a complex association with the hypothalamic-pituitary-gonadal (HPG) axis and FSH, with an initially positive association at around 3-6 months of age and prior to the onset of puberty, followed by a negative feedback loop (25).

TESTICULAR HORMONE PRODUCTION IN FETAL AND EARLY POSTNATAL LIFE

Testosterone production by the human fetal testis begins around 8 weeks gestation, with a peak between 14-17 weeks and then a sharp decline, so that in late pregnancy the serum concentration of testosterone is similar in male and female foetuses (**Figure 1**) (2, 26). Gonadotrophins are not required to initiate steroid

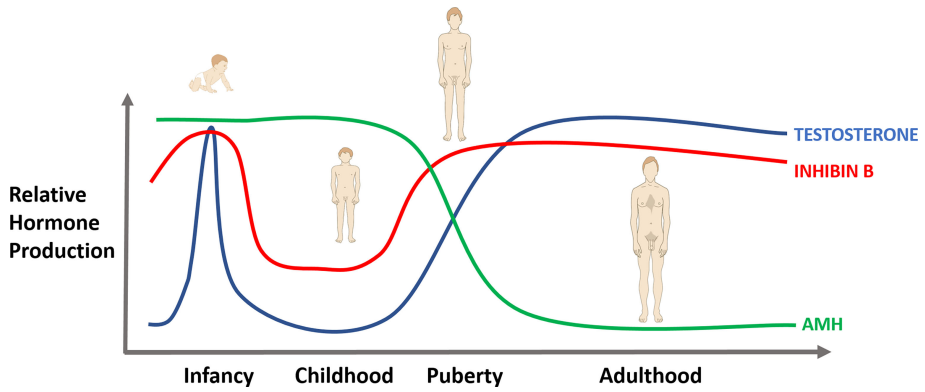

FIGURE 1 | Levels of testosterone, AMH and inhibin B from fetal life to adulthood.

synthesis during this time, but levels of testosterone are closely correlated with human chorionic gonadotrophin (hCG) levels during the early gestational period (2). Levels of testosterone and gonadotrophins are low towards the end of pregnancy and at birth before increasing in the early postnatal period, the so-called 'mini-puberty', and can be used as a window to assess the activity of the HPG axis in the first few months of postnatal life. However, they are of limited use to assess testicular function in the prepubertal boy, because of the relative inactivity of the axis during childhood.

AMH is produced at high levels in early fetal life, but is not measurable in amniotic fluid (27, 28). As the testes differentiate during the 7th week of gestation, Sertoli cells start producing AMH, which binds to the specific AMH Receptor Type II (AMHR2) on the Müllerian ducts, resulting in their regression before week 10 (29). AMH levels rise progressively from this point and then decline in the second year of life (**Figure 1**). AMH then remains stable until puberty, at which point it declines to adult levels, which are 3-4% of those in infancy (30). AMH is therefore an excellent marker of Sertoli cell function in infancy and early childhood, but its use becomes more difficult to interpret in adolescents with lower levels, as at this stage, reduction in AMH is an indicator of pubertal terminal differentiation and failure of the Sertoli cells (31). Serum levels of AMH are 50-fold lower in girls than boys at birth (32) and as such, it is a useful marker to confirm the presence of testicular tissue. No extragonadal sites of AMH production have been reported to date. Given that levels do vary with age, care must be taken with interpretation of AMH to ensure the correct reference range is used.

Inhibin B has been detected from 14-16 weeks gestation (33). and has been detected in umbilical cord samples in boys but not girls (34). Between 3-6 months of age, inhibin B rises in line with FSH and a concurrent increase in Sertoli cell number (35) (**Figure 1**). Levels persist after testosterone, LH and FSH start to fall (25). Levels then remain consistent until approximately 8 years of age, peaking at around age 17 before a slow decline to adult levels (36). Inhibin B is therefore a useful indicator of spermatogenesis and Sertoli cell function in adults, when AMH is no longer measurable.

THE ROLE OF FETAL SERTOLI CELL HORMONES IN DSD

Differences in AMH and inhibin B can be seen in many forms of DSD. These changes have been reviewed extensively elsewhere (37) but this review will discuss some specific DSD conditions.

Sex Chromosome DSD

Klinefelter syndrome, 47,XXY, is the most common sex chromosome anomaly, affecting around 1 in 660 live male births (38). Men with this condition have hypergonadotrophic hypogonadism, as well as being 6 times more likely to have cryptorchidism, which will also impact on Sertoli cell function (39). A study by Aksglaede et al., measured AMH levels in a cohort of 95 men with non-mosaic Klinefelter syndrome and demonstrated that levels remained within the population reference range in infancy, childhood and into adolescence (40). In 47,XXY infants, levels of inhibin B have also been shown to remain in the normal reference range for age (41). Based on these findings, it is presumed that fetal levels of AMH and inhibin B will also be similar between boys with Klinefelter syndrome and controls. However, levels of inhibin B do reduce significantly with age once adulthood is attained, in addition to low AMH levels reflecting the progressive degeneration of the seminiferous tubules (40).

A recent study by Spaziani et al. (10.1007/s40618-020-01281-x) has also found that inhibin B and AMH can be high in early childhood and mini-puberty, suggesting that further research is required in this cohort of patients to confirm expected biochemical findings.

46,XX DSD

Ovotesticular DSD

About 65% of those with ovotesticular DSD have a 46,XX chromosome complement and the ability of the ovotestes to function will be variable. As such, AMH and inhibin B levels in fetal life will vary from being high for female but low for male to normal for male, depending on the clinical phenotype and assays used. Generally, in fully virilised 46,XX males, AMH, inhibin B and testosterone will be within the normal male range in childhood but the germ cells will fail to undergo complete meiosis and undergo apoptosis at puberty, resulting in low testicular volumes (15). In 46,XX children with atypical genitalia, AMH levels above the normal female range are highly suggestive of ovotesticular DSD and exclude the differential diagnoses of congenital adrenal hyperplasia, aromatase defects or virilising tumours.

SOX9 gene variants have been associated with ovotesticular DSD and skeletal dysplasias (42). The SRY gene upregulates SOX9 expression and once levels of SOX9 have reached a critical threshold, several positive regulatory loops are initiated, including autoregulation of SOX9 expression and formation of feed-forward loops *via* FGF9 or PGD2 signalling, which are required for the maintenance and sustained function of Sertoli cells (43). During testicular development, SOX9 functions by regulating the production of AMH from Sertoli cells, and possibly by repressing genes involved in ovarian development such as *Wnt4* and *Foxl2* (44). Studies regarding boys with SOX9 variants, report AMH levels, which are low to low normal (45, 46), with insufficient data regarding inhibin B levels to date.

46,XY DSD

Disorders of AMH and AMH Receptor Defects

From around 7 weeks gestation, the AMH gene is activated by SF1 in Sertoli cells. This leads to the regression of Müllerian structures in the developing male fetus (47). Where there is a mutation in the AMH gene, or in AMHR2, Persistent Müllerian Duct Syndrome (PMDS) occurs. Boys most commonly present with cryptorchidism, inguinal herniae and later infertility. Orchidopexy can be challenging, likely because the testes often have an excessively elongated gubernacular cord, potentially due to the mechanical effects of the retained uterus, or because AMH has an effect on shortening the gubernacular cord (14). A longitudinal study of 157 men with PMDS demonstrated that testicular malignant transformation occurs in 33% of individuals with PMDS (48). Malignancy has been reported in cases of PMDS with cryptorchidism and transverse testicular ectopia but has also been seen in normal testis in patients with PMDS, suggesting that the mechanism for malignancy is not just related to mechanical cryptorchidism (49). In approximately 8% of cases, malignant transformation of the Müllerian remnants can also occur, particularly after puberty, although again the mechanism for this is unknown (50). Treatment is primarily surgical, with excision of Müllerian remnants to allow for orchidopexy. Leydig cell function is typically normal but AMH levels will be low or undetectable in those with an AMH gene variant. In contrast, those with a mutation in AMHR2 will have normal-for-age AMH levels (48).

Disorders of Gonadal Development

Approximately 15% of all cases of 46,XY complete gonadal dysgenesis (CGD) result from a deletion in SRY, with the majority being located within the HMG domain (51). AMH and inhibin B levels will be low/undetectable in individuals with CGD, with the absolute level correlating to the amount of gonadal tissue and number of functioning Sertoli cells present. As such individuals with partial gonadal dysgenesis (PGD) tend to have higher levels than those with CGD (52).

Disorders of Androgen Action

Complete and partial androgen insensitivity syndrome (CAIS and PAIS respectively) are characterised by mutations in the AR gene. Testis differentiation and development, as well as gene expression patterns of AMH and AMHR are independent of AR action up to the second trimester of pregnancy. This has been confirmed by post-mortem examinations of fetuses with AR defects and expression of AMH, AMH2 and testicular differentiation markers (53). During childhood, AMH levels are usually within the normal range in boys with PAIS. By puberty, testicular AMH increases in young people with AIS, in tandem with FSH and oestradiol levels. The expression of Oestrogen Receptor α (ERα) has been confirmed in Sertoli cells from patients with CAIS (54). AMH may be a useful tool to distinguish CAIS and PAIS. A recent study of 29 AIS patients under the age of 11 years reported lower AMH levels in individuals with CAIS compared to PAIS, although still within the normal range for men (55).

Inhibin B levels have been measured in different cohorts of boys with PAIS, with median levels being lower in these boys compared to controls at all ages but usually still within the normal range and higher than in other forms of XY DSD (56, 57). No statistically significant differences are reported in inhibin B between individuals with CAIS and PAIS (55).

Disorders of Androgen Synthesis

Studies of inhibin B and AMH in boys with 5α-reductase type 2 deficiency (5ARD2) compared to other DSD conditions and controls have found that boys with 5ARD2 had lower levels of both hormones compared to controls (56).

Non-Specific Disorders of Undermasculinisation

Cryptorchidism occurs due to failure of descent of the testes and is a common congenital disorder, reported to affect up to 9% of male infants in some populations (58). Boys with both unilateral and bilateral cryptorchidism have been demonstrated to have lower AMH levels than control boys, indicating testicular dysfunction in childhood. Postnatal maturation of Sertoli cells is altered in cryptorchidism and a study of 40 infants with cryptorchidism aged 4-35 months showed strong positive correlations between inhibin B, LH and FSH with Sertoli cell number (59)

A recent retrospective study of 310 prepubertal boys with cryptorchidism confirmed that whilst low AMH was prevalent in boys with both unilateral and bilateral cryptorchidism, lower levels were seen in boys with bilateral undescended testes (60). In addition, in terms of treatment, testicular descent was more likely to be successful in response to treatment with hCG in those with a higher AMH at baseline, suggesting this may be a useful predictive marker when counselling families regarding the advantages and disadvantages of hormonal versus surgical management (60).

In boys with undermasculinisation, hCG stimulation may be used to assess Leydig cell function in tandem with an AMH measurement to assess Sertoli cell function. In 138 children with a non-specific XY DSD, a normal AMH was predictive of a normal testosterone response to hCG, suggesting that where Sertoli cell function is preserved, Leydig cell function is also likely to be (61). Of the 138 boys in the study cohort, 53 (38%) had combined genital anomalies; 47 (34%) had isolated bilateral undescended testes and 29 (21%) had isolated proximal hypospadias. Boys with isolated hypospadias had a higher AMH and higher testosterone after stimulation with human chorionic gonadotrophin (hCG) compared to children with isolated bilateral undescended testes ($p=0.0001$) or children with combined anomalies including undescended testes ($p<0.0001$) (61). Children with undescended testes but no other genital anomalies had the lowest AMH and amongst those with bilateral undescended testes, children with impalpable testes had a lower median AMH than children with inguinal testes (470 (1.5, 1926) vs 832 (72, 2280) pmol/l ($p=0.04$) (61).

Testicular dysgenesis syndrome (TDS) is a term used to describe a group of associated male reproductive disorders that arise as a result of impaired androgen production or action during a critical period of fetal testicular development (62). The reduced androgen exposure is associated with cryptorchidism, hypospadias, testicular germ cell tumours and subfertility. Dysgenesis (often focal) within the testis is a frequent finding in men with these disorders, which includes undifferentiated Sertoli cells (63, 64). Animal models have been used to study Sertoli cell development and maturation in this cohort. Pregnancy exposure to di(n-Butyl) phthtalate in rats produces a similar phenotype and demonstrate altered Sertoli cell maturation, with a worse phenotype seen in cryptorchid testes compared to scrotal testes (65).

In anorchia, AMH and inhibin B levels are both undetectable, usually in combination with raised FSH levels (66).

Specific Gene Variants

NR5A1 (also known as Steroidogenic Factor 1 or SF1) is a nuclear receptor transcription factor whose expression commences in the coelomic epithelium and continues in steroidogenic cells. Its expression has been demonstrated in the bipotential gonad from 32 days post conception and following testis determination (around 42 days onward), its expression is maintained in the somatic cells of the early testis, which suggests it may play a role in supporting SOX9 expression (67). It is also known to activate the expression of AMH in Sertoli cells from around 7 weeks gestation, resulting in the regression of Müllerian structures in the developing male foetus and is responsible for activating the expression of steroidogenic enzymes from 8 weeks gestation, resulting in the androgenisation of the external genitalia (9). AMH and inhibin B levels in individuals with NR5A1 gene variants have been reported as normal to low-normal (68, 69).

Early studies of simulated FSH deficiency *via* administration of hCG demonstrated that FSH is required for normal spermatogenesis, although sperm production was not entirely suppressed in the absence of FSH (70). Babies born with FSH receptor mutations, have Sertoli cell hypoplasia and small testis resulting in low spermatogenesis and low inhibin B in adulthood. Müllerian structures do regress however suggesting AMH levels are likely to be normal in early fetal life (71).

GATA-binding protein 4 (GATA4) is a transcription factor which is known to be involved in the development of some forms of congenital heart disease (72). Studies with GATA4 mutations have demonstrated its likely involvement in gonadal development in conjunction with its cofactor, Friend of GATA2 (FOG2) (73). These genes are upstream of SRY and when mutated cause significant reductions in SRY expression [Sekido and Lovell-Badge, 2013]. In a family of individuals with mutations in GATA4, 46,XY DSD and congenital heart disease, AMH levels were consistently low and functional analysis demonstrated that mutations in GATA4 may reduce the action of the AMH promoter (74).

Hypogonadotrophic Hypogonadism

In cases of congenital hypogonadotrophic hypogonadism, deficient LH and FSH does not affect Müllerian regression and early sex development, but does impair genital development, which is dependent on testosterone from mid to late pregnancy. This may result in small testes as a result of FSH deficiency and micropenis +/- cryptorchidism arising from LH deficiency (75). A study of 8 men with hypogonadotrophic hypogonadism (4 with Kallmann's syndrome, 4 idiopathic), demonstrated that AMH was high for age, because serum testosterone remained low and therefore did not downregulate AMH. Treatment with recombinant human FSH increased serum AMH, Further treatment with hCG increased testosterone and reduced AMH and inhibin B (76).

Current Research Gaps

Overall, whilst many studies have considered AMH concentrations as an indicator of testicular development in children and young people with DSD, very few have focussed on inhibin B. Therefore, the use of Inhibin B as a marker of Sertoli cell function should be a research priority in future years, particularly when assessing adolescents with DSD, in whom AMH levels are more difficult to interpret.

In young children with non-specific 46,XY disorders of undermasculinisation, AMH and inhibin B levels both show

good correlation with post-hCG testosterone (61, 77), potentially obviating the need for a stimulated test. hCG stimulation tests are invasive for the child and logistically difficult for the healthcare team and family and as such, studies assessing the clinical utility of AMH or inhibin B levels as an alternative to hCG testing to assess gonadal function will be invaluable.

Summary and Conclusions

To summarize, fetal Sertoli cell hormones are crucial for normal sex development. In particular, AMH is responsible for regression of Müllerian structures. Both AMH and inhibin B represent useful biomarkers in children with DSD conditions, as they allow for confirmation of the presence of testicular tissue, as well as monitoring of gonadal function. However, their secretion is age-dependent, requiring specific reference ranges and reliable assays if they are to be used in clinical practice. Serum levels of both, in conjunction with those of androgens and gonadotrophins, can, however, be helpful in the diagnosis of DSD conditions, with specific patterns being more likely to be seen in certain disorders.

AUTHOR CONTRIBUTIONS

Both authors contributed sugnificantly to the conception and writing of this manuscript. Both authors agreed on the final version.

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Distal-less homeobox genes *Dlx5/6* regulate Müllerian duct regression

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Dlx5 and *Dlx6* encode distal-less homeodomain transcription factors that are present in the genome as a linked pair at a single locus. *Dlx5* and *Dlx6* have redundant roles in craniofacial, skeletal, and uterine development. Previously, we performed a transcriptome comparison for anti-Müllerian hormone (AMH)-induced genes expressed in the Müllerian duct mesenchyme of male and female mouse embryos. In that study, we found that *Dlx5* transcripts were nearly seven-fold higher in males compared to females and *Dlx6* transcripts were found only in males, suggesting they may be AMH-induced genes. Therefore, we investigated the role of *Dlx5* and *Dlx6* during AMH-induced Müllerian duct regression. We found that *Dlx5* was detected in the male Müllerian duct mesenchyme from E14.5 to E16.5. In contrast, in female embryos *Dlx5* was detected in the Müllerian duct epithelium. *Dlx6* expression in Müllerian duct mesenchyme was restricted to males. *Dlx6* expression was not detected in female Müllerian duct mesenchyme or epithelium. Genetic experiments showed that AMH signaling is necessary for *Dlx5* and *Dlx6* expression. Müllerian duct regression was variable in *Dlx5* homozygous mutant males at E16.5, ranging from regression like controls to a block in Müllerian duct regression. In E16.5 *Dlx6* homozygous mutants, Müllerian duct tissue persisted primarily in the region adjacent to the testes. In *Dlx5-6* double homozygous mutant males Müllerian duct regression was also found to be incomplete but more severe than either single mutant. These studies suggest that *Dlx5* and *Dlx6* act redundantly to mediate AMH-induced Müllerian duct regression during male differentiation.

KEYWORDS

sex differentiation, anti-Müllerian hormone, reproductive tract development, *Dlx5*, *Dlx6*, *Amhr2*

Introduction

The male reproductive tract organs include the vas deferentia, epididymides, and seminal vesicles. These structures provide the conduit for movement and maturation of spermatozoa from the testes for sexual reproduction. While formation and differentiation of these male reproductive tract organs are essential for reproduction, another process which eliminates a progenitor organ system termed Müllerian duct regression is also required for male development (1). These developmental processes are regulated by the presence or absence of fetal hormones (2).

The reproductive tract organs of mammals are derived from two pairs of epithelial tubes surrounded by mesenchyme called the Wolffian ducts (progenitor male reproductive tract) and Müllerian ducts (progenitor female reproductive tract). The Wolffian ducts differentiate into the vas deferentia, epididymides, and seminal vesicles. The oviducts, uterus and upper vagina are derived from the Müllerian ducts. Interestingly, both the Wolffian and Müllerian ducts are formed regardless of the genetic sex of the embryo. Differential hormone signaling after the formation of these genital ducts results in Wolffian duct loss in female embryos and Müllerian duct regression in male embryos.

Müllerian duct regression is mediated by the TGF-β family member anti-Müllerian hormone (AMH) secreted by Sertoli cells of the fetal testes (3). AMH signaling acts in the Müllerian duct mesenchyme through ACVR1 or BMPR1A type 1 receptors (shared with the bone morphogenetic protein (BMP) signaling pathway) and a sole anti-Müllerian hormone type 2 receptor (AMHR2) (4, 5). *Amhr2* is expressed in the Müllerian duct mesenchyme and in the somatic cells of the gonads (6). In the Mullerian duct mesenchyme, AMH binds AMHR2 which then activates its type 1 receptors. This AMH ligand receptor complex subsequently phosphorylates R-SMAD1, 5 and 8 (also shared with the BMP signaling pathway) (4). These redundant phosphorylated R-SMADs then presumably activate downstream effectors of AMH signaling.

Amh and *Amhr2* are each required for Müllerian duct regression. Male mice homozygous for either *Amh* or *Amhr2* null alleles retain Müllerian duct derived structures, including a complete uterus and oviducts (7, 8). Further, mutations of either the *AMH* or *AMHR2* genes in human males result in Persistent Mullerian Duct Syndrome (PMDS). Like mouse models with *Amh* and *Amhr2* loss, patients with PMDS have testes and male secondary sex characteristics but retain female reproductive tract organs associated with their male reproductive tract (9–11). A mutation in *Amhr2* in dogs is also known to result in PMDS (12). Males of these species with loss of either *AMH* or *AMHR2* also have subfertility likely due to a combination of the effects of cryptorchidism (observed in humans and dogs) and the presence of superimposed Müllerian duct-derived tissues impeding sperm passage (mouse, human and dog) (9, 12). *AMH* alone is also sufficient for Müllerian duct regression in female embryos. Transgenic female mice with widespread expression of human AMH have complete regression of the Müllerian ducts and lack a uterus and oviducts (13).

Few downstream effectors of AMH have been identified. In mice, loss of the signaling molecule beta-catenin encoded by *Ctnnb1* in the Müllerian duct mesenchyme results in the complete retention of the Müllerian duct in newborn males (14). In a recent RNA-seq study we compared male and female mouse Müllerian duct mesenchyme transcriptomes soon after the initiation of AMH signaling in males to identify potential AMH-induced target genes. In that study we identified the BMP target *Osterix/Sp7* (*Osx*) (15, 16). We found that AMH signaling is necessary and sufficient for *Osx* expression. *Osx* null male mice have a delay in Müllerian duct regression but later Müllerian duct derived tissues are eliminated (16).

This transcriptome analysis also revealed differential upregulation of BMP target genes *Dlx5* and *Dlx6* in the Müllerian mesenchyme of males in comparison to females. Based on this transcriptome data and that the BMP pathway shares multiple signaling components with the AMH signaling pathway, we further characterize *Dlx5* and *Dlx6* expression and function during Müllerian regression. We show that AMH signaling is necessary for *Dlx5* and *Dlx6* expression in Müllerian duct mesenchyme. Loss of *Dlx5* or *Dlx6* results in partial retention of the Müllerian ducts in male mice, whereas double mutant males consistently retain more Müllerian tissue. This study identifies *Dlx5* and *Dlx6* as redundant downstream effectors of AMH signaling for Müllerian regression.

Materials and methods

Mice

Swiss outbred mice were obtained from Taconic Biosciences. C57BL/6J mice were obtained from the Jackson Laboratory. *Dlx6*^{tm1Jlr} mice were obtained from the MMRRC (Mutant Mouse Resource and Research Center). *Amhr2*$^{tm3(cre)Bhr}$ [*Amhr2-Cre* (17),], *Amhr2*^{tm2Bhr} (*Amhr2-lacZ* (18), *Dlx5*^{tm1Levi} [*Dlx5-lacZ* (19),], *Dlx6*^{tm1Jlr} (*Dlx6-lacZ* (20), *Dlx5/Dlx6*^{tm1Levi} [*Dlx5-6* (21),] mice were maintained on a predominantly C57BL/6J genetic background. *Amhr2-Cre, Amhr2-lacZ*, *Dlx5-lacZ*, *Dlx6-lacZ*, and *Dlx5-6* mice were genotyped by PCR as described previously. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. Studies were performed consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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β-galactosidase staining

Dlx5-lacZ and *Dlx6-lacZ* heterozygous males were bred with Swiss females to establish timed matings for β-galactosidase (β-gal) staining as previously described (16).

Immunofluorescence

Immunofluorescence was performed as previously described (22). Rabbit anti-DLX5 polyclonal antibody (Sigma-Aldrich Cat# HPA005670, RRID : AB_1078681, 1:50). Rabbit anti-PAX2 polyclonal antibody (Thermo Fisher Scientific Cat# 71-6000, RRID : AB_2533990, 1:100). Primary antibodies were detected with goat anti-rabbit IgG (H+L) Alexa Fluor Plus 488 rabbit (Thermo Fisher Scientific Cat# A32731TR, RRID : AB_2866491, 1:200). At least three specimens of each genotype were analyzed.

Microscopy and image analyses

Z-stack images of wholemount immunofluorescent staining for PAX2 were acquired using an A1 Nikon confocal microscope. Linear length in microns of Wolffian duct epithelium and retained Müllerian duct epithelium marked by PAX2 immunofluorescent staining in 3D volume rendered confocal images were measured in Imaris (Bitplane). Length of Wolffian duct epithelium was measured from point of fusion with seminal vesicle to start of coiling in epididymis on left and right sides. Retained Müllerian duct epithelium lengths were measured along the Wolffian duct epithelium on left and right sides beginning at the point of Müllerian duct fusion at the urogenital sinus.

Statistical analyses

Measurements of retained Müllerian duct epithelium length to Wolffian duct epithelium length ratios in *Dlx5-6* homozygous mutant and control males were subjected to a Welch's t-test (t-test; two-sample assuming unequal variances) using Microsoft Excel. A *P* value of less than .05 was considered statistically significant.

Results

Dlx5 and *Dlx6* expression in the developing reproductive organs is sexually dimorphic

Previously, we generated transcriptomes by RNA-seq of FACS-purified Müllerian duct mesenchyme cells from E14.5 male and female embryos to identify candidate genes induced by AMH that mediate Müllerian duct regression (16). At this stage of development, male mesenchymal cells are responding to AMH secreted by the testes, whereas in female embryos these cells are naïve to AMH because the fetal ovaries do not express AMH. Analysis of the bulk RNA-seq results showed that *Dlx5* transcripts were 6.7-fold higher in males compared to females and *Dlx6* transcripts were found only in males, suggesting these genes may respond to AMH signaling to mediate Müllerian duct regression (**Table 1**).

Previous studies in the mouse showed that *Dlx5* is expressed in the epithelium of the E18.5 uterus and subsequently in the luminal and glandular epithelia of the postnatal and adult uterus (23). In that study, expression was also detected earlier in the Müllerian ducts at E15.5 and E16.5. We examined DLX5 expression in the Müllerian ducts of E14.5 male and female embryos by immunofluorescence (**Figure 1**). In males, DLX5 was detected in the mesenchymal cells surrounding the Müllerian duct epithelium (**Figure 1A**). In contrast, but consistent with previous studies (23), DLX5 expression was detected in the epithelial cells of the Müllerian duct of E14.5 female embryos (**Figure 1B**).

We also used a *Dlx5-lacZ* knock-in allele to examine *Dlx5* expression in the developing reproductive organs (19, 20). *Dlx5-lacZ* expression was detected at E14.5 in the Müllerian ducts of both male and female embryos (**Figures 1C, D**). In male and female embryos, β-gal staining was observed throughout the entire length of the Müllerian ducts. At E15.5 and E16.5, *Dlx5-lacZ* expression persisted in the residual Müllerian ducts of male embryos (**Figures 1E, G**). Consistent with earlier studies, in *Dlx5-lacZ* female embryos at E15.5 and E16.5, β-gal staining was detected throughout the entire length of the Müllerian ducts with strongest staining caudally (**Figures 1E, G**) (23). These data indicate that *Dlx5* is expressed in the male Müllerian duct mesenchyme and female Müllerian duct epithelium. Thus, *Dlx5* exhibits a sexually dimorphic pattern of expression in the developing Müllerian system.

We used a *Dlx6-lacZ* knock-in allele (20) to examine *Dlx6* expression in the developing reproductive organs (**Figure 2**). *Dlx6-lacZ* expression in male embryos was detected at E14.5 and E15.5 throughout the entire length of the Müllerian ducts and later at E16.5 as the Müllerian duct was regressing (**Figures 2A-C**). Histological analysis showed that β-gal expression was localized to the Müllerian duct mesenchyme (**Figure 2B'**). No

TABLE 1 *Dlx5* and *Dlx6* RNA-Seq mean of counts normalized for sequencing depth and *Dlx5* Log2foldchange.

Gene	Mean Count Male	Mean Count Female	Log2FoldChange
Dlx5	715.73	6.95	6.69
Dlx6	160.00	0.00	NC

Dlx6 Log2foldchange not calculated (NC) because *Dlx6* female mean counts are zero [Data from Mullen et al., 2018 (16)].

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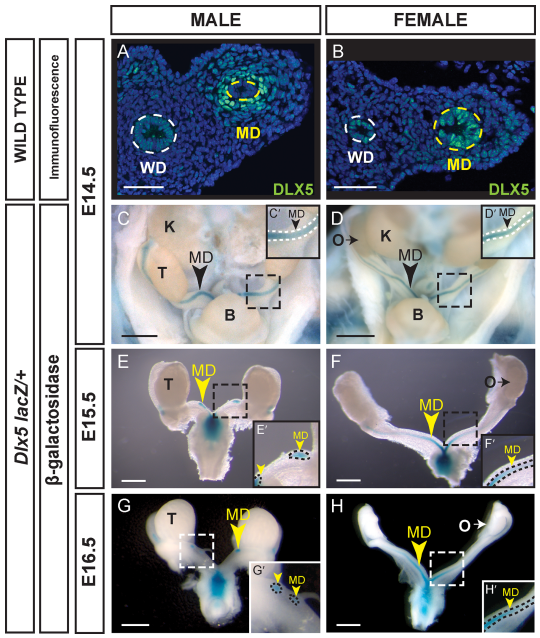

FIGURE 1
Sexually dimorphic expression of DLX5 in the male Müllerian duct mesenchyme and female Müllerian duct epithelium. Immunofluorescent staining of DLX5 in cross sections of the mesonephros from E14.5 male **(A)** and female **(B)** embryos. Dotted lines surround MD and Wolffian duct (WD) epithelium. Scale bars = 50 μm. *Dlx5-lacZ* expression in male MD is lost as MD regresses. β-gal-stained urogenital organs from E14.5 to 16.5 *Dlx5-lacZ* heterozygous male **(C, E, G)** and female **(D, F, H)** embryos. Dashed square boxes show location of higher magnification insets. Arrowheads, Müllerian ducts. MD, Müllerian duct; WD, Wolffian duct; B, bladder; K, kidney; O, ovary; T, testis. Scale bars = 500 μm. **(C'- H')** Higher magnification insets. Arrowheads, Müllerian ducts. MD. Dotted lines surround MD. Each sex and genotype analyzed n≥3.

β-gal staining was detected in the Müllerian ducts of female embryos at each time point examined (E14.5 to E16.5) (**Figures 2D-F**). These results show that *Dlx6* is expressed in the male Müllerian duct mesenchyme. The male-specific expression of *Dlx6* in the developing reproductive organs demonstrates that it is expressed in a sexually dimorphic pattern. These findings also show that *Dlx5* and *Dlx6* are co-expressed in the male Müllerian duct mesenchyme at the initiation and during ductal regression.

AMH is necessary for *Dlx5* and *Dlx6* expression in the Müllerian duct mesenchyme

The temporal and spatial patterns of expression for *Dlx5* and *Dlx6* in the developing male Müllerian duct mesenchyme are consistent with the idea that they are induced by AMH signaling. AMH is expressed in the fetal testis at ~E12.0 (24). At ~E13.5 *Amhr2* expression is activated in the Müllerian duct mesenchyme initiating AMH signaling (18). We first observe *Dlx5* and *Dlx6* expression in the Müllerian duct mesenchyme at ~E14.5, a timing consistent with their activation by the AMH-signaling pathway. To test this idea, we performed genetic experiments examining *Dlx5* and *Dlx6* expression in the absence of AMH signaling in male embryos.

To determine if AMH signaling is required for *Dlx5* expression, we performed DLX5 immunostaining of the Müllerian ducts of *Amhr2 Cre/lacZ* mutant males at E14.5 (**Figure 3**). *Amhr2-Cre* and *Amhr2-lacZ* are knock-in alleles that are also loss-of-function alleles (17, 18). Thus, *Amhr2 Cre/lacZ* mutants lack *Amhr2* function, blocking AMH signaling, resulting in males with a retained and fully developed Müllerian system (8). DLX5 positive Müllerian duct mesenchyme cells were detected in wild-type male controls (**Figure 3A**). No DLX5 immunostaining was detected in the Müllerian duct mesenchyme of *Amhr2 Cre/lacZ* mutant males (**Figure 3B**). This suggests that DLX5 expression requires AMH signaling.

To determine if AMH signaling was required for *Dlx6* expression, we generated E15.5 *Dlx6-lacZ/+; Amhr2-Cre/Cre* male embryos (**Figure 4**). β-gal staining was detected in the residual Müllerian duct tissue of *Dlx6-lacZ/+* embryos (**Figure 4A**). However, no β-gal staining was observed in the Müllerian ducts of E15.5 *Dlx6-lacZ/+; Amhr2-Cre/Cre* male embryos that retained a complete Müllerian system due to the block in AMH signaling (**Figure 4B**). This suggests that *Dlx6-lacZ* expression requires AMH signaling.

Dlx5 and *Dlx6* regulate Müllerian duct regression

Dlx5-lacZ homozygotes die shortly after birth with craniofacial defects (19). In wild-type male embryos, most of the Müllerian duct is regressed at E16.5 and regression is complete by E18.5. We initially screened for the presence of residual Müllerian duct-derived tissues (uterus) by histological analysis of *Dlx5-lacZ* homozygous mutants at E18.5 (**Figures 5A, B**). None of the 4 *Dlx5-lacZ* homozygous mutant males screened by histological sectioning had uterine tissue (**Figure 5B**). We next examined Müllerian duct regression in *Dlx5-lacZ* homozygous mutant males and controls at E16.5, using *lacZ* expression to mark the Müllerian duct. Five of 6 *Dlx5-lacZ* homozygous mutant males analyzed had Müllerian duct regression like controls (**Figure 5C**). However, one of the mutants retained a significant amount of Müllerian duct (**Figure 5D**). These results indicate that *Dlx5* contributes to Müllerian duct regression.

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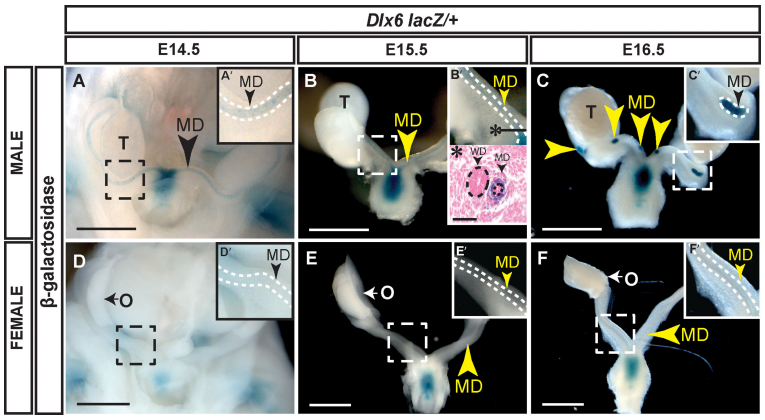

FIGURE 2
Dlx6-*lacZ* expression in male Müllerian duct mesenchyme but not in female Müllerian ducts. Wholemount β-gal-stained urogenital organs from E14.5 to 16.5 *Dlx6-lacZ* heterozygous male **(A-C)** and female **(D-F)** embryos. Arrowheads, Müllerian ducts. MD, Müllerian ducts; O, ovary; T, testis. Dashed square boxes show location of higher magnification insets. Scale bars = 500 µm. **(A'-F')** Higher magnification insets. Arrowheads, Müllerian ducts. MD, Müllerian duct. Dotted white lines surround MD. **(B'*)** Eosin counterstained paraffin section (10 µm) of wholemount β-gal-stained E15.5 *Dlx6-lacZ* heterozygous male. Asterisk denotes location of paraffin cross section. Arrowheads, Müllerian ducts. MD; Wolffian ducts, WD. Dotted lines surround MD and WD epithelium. Scale bars = 50 µm. Each sex and genotype analyzed n≥3.

Dlx6-lacZ homozygous mutants die within a day after birth also with craniofacial abnormalities (20). Like the *Dlx5-lacZ* mutants, we examined Müllerian duct regression by *lacZ* expression in mutants and controls at E16.5 and E18.5. At E16.5, control males showed very small β-gal positive regions, indicating residual Müllerian duct tissue (**Figures 6A, C**). In contrast, in E16.5 *Dlx6-lacZ* homozygous mutant male embryos there were still significant stretches of β-gal staining notably in the Müllerian ducts adjacent to the testes (**Figures 6B, D**). However, by E18.5, Müllerian duct regression in *Dlx6-lacZ* homozygous mutant male embryos was comparable to control males (**Figures 6E, F**). These results suggest that loss of *Dlx6* results in a delay in Müllerian duct regression and *Dlx6* contributes to Müllerian duct regression.

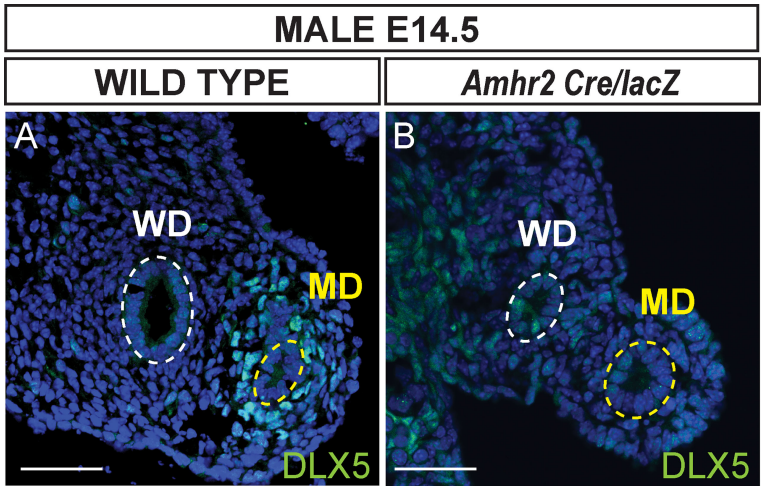

FIGURE 3
AMH signaling is required for DLX5 expression. Immunofluorescent staining of DLX5 in cross sections of the mesonephros from E14.5 wild-type **(A)** and *Dlx5-lacZ/+; Amhr2-Cre/lacZ* **(B)** male embryos. DLX5 is not expressed in *Amhr2-Cre/lacZ* males, indicating AMH signaling is required for DLX5 expression in the male Müllerian duct (MD). Yellow dotted lines surround the MD epithelium; white dotted lines surround the Wolffian duct epithelium (WD). MD, Müllerian duct; WD, Wolffian duct. Scale bars = 50 µm. Each genotype analyzed n=3.

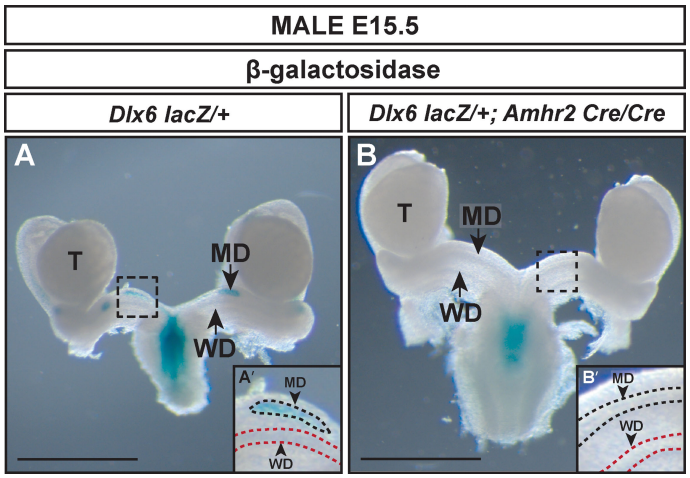

FIGURE 4
AMH signaling is required for *Dlx6-lacZ* expression in the Müllerian duct. Wholemount β-gal-stained urogenital organs from E15.5 *Dlx6-lacZ/+* **(A)** and *Dlx6-lacZ/+; Amhr2-Cre/Cre* **(B)** male embryos. Arrow in **(A)**, residual β-gal-stained Müllerian duct (MD) tissue. Arrow in **(B)**, absence of β-gal-stained Müllerian duct tissue in fully retained Müllerian duct caused by loss of *Amhr2*. Dashed square boxes show location of higher magnification insets. MD, Müllerian duct; T, testis; WD, Wolffian duct. Scale bars=1000 μm. (**A′, B′**) Higher magnification insets. Arrowheads, Müllerian ducts, MD; Wolffian ducts, WD. Dotted lines surround MD (black) and WD (red). Each genotype analyzed n=3.

Müllerian duct regression in *Dlx5* male homozygous mutants was variable, whereas in *Dlx6* male homozygous mutants it was delayed. *Dlx5* and *Dlx6* are co-expressed in the male Müllerian duct mesenchyme, suggesting that they may act together for Müllerian duct regression. Therefore, we examined Müllerian duct regression in *Dlx5-6* homozygous mutant males. The *Dlx5-6* allele (*Dlx5/Dlx6*^{tm1Levi}) is a deletion of both *Dlx5* and *Dlx6* coding regions that also contains a *lacZ* reporter (21).

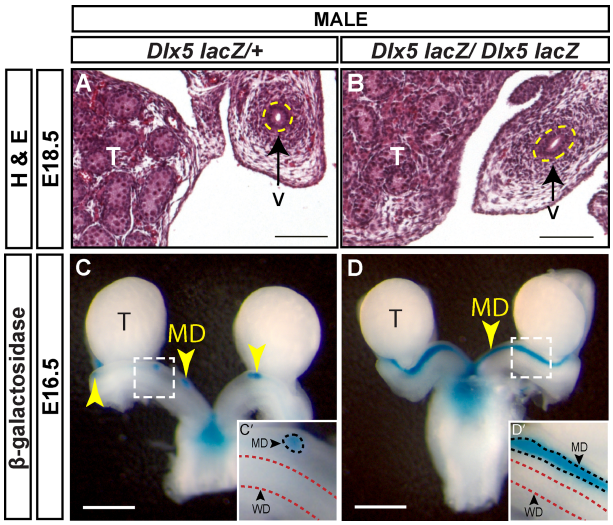

FIGURE 5
Defects in Müllerian regression are observed in some *Dlx5* mutants at E16.5 but are resolved by E18.5. Hematoxylin (H) and Eosin (E) stained cross sections of the vas deferens (v) adjacent to the testis (T) from E18.5 *Dlx5-lacZ/+* **(A)** and *Dlx5-lacZ/Dlx5-lacZ* **(B)** male embryos. Yellow dotted lines surround the vas deferens epithelium. Scale = 100 μm. Wholemount β-gal-stained urogenital organs from E16.5 *Dlx5-lacZ/+* **(C)** and *Dlx5-lacZ/Dlx5-lacZ* **(D)** male embryos. Yellow arrowheads, Müllerian ducts. T, testis; MD, Müllerian duct. Scale bars = 500 μm. **(C′, D′)** Higher magnification insets. Arrowheads, Müllerian ducts, MD; Wolffian ducts, WD. Dotted lines surround MD (black) and WD (red). E18.5 each genotype analyzed (n=4); E16.5 *Dlx5-lacZ/+* (n=5); E16.5 *Dlx5-lacZ/lacZ* (n=6).

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However, the *lacZ* reporter in this allele is not functional. *Pax2* is expressed in the developing Müllerian duct epithelium (25). Therefore, we followed Müllerian duct regression by wholemount immunostaining for PAX2. At E16.5, much like the *Dlx5* male homozygous mutants, one *Dlx5-6* homozygous mutants had near complete retention of the Müllerian duct epithelium while others were comparable to wild type (**Figures 7A, B**). To quantify the portion of Müllerian duct epithelium retained in *Dlx5-6* homozygous mutants and controls we calculated the ratio of the length of retained Müllerian duct epithelium to Wolffian duct epithelium from the whole mount images (**Figure 8**). Ratios for *Dlx5-6* homozygous mutants were 0.70 (**Figure 7B**), 0.32 and 0.27 in comparison to ratios of controls 0.38, 0.22, 0.24 and 0.19 (**Figure 7A**). At E18.5 ratios of retained uterine epithelium to vas deferens epithelium for *Dlx5-6* homozygous mutants were significantly increased in comparison to controls (**Figure 7C** (ratio 0.03), **Figure 7D** (ratio 0.35) and **Figure 8**). Consistent with these results, initial histology at E18.5 detected retained uterine tissue in 1 of 4 *Dlx5-6* homozygous mutant males examined (**Figures 7E, F**). These results suggest that *Dlx5* and *Dlx6* contribute to Müllerian duct regression and may be functioning redundantly.

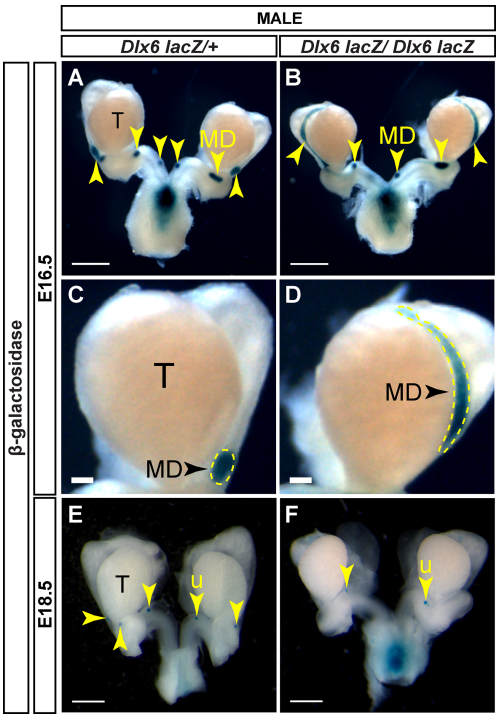

FIGURE 6
Loss of *Dlx6* results in a delay in Müllerian duct regression. Wholemount β-gal-stained urogenital organs from E16.5 *Dlx6-lacZ/+* **(A)** and *Dlx6-lacZ/Dlx6-lacZ* **(B)** male embryos. Higher magnification of testis and adjacent Müllerian duct from E16.5 *Dlx6-lacZ/+* **(C)** and *Dlx6-lacZ/Dlx6-lacZ* **(D)** male embryos. Wholemount β-gal-stained urogenital organs from E18.5 *Dlx6-lacZ/+* **(E)** and *Dlx6-lacZ/Dlx6-lacZ* **(F)** male embryos. Arrowheads, residual Müllerian ducts **(A-D)** and uterus **(E, F)**. Yellow dotted lines surround Müllerian ducts **(C,D)**. T, testis; MD, Müllerian duct; u, uterus. E16.5 *Dlx6-lacZ/+* (n=5); E16.5 *Dlx6-lacZ/lacZ* (n=4); E18.5 *Dlx6-lacZ/+* (n=5); E18.5 *Dlx6-lacZ/lacZ* (n=4). Scale bars = 500 μm **(A, B, E, F)**, 100 μm **(C,D)**.

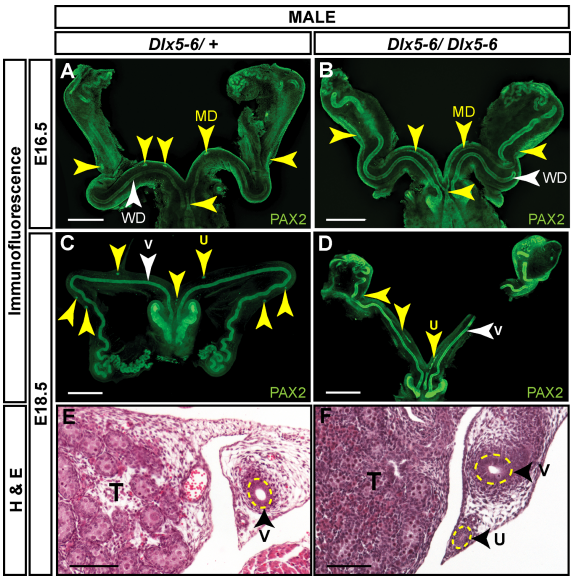

FIGURE 7
Dlx5-6 mutant males retain significant portions of uterine tissue at E18.5 compared to controls. Wholemount PAX2 immunofluorescent staining of E16.5 *Dlx5-6/+* **(A)** and *Dlx5-6/Dlx5-6* **(B)** male embryos and E18.5 *Dlx5-6/+* **(C)** and *Dlx5-6/Dlx5-6* **(D)** male embryos. Yellow arrowheads, Müllerian ducts **(A, B)** and uterus **(C, D)**. White arrowheads, Wolffian ducts **(A, B)** and vas deferens **(C, D)**. MD, Müllerian duct; WD, Wolffian duct; T, testis; v, vas deferens; u, uterus. Scale bars = 500 μm. Hematoxylin **(H)** and Eosin **(E)** stained cross sections of the vas deferens (v) adjacent to the testis (T) from E18.5 *Dlx5-6/+* **(E)** and *Dlx5-6/Dlx5-6* **(F)** male embryos. u, uterus. Scale bars =100 μm. E16.5 *Dlx5-6/+* (n=4); E16.5 *Dlx5-6/Dlx5-6* (n=3); E18.5 *Dlx5-6/+* (whole mount n=6, histology n=4); E18.5 *Dlx5-6/Dlx5-6* (whole mount n=3, histology n=3).

Discussion

Previously, we compared the transcriptomes of FACS-isolated E14.5 male and female Müllerian duct mesenchyme cells in a screen for AMH-induced genes that mediate Müllerian duct regression during male differentiation (16). In our initial analysis, we identified *Osx* as an AMH-induced gene that is expressed in the male Müllerian duct mesenchyme to regulate Müllerian duct regression. Upon further analysis of the transcriptomes, we found that the distal-less homeobox genes *Dlx5* and *Dlx6* were upregulated in males relative to females. We found that both genes are expressed in the male Müllerian duct mesenchyme and are dependent on AMH signaling. The *Dlx5*-null and *Dlx6*-null male embryos (19, 20) showed variable

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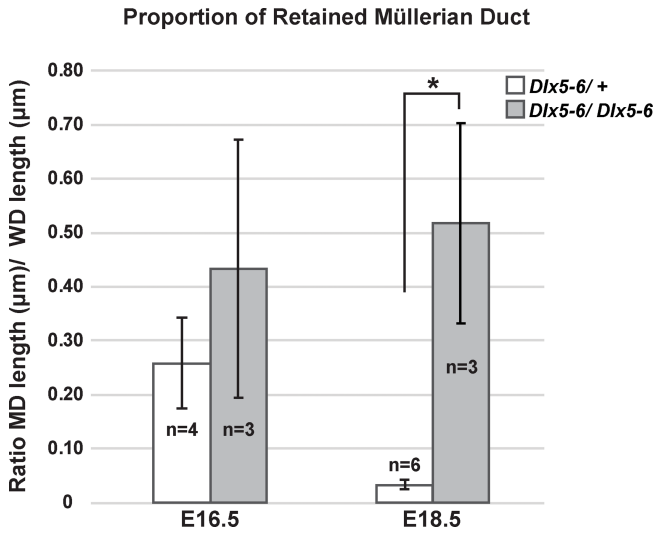

FIGURE 8
Proportion of retained Müllerian duct in *Dlx5-6* mutant males is increased at E18.5 in comparison to controls. Quantification of the ratio of retained Müllerian duct epithelium length to Wolffian duct epithelium length in *Dlx5-6/+* and *Dlx5-6/Dlx5-6* males at E16.5 and E18.5. Data are presented as mean ± standard deviation. Asterisk (*) indicates statistically significant difference ($p \leq 0.05$).

retention of the Müllerian ducts and a delay in Müllerian duct regression, whereas the *Dlx5-6*-null male embryos (21) retained more Müllerian duct tissue compared to each single mutant. These findings place a new pair of transcription factors that act together in the gene regulatory network to mediate AMH-induced Müllerian duct regression during male differentiation (26).

AMH signaling is required for *Dlx5* and *Dlx6* expression in the male Müllerian duct mesenchyme

We found that both *Dlx5* and *Dlx6* are expressed in the male Müllerian duct mesenchyme during the embryonic stages coinciding with the initiation and progression of Müllerian duct regression. *Dlx* genes are organized as linked pairs in the mammalian genome (27). These *Dlx* gene pairs are typically co-expressed in various tissues, suggesting common cis regulation. Perhaps *Dlx5* and *Dlx6* share Müllerian duct mesenchyme-specific regulatory elements that respond to AMH signaling.

Previously, we discovered that *Osx* was expressed in the male but not in female Müllerian duct mesenchyme (16). Our observations of *Dlx5* and *Dlx6* expression add to a growing list of genes that exhibit sexually dimorphic patterns of expression in the Müllerian ducts. We found that the expression of *Dlx5*, and *Dlx6* in the Müllerian duct mesenchyme was dependent upon AMH signaling through AMHR2. Similar results were found for *Osterix* expression (16). *Amhr2* is expressed in the Müllerian duct mesenchyme of both male and female embryos (6, 17, 18). The female Müllerian duct mesenchyme is competent to respond to AMH because ectopic AMH can induce Müllerian duct regression, eliminating the development of the uterus and oviducts (13). Thus, the sexually dimorphic expression of *Osx*, *Dlx5*, and *Dlx6* expression in the Müllerian duct mesenchyme mirrors the sexually dimorphic expression of fetal AMH.

Interestingly, *Dlx5* is also expressed in the Müllerian duct of female embryos. However, in contrast to mesenchymal expression in male embryos, expression in female embryos was localized to the Müllerian duct epithelium. This epithelial expression persists at later stages of embryogenesis and postnatally in the luminal and glandular epithelium of the uterus (23). We found that *Dlx6* expression in the Müllerian duct was only in male embryos; no expression was detected in female embryos. However, *Dlx6* expression is detected later in the postnatal uterine epithelium (23). These observations suggest that the expression of *Dlx5* in the epithelium of the female Müllerian duct is independent of AMH signaling, suggesting that other factors direct expression in this tissue compartment.

Dlx5 and *Dlx6* mediate Müllerian duct regression

Simultaneous deletion of *Dlx5* and *Dlx6* in the postnatal uterus leads to alterations in uterine adenogenesis and infertility, suggesting that they act redundantly for epithelial morphogenesis in the uterus (23). In addition to the postnatal uterus, redundant functions for *Dlx5* and *Dlx6* have also been reported for limb and craniofacial development (19–21, 28, 29).

The overlapping expression patterns of *Dlx5* and *Dlx6* suggested that they functioned redundantly in the male Müllerian duct mesenchyme. Male homozygotes for each single mutation retain variable amounts of Müllerian tissue and male homozygous double mutants retain more Müllerian tissue. Interestingly, the *Dlx5-6* double mutant males did not show complete retention and differentiation of the Müllerian system like *Amh* and *Amhr2* mutant males (7, 8). The incompletely penetrant Müllerian duct regression phenotypes in the *Dlx5-6* double mutant male embryos suggests that there are other mediators of AMH-induced Müllerian duct regression that act together with *Dlx5-6* (e.g. *Osx*). Previous studies suggest that there are significantly excess levels of AMH produced for Müllerian duct regression because *Amh* levels must be reduced to ~10% of wild type to uncover partial Müllerian duct regression phenotypes (18). Perhaps downstream target genes of AMH signaling must be collectively inactivated for a complete block to Müllerian duct regression (30,31).

Dlx genes are known targets of BMP signaling (26). Interestingly, AMH signal transduction shares receptors (BMPR1A and ACVR1) and downstream R-SMAD proteins (SMAD1, 5, 8) with BMP signaling (4, 5). Considering the temporal and spatial expression of *Dlx5* and *Dlx6* in the Müllerian duct mesenchyme requires AMH signaling it is possible that they are direct transcriptional targets. *Osx* was discovered as a BMP-induced gene (15). *Osx*, *Dlx5*, and *Dlx6* each have roles in skeleton development and Müllerian duct regression (15, 16, 19–21, 28, 29). Do these transcription factor genes regulate the same downstream effectors in skeleton-forming and Müllerian duct mesenchyme cells, or do they regulate different sets of target genes? This question can be addressed by identifying tissue specific cis regulatory elements that are bound by OSX, DLX5, and DLX6 and regulate transcription.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Author contributions

RM and RB conceived the study and designed experiments. RM performed experiments and analyzed data. BB and GL generated mutant embryos. RM and RB wrote the paper with input from all authors. All authors contributed to the article and approved the submitted version.

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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

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Sexual dimorphism through androgen signaling; from external genitalia to muscles

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Sexual dimorphisms can be seen in many organisms with some exhibiting subtle differences while some can be very evident. The difference between male and female can be seen on the morphological level such as discrepancies in body mass, presence of body hair in distinct places, or through the presence of specific reproductive structures. It is known that the development of the reproductive structures is governed by hormone signaling, most commonly explained through the actions of androgen signaling. The developmental program of the male and female external genitalia involves a common anlage, the genital tubercle or GT, that later on develop into a penis and clitoris, respectively. Androgen signaling involvement can be seen in the different tissues in the GT that express Androgen receptor and the different genes that are regulated by androgen in the mesenchyme and endoderm component of the GT. Muscles are also known to be responsive to androgen signaling with male and female muscles exhibiting different capabilities. However, the occurrence of sexual dimorphism in muscle development is unclear. In this minireview, a summary on the role of androgen in the sexually dimorphic development of the genital tubercle was provided. This was used as a framework on analyzing the different mechanism employed by androgen signaling to regulate the sexual dimorphism in muscle development.

KEYWORDS

androgen, muscle, external genitalia, sexual dimorphism, developmental mechanism

Introduction

Biological differences are evident in extant species to date, accounting for great diversity in physiology, reproductive strategies, anatomy, and morphology. Initially, dimorphic organs have similar precursor structures that undergo the same early developmental program which includes mitotic growth and cellular differentiation. This will then be followed by a stage of divergent developmental process which usually involves sex

hormones, androgens and estrogens, which leads to sexual dimorphism. This sexual dimorphism is important in establishing male- and female-specific organs to ensure greater reproductive success. Among the apparent manifestations of sexual dimorphism can be determined morphologically in terms of size, shape, colors, and development of appendages (1). In many mammalian species such as elephants, sea lions, and kangaroos, adult males are larger than females. Neck crests are also prominent in male bulls and stallions due to greater muscle build-up in the head, neck, and shoulder regions as compared to their female counterparts and has been attributed to androgen-facilitated muscle deposition patterns (2). However, the developmental program underlying sexual dimorphisms are not yet fully understood.

One of the most notable sexually dimorphic structures in mice are the features of the external genitalia (ExG) and the anogenital distance (AGD) in the perineum, area between the anus and genitals (3). Male mice have longer and more pronounced ExG and AGD as compared to female mice. Some distinguishing features of the ExG between the males and females include the presence of erectile tissue (diffused in clitoris), organ size (larger for the penis), bone (larger for the penis), cartilage (absent in the clitoris), surface spines (absent in the clitoris), urethral position (completely within the penis for males), and cross-sectional shape (U-shaped for the clitoris and circular for the penis) (4). The penis and clitoris of the ExG both develop from the ambisexual genital tubercle (GT), from embryonic day 11 or E 11, and become sex-specific at E 16 in mice (5). The longer AGD is used in sexing mice pups as early as E16.5. The perineum region is longer in male mice which may be attributed to the presence of well-developed bulbocavernosus/levator ani (BC/LA) muscle complex (3).

Androgen signaling has been identified as the primary factor that establishes male characteristics. The ligands for androgen signaling are testosterone (T) and dihydrotestosterone (DHT). T is the primary circulating androgen that is secreted by the Leydig cells of the testis while DHT is a product of the conversion of T *via* 5a-reductase in several reproductive tissues (6). The androgen receptor (*Ar*) is composed of different functional domains: N-terminal domain, DNA-binding domain, hinge region and C-terminal or ligand binding domain (7). Androgen signaling is initiated by the binding of androgens, (T) or dihydrotestosterone (DHT) to androgen receptor resulting to the dimerization of Ar and its nuclear translocation. The Ar can bind to the DNA of target genes through androgen response elements (ARE) (8). Ar is known to regulate transcription of different genes and more recently, non-genomic functions of AR have been evident (ADD 2 here).

The developmental program of the ExG formation has been explained through the dominant effect of androgen signaling. Muscle development and physiology is known to be responsive to the effect of testosterone. The understanding of the masculinization process of the external genitalia and AGD might help in identifying the occurrence of sexually dimorphic muscle development.

The study of sexual dimorphism in the external genitalia

The development of the ExG starts with a common anlage known as the genital tubercle or GT (Figure 1) which will later develop into the penis and clitoris. The GT is composed of different tissues originating from the ectoderm, endoderm, and mesoderm. The epidermis of the GT is mainly comprised of ectodermal cells while the urethral tube is of endodermal origin. The mesenchyme of the GT is derived from the pericloacal mesenchyme which is primarily from the mesoderm. Each of these tissue types contribute to the development of the GT and towards a sexual dimorphic structure of a penis or clitoris. One of the most notable structure that distinguish the male and female GT is the fusion of urethral folds. Failure of urethral folds fusion leads to hypospadias, a human penile condition wherein the urethral opening is not located at the tip of the penis. This condition can be replicated in mice when androgen signaling is disrupted during the stage of urethral tube fusion (9, 10). Additionally, exclusive dysregulation of signaling in the ectoderm, endoderm,or mesenchyme exhibits a hypospadias-like phenotype, in varying degree, due to the failure in the proper fusion of the urethral folds (10–13). This shows that each of this tissue contributes to GT masculinization.

Androgen signaling has been a consistent variable in the study of a masculinized GT. Androgen receptor (AR) expression is observed in the mesenchyme of the urethral folds and the urethral tube while its expression in the ectoderm is not detected (10, 12). Only the mesenchyme -specific knock-out (KO) of AR results to hypoplasia of the urethral folds. This indicates that the influence of androgen signaling during male-specific GT development may depend on which AR-expressing tissue is most important in the masculinization process (ADD 10 or 12). A microarray analysis comparing the GT ventral mesenchyme and urethra of male and female shows some genes that are differentially expressed at the onset of sexually dimorphic features. *Cyp1b1*, *Fkbp51* (FK506 binding protein 5) and *Mafb* (v-maf masculoaponeurotic fibrosarcoma oncogene family protein B) are differentially expressed in male and female GTs (14). *Mafb* is more prominently expressed in the male GT mesenchyme than in females. Additionally, exogenous exposure to androgen induced *Mafb* expression in females while ArKO (Androgen receptor knockout) mice downregulated its expression. Interestingly, androgen exposure in the absence of *Mafb* resulted to failed masculinization of the urethra and *Mafb* KO mice exhibited defective urethra which is similar to hypospadias (10). This implies that androgen is essential in

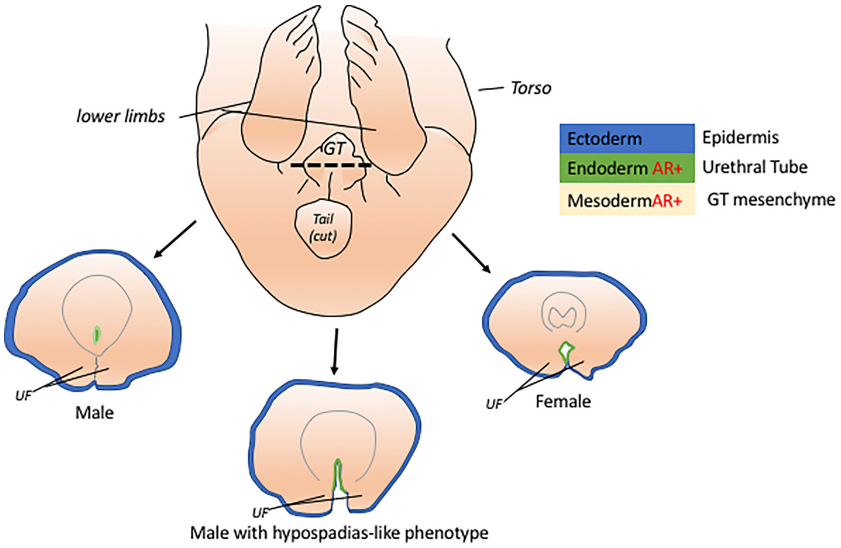

FIGURE 1
The genital tubercle or GT is the common anlage of the penis and clitoris. The GT has cells from 3 embryonic layers, ectoderm, mesoderm, and endoderm. At early embryonic stage, the male GT is distinguished from female GT through the fusion of the urethral folds (UF) which can be viewed histologically through a cross-section of the GT (dotted line). The masculinization of the male GT is attributed to the active androgen signaling in the endoderm and mesoderm tissues of the GT. Unfused UF in males, hypospadias-like phenotype, are due to disruptions in the signaling of the endoderm and mesoderm of the GT.

inducing and regulating Mafb action in the masculinization of the urethral folds. Another signaling pathway that is influenced by androgen signaling is the Wnt signaling. Beta-catenin (*B-cat*), a downstream effector of Wnt-signaling, is found to be highly expressed in the male ventral mesenchyme. Such expression pattern can be replicated in the female through the administration of testosterone propionate, a testosterone analogue. KO of *Ar* and *B-cat* in the urethral ventral mesenchyme both leads to hypospadias-like phenotype (12).

From the studies of GT development mentioned above, there are some investigative strategies that can be used as a framework in identifying sexually dimorphic muscle formation. First, it is important to identify which cell or tissues are necessary for the proper sexually dimorphic development of tissues or organs. And also, which of these tissues express *Ar* and which tissue-specific *Ar* deletion results to drastic changes in phenotype. Second, all the above-mentioned studies include comparison between male and female structures and differential gene expression patterns. This method is necessary to elucidate which developmental mechanism is necessary for general development of structures and which is necessary for male- or female- specific developmental program. It is important that identified mechanism may be used to manipulate the female GT development which will result to structures or expression pattern that will resemble that of the male, or vice versa. And lastly, genes that are differentially expressed between male and females need to be validated on how androgen signaling might influence its expression pattern. This can be done by using different hormonal modulation experiments (see Diff review paper) and analyze how gene expression patterns are affected.

Muscle development

Muscle development starts with the specification of a somite region known as dermomyotome which leads to primary myotome formation. The myotome contains muscle cells or myoblasts that migrate to different parts of the body to serve as muscle precursor cells for the rib and back muscles, body walls, limbs and tongue. This process occurs between E9 – E12 in mice (15). During this time, the development of the muscles is not observed to be androgen-dependent nor is it different between male and female embryos.

The regulation of myogenesis is dependent on the expression of members of the basic helix-loop-helix domain-containing myogenic regulatory factors (MRFs) (Figure 2A). MRFs include myogenic factor 5 (*Myf5*) and 6 (*Myf6*), myogenic differentiation 1 (*MyoD*), and myogenin (*Myog*) (16). *Myf5* and *MyoD* are suggested to act redundantly and upstream of both *Myf6* and *Myog*. *Myf5* mutants exhibit initial delayed muscle development which was rescued once *MyoD* begins to be expressed resulting in an overall normal myogenesis (17, 18). Similarly, *MyoD* mutants possess normal skeletal muscle development with a notable persistent expression of *Myf5*, which is normally down-

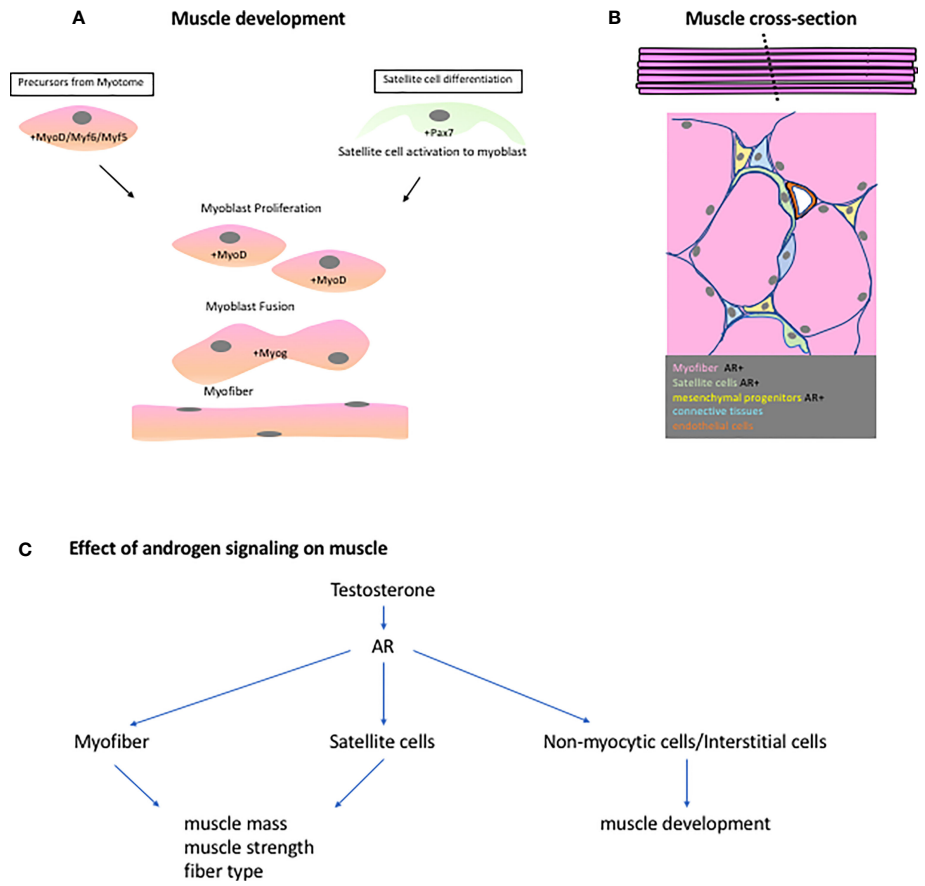

FIGURE 2
(A) Muscle embryonic development is highly regulated by the MRFs (Myf5, Myf6,MyoD and Myog). Precusor myoblast from the myotome travel to different parts of the body to establish the different muscle groups. These precursors or myoblast, expressing MyoD, will proliferate, fuse, and differentiate to form syncytial myofibers. During regeneration, the resident stem cells of the muscles, satellite cells, are activated and begin to express MyoD. It will then follow the same steps as in the embryonic muscle development. **(B)** A cross-section of the muscle shows the different cell population found in the muscles. Some of these cells express AR and are instrumental to the androgen-responsive nature of some muscle groups. **(C)** A scheme showing the effects of androgen signaling based on ArKO studies on different muscle groups.

regulated after day 14 of gestation (18). This suggests that some functional redundancy between *MyoD* and *Myf5* exists. *Myog* null mutation results in normal initiation of myogenesis but defects in myocyte differentiation and myotube formation were observed (19, 20). These MRFs act downstream of the paired domain and homeobox-containing transcription factors paired box gene 3 (*Pax3*) and 7 (*Pax7*) (21, 22). *Pax3* mutants display failure in the proper specification of the dermamyotome and myotome compartments of the somite which may lead to defective body wall musculature development (23). *Pax 7* could substitute for some of the function of Pax3 in somite development but not the functions on myogenic specification (24). The primary role of *Pax7* is suggested to be implicated with the specification of myogenic satellite cells (22). The satellite cells contribute to the development, growth and repair of muscles. During development and muscle regeneration, the satellite cells are activated and start to express *MyoD* (Figure 2A). Activated satellite cells become the myoblasts and undergo proliferation. Post-mitotic myoblasts will further differentiate and express *Myog* or *Myf6*. These transcription factors will regulate the terminal differentiation of myocytes and myotube formation (25, 26). The regulatory mechanism of myogenesis is considered as one of the active areas of research and more findings are being accumulated that could specify the exact hierarchy and players during muscle development.

Androgen effects on muscle development

The most recognized effect of androgens on muscles is studied through physiological changes on athletes taking

testosterone (T) and other related steroids. It was discovered that intramuscular injection of T results in an increase in the net protein synthesis followed by enlargement of the muscle fibers (27). The mechanism of hypertrophy is explained through the concepts of nuclear domain and ceiling theory. A muscle fiber contains numerous myonuclei that sustain protein synthesis over a finite number of cytoplasm (nuclear domain). Upon T treatment, the protein synthesis becomes too high to be sustained by one myonuclei (ceiling theory) hence promoting myonuclear accretion. This process will create an enlargement of the muscle fiber leading to muscle hypertrophy (28–30). This could possibly be attributed to the ability of testosterone, through the androgen receptor, to increase the expression of *Myog* and *mTOR* and decrease the expression of myostatin, a negative regulator of muscle mass (31). Additionally, it has been reported that androgen can regulate the expression of polyamine biosynthesis genes such as *Odc1* and *Amd1*. This may also mediate the anabolic actions of androgens on muscle mass (32).

To understand the effect of androgen on the sexual dimorphism in muscle development, the points discussed in studying sexual dimorphism in GT can be used as perspective for summarizing the role of androgen on muscles. First is to identify the type of tissues that are necessary for muscle development and how Ar is expressed and utilized in those cells. A summary of this can be seen in Supplementary Table 1. The effect of androgen signaling is very diverse since the muscle cell population is also composed of different types of cells (Figure 2B, C). A cross section of a skeletal muscle will reveal the presence of different cell such as myofibers, satellite cells, connective tissues, pericytes, endothelial cells, and other mesenchymal cells.

Satellite cells (SC) are the resident stem cells of the muscles and testosterone treatment results in the increase of SC number in humans, rats and pigs leading to muscle hyperthrophy (33–36). SC are described as targets of androgen actions since *Ar* expression is evident on SC in the LA muscle (37). In a study using depletion of satellite cells and testosterone treatment, it was discovered that muscle hypertrophy occurred in testosterone-treated female mice regardless if the mice were depleted with SC ($Pax7^{CreER/CreER}$+;$Rosa26^{DTA/DTA}$) or with the normal number of SC (38). This was further confirmed when satellite cell-specific AR KO ($Pax7^{CreERT2}$+;AR^{flox})female mice exhibited increased mass upon testosterone treatment (39). In the same study, a myofiber-specific ArKO or mArKO ($Ar^{flox/flox}$; *HSA-Cre*) was observed to have increased muscle mass upon testosterone treatment while there was a loss in grip strength. This indicates that muscle mass is not controlled by AR in the myofiber or satellite cell but muscle strength is directly affected by androgen signaling. An RNA seq of the mARKO suggests that glucose/amino acid metabolism and muscle sarcomeric gene regulation, such as *Mylk4*, is regulated by androgen signaling (39).

Aside from satellite cells, a population of uncommitted progenitor cells of mesenchymal (40) and adipocytes origin (41) acts as reservoir of satellite cells during muscle regeneration or hypertrophy. Ar deletion targeted to mesenchymal stem cells resulted in upreglation of key adipogenic transcription factors such as Peroxisome proliferator-activated receptor gamma (*PPARγ*) and CCAAT/enhancer binding protein alpha (*C/EBPα*) (42). Additionally, testosterone treatment of CH3 10T1/2 cells result in the upregulation of myogenic differentiation markers such as *MyoD* and myosin heavy chain II (MyHC) and downregulation of markers for adipogenic differentiation such as *PPARγ*and *C/EBPα* (43). These studies suggest that androgen can shift the commitment of cells from adipocytes or vascular fate to myogenic cells (44). Other cells found in the interstitial compartment such as connective tissues may play a role in regulation of muscle development. A ligament known as gubernaculum which connects the gonad to the future inguinal canal is known to work with the cremaster muscle in the proper descent of the testis. Gubernaculum-specific *Ar* deletion resulted in increased *Pax7* expression and decreased expression of skeletal muscle actin suggesting defective development of the cremaster muscle. However, cremaster muscle-specific *Ar* deletion did not show any observable phenotypes. The authors suggested that the deficiency in AR results in the loss of Ar-induced paracrine signals from the gubernacular cells to the myoblasts (45).

Interestingly, another non-myocytic cell is found to be necessary for the sexually dimorphic development of muscles in the perineum, the BC/LA muscle. The BC is a striated muscle complex inserted into the urethral bulb or bulb of the penis (46, 47) which possesses a posterior extension which is the LA. The functions of these muscles in mouse have not yet been completely studied but equivalent structures in human are suggested to be involved for sustaining erection (BC) and defecation (LA) (48–51). BC/LA complex is known to be sensitive to androgen signaling (52) which is evident in the table, BC/LA is the muscle complex that is mostly affected by muscle-specific AR deletion. A study on the embryonic development of the BC revealed that the *Ar* expressed in the mesenchyme surrounding the BC muscle cells are more important for masculinized BC and not the *Ar* in the myoblast (53). The study mentioned a possible paracrine regulation from non-myocytic cells to myoblast that controls proliferation of myoblast leading to a bigger BC in male. Such well-developed BC in male was feminized after mesenchyme-specific *Ar* deletion.

The second point in studying the sexually dimorphic organ is to identify the inherent difference between male and female muscle development. There are a few studies that compare the development of muscles between male and female or even to compare the difference in muscle markers or gene players in the normal development of sexually dimorphic muscles. The formation of BC and LA are more prominent in male than in

female and ArKO mouse exhibits defective formation in such muscles (46, 51). A study discovered that there are more satellite cells in male LA as compared to female and prenatal exposure of female to exogenous testosterone analogue resulted in the increase of satellite cells and increased LA muscle mass. Additionally, a study observed that the formation of BC in male and female was similar until E.16.5 when the myoblast number in males become more prominent than in the female. ArKO in the mesenchyme of the BC resulted into feminized BC muscles (53). In a later stage (E17), the BC/LA of the female undergoes higher apoptosis rate which leads to an underdeveloped perineum muscle in female (51). These studies show that examining development of BC/LA muscles at embryonic stage,while comparing male and female, can provide a clear picture on the role of androgen in the differential muscle mass of male and female BC/LA.

The male and female difference in the development of other muscle groups has not been studied at embryonic stages or whether a sensitive time window of the sexually dimorphism of muscle exists. Many publications show the control of androgen on varying processes of myogenesis (CITE here) but not on the inherent difference between male and female muscle properties. There are some reports of differences in myogenic properties, and its dependency on androgen signaling between males and females that were observed in adult mice. It was identified that global-KO of *Ar* affected only the muscle mass of male mice and not that of the female. A reduction in the muscle mass of the tibialis anterior (TA) extensor digitorum longus (EDL), gastronomicus (GAST), and soleus (SOL) was evident in male ArKO mice and the same muscles were unaffected in female ARKO (54). Furthermore, muscle contractile strength was also reduced in the EDL of male ARKO, becoming more similar to that of the female. There are studies suggesting that the type of fibers between male and females are inherently different from each other. Muscle fiber types is distinguished by the (MyHC) expressed in the fiber and is correlated with the morphology and abundance of glycolytic enzymes. This translates into contractile velocity of the muscles with type IIB having a higher relative velocity than IIA (55) (Resnicow 2010). In mouse masseter, or muscles of mastication, the female has more Type IIA fibers (expressing MyHC-IIA) while male exhibit more type IIB fibers. The expression of MyHC-IIA is higher in males in the SOL and TA but in the plantaris, IIA is higher in females than in males (56). Another study reported that Mylk4 is differentially expressed in fast-type muscles of male and female mice. *Mylk4* is a gene implicated with increased muscle strength and it was found to be more expressed in male fat-type muscles compared to female. Additionally, *Mylk4* expression can be stimulated by androgen treatment (39).

The third perspective on GT development, confirm the androgen-responsive nature of the genes that were found to be inherently different between male and females, is more difficult to be applied in this review. There are numerous studies measuring the androgen responsiveness of varying key players on myogenesis and muscle physiology,which can be found elsewhere (57–59). These reports are extremely helpful in understanding the possible use of androgens on muscle dystrophy and muscle regeneration. These may be used as initial candidates in understanding the inherent difference in male and female muscles. This will require examining the role of these genes or processes in the natural setting of male and female muscle development and growth.

Conclusion

In terms of musculature, are males and females created equal? The investigation on the sexual dimorphism of the muscles could utilize the perspectives used in discovering the formation of a gender-specific ExG. It would be helpful to identify what are the unique properties of male and female muscles and identify whether these properties are responsive to androgen. It is also important to recognize the different tissues or cells involved in the development and/or physiology of muscles. Each cell type might contribute to the muscle growth through autocrine and paracrine fashion. However, the muscle, as an organ, has its own complexity different from the ExG. Muscles have different muscle fiber types, with each muscle complex comprised of different ratio of such fiber types. These contributes to the differential androgen responsiveness/ dependency of numerous muscle groups. Unlike the ExG, muscles can regenerate and such process, its fruition and implementation, adds another vantage point in understanding androgen`s influence. Muscles has many other properties that goes beyond structure such as muscle strength, contractile velocity, and endurance which cannot be easily distinguished as dimorphic or not. This makes the study of muscle development dynamic and,dare I say, exciting.

Author contributions

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

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.940229/full#supplementary-material

SUPPLEMENTARY TABLE 1
A summary of studies detailing the effect of androgen on muscle as mediated by different cell population found in the muscles.

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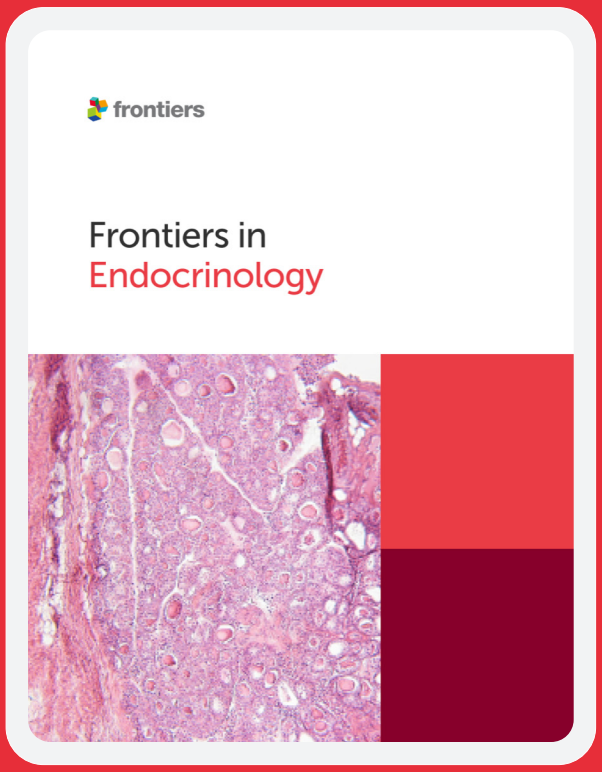

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