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Spatiotemporal map of key signaling factors during early penis development

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Abstract

The formation of the external genitalia is a highly complex developmental process, considering it involves a wide range of cell types and results in sexually dimorphic outcomes. Development is controlled by several secreted signalling factors produced in complex spatiotemporal patterns, including the hedgehog (HH), bone morphogenic protein (BMP), fibroblast growth factor (FGF) and WNT signalling families. Many of these factors act on or are influenced by the actions of the androgen receptor (AR) that is critical to masculinisation. This complexity of expression makes it difficult to conceptualise patterns of potential importance. Mapping expression during key stages of development is needed to develop a comprehensive model of how different cell types interact in formation of external genitalia, and the global regulatory networks at play. This is particularly true in light of the sensitivity of this process to environmental disruption during key stages of development. The goal of this review is to integrate all recent studies on gene expression in early penis development to create a comprehensive spatiotemporal map. This serves as a resource to aid in visualising potentially significant interactions involved in external genital development.

KEYWORDS

androgen receptor, bone morphogenic protein, fibroblast growth factor, genital tubercle, hedgehog, localization, penis, Wnt

1 | DEVELOPMENT OF THE EXTERNAL GENITALIA

There are two distinct phases of external genital development. The first is responsible for patterning and outgrowth of the sexually indifferent genital tubercle (GT) from which the external genitals will develop. The second phase involves the sexually dimorphic development of the GT into either the penis in males or the clitoris in females, with the former being dependent on the actions of the androgen receptor (AR) (Figure 1).

In the mouse, GT development is initiated at embryonic day (E)10.25 at which time lateral plate mesoderm-derived buds form on either side of the cloaca. The cloacal endoderm extends between these paired buds and forms the urethral plate epithelium (UPE) while the left and right buds merge and form a single GT.[1,2] In the
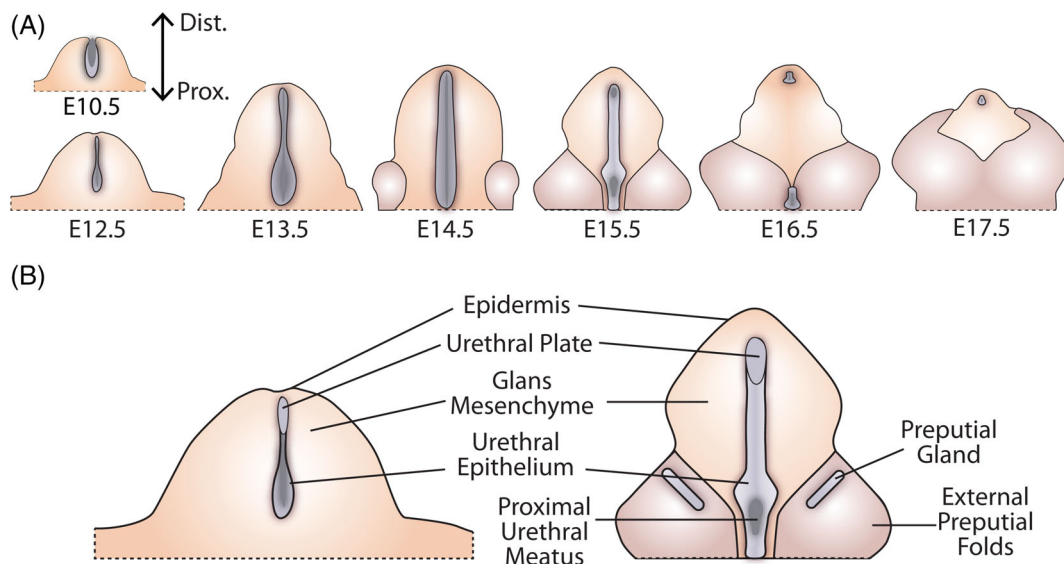
FIGURE 1 (A) Morphological development of the early mouse penis from E10.5 to E17.5. (B) Identification of structures for gene localizations presented in Figure 2 onwards. Preputial gland at E15.5 to E16.5 develop within the preputial mesenchyme and are not physically visible in the genital tubercle from this view

penis, the cloaca-derived UPE canalizes to form a urethral tube.

At E11.5, the lateral swellings have merged to form a single GT. By E12.5, the urethral epithelium appears as a seam at the ventral GT midline, while the GT mesenchyme is yet to undergo spatial differentiation. At E13.5, lateral edges of the GT form secondary outgrowths, growing laterally and ventrally to form the prepuce and later the preputial glands.[1] At this time, dorsolateral condensations of mesenchyme begin to differentiate to form corporeal bodies (spongiosum and cavernosa) that further develop proximo-distally at E14.5. Outgrowth of the dorsal GT results in a marked ventral positioning of the UPE, and the formation of a pit at the interface between dorsal and ventral aspects of the most distal portion of the GT.[1,3,4] At this stage, canalization of the UPE is apparent in the proximal but not distal GT.[5–7] From E16 onwards, the preputial swellings expand distally to encase the GT, fusing in the ventral midline.

2 | HEDGEHOG FACTORS

The hedgehog (HH) family of secreted cell signaling proteins, made up of Sonic (Shh), Indian (Ihh), and Desert hedgehog (Dhh), is critical to many developmental events including those of all appendages including limbs (reviewed in Refs. 8 and 8). HH factors are thought to travel a distance of about 300 μm from their producing cells and can be released from a cell in one of four ways[1]: by association with transmembrane Dispatched protein

as well as the secreted Scube2 protein,[9,10] by self-association of HH protein into large soluble monomers,[11,12] through interaction with glypicans and apolipoproteins to form lipoprotein particles,[13,14] or[4] by release from the cell surface as exovesicles.[15,16] This diversity raises the possibility that different mechanisms for release of HH proteins, and the resulting differences in stability/accessibility, may serve as ways of differentially regulating the actions of individual HH family members. A specific example of this is in the *Drosophila melanogaster* imaginal disk where multiple pools of secreted HH factors are secreted from different tissue regions that possess different ranges of activity.[17] This is in addition to the involvement of membrane proteins in HH-responsive cells, such as patched-1 (Ptch1) and HH interacting protein 1, that limit the diffusion of HH proteins by sequestering them at the cell surface and significantly reducing their range and availability.[18,19]

2.1 | Shh localization

Before the initiation of genital outgrowth in the mouse (E10.5), *Shh* mRNA is expressed in the hindgut endoderm that will contribute significantly to the urethra and UPE. Endodermal *Shh*-expressing cells also contribute extensively to the gut endoderm and ectoderm of the perineum. As GT budding is initiated, *Shh* expression persists throughout urethral endoderm and urethral epithelium, including the distal UPE[1,20–23] (Figure 2).
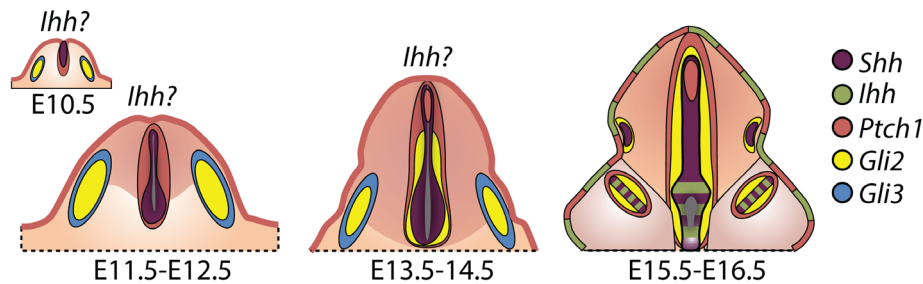
FIGURE 2 Expression and cellular localization of hedgehog ligands and associated factors. While the general view is that hedgehog target genes are expressed in mesenchyme adjacent to UPE, Ptch1 (red) and Gli1 (not shown) are also present in the UPE, suggesting direct hedgehog signaling in this population. *Ihh* has only been localized in mouse genital tubercle (GT) at E17. Expression patterns relate to structures identified in Figure 1. Horizontal bars denote co-localization within the urethral epithelium or preputial glands. Lower expression is indicated by pale colors. Concentric circles denote co-localization in mesenchyme. Expression patterns relate to structures identified in Figure 1

By E12.5 in the GT, *Shh* expression is restricted to the ventral portion, spanning the urogenital sinus epithelium and distal UPE. By E13.5, *Shh* expression is still restricted to urethral and UPE in the most ventral portion of the GT through expansion of the dorsal portion. At E13.5 to E14.5, *Shh* expression is also focally observed in the swellings of the developing preputial glands.[1,24] At E15.5, *Shh* continues to be expressed throughout the urethral and UPE, though in transverse view *Shh* is most highly expressed in the dorsal population of UPE cells.[3,25] High *Shh* expression continues to be observed in the developing preputial glands, a pattern continuing through E17.5 onwards.[25,26]

Recently, an additional member of the HH family—Indian hedgehog (Ihh)—has been implicated in external genital development and its expression pattern is beginning to be characterized. One study showed that *Ihh* mRNA localizes to the epithelial cells of the urethra, prepuce, and glans at E17, with expression higher in the male GT than in females. No analysis of *Ihh* expression at earlier stages of mouse GT development has been reported (Figure 2). In this study, *Ihh* was a downstream target of androgen signaling in GT development,[27] involved in the masculinization stage of GT development. *Ihh* knockout mice have a smaller penis, as well as a smaller mouse urethral mating protuberance (MUMP) and MUMP ridges in the glans. In a marsupial, the tammar wallaby that undergoes a long postnatal process of penis development, Ihh protein localizes to proximal urethral epithelial cells but is absent from the UPE and the distal GT during early development (postnatal day 60; equivalent to E15 in the mouse). Strong Ihh expression is also identified in the ectoderm overlying the urorectal septum (URS). By the time urethral closure is complete (postnatal d150), Ihh expression is observed in distal epithelium. In all cases in the wallaby, Ihh expression is restricted to the outermost layers of urethral and ectodermal epithelium that represent the most differentiated population (ie, squamous and stratified). In contrast, Shh protein was highest in cells occupying an intermediate position within the urethral epithelium and UPE. This mimics the expression of Shh and Ihh in the developing gut epithelium in mice.[20] While the discrete localization of Shh and Ihh observed in the wallaby may reflect different states of differentiation within urethral epithelium, it also raises the possibility that discrete compartments within the same epithelium are serving distinct functions. Of note, *Ihh* RNA localized to preputial epithelium in mouse at E17.5, concentrating in basal cells, except on the ventral preputial surface ectoderm. The latter is consistent with protein localization data from the wallaby.[27,28] Altogether, spatiotemporal data from HH ligand studies reveal potential additional complexities in how these factors signal, not only between epithelium and mesenchyme but also between different epithelial populations.

2.2 | Response to HH proteins

The classic HH receptor is Ptch1, which binds HH proteins at the cell surface and in the absence of ligand constitutively represses HH signaling.[29] The transmembrane protein Smoothened (Smo) is released from intracellular sequestration by Ptch1 binding to HH proteins, and thus executing intracellular activation of HH signaling. The release of cell surface Smo results in de-repression and activates transcription factors of the Gli zinc finger protein family (*Gli1-Gli3*). These transcription factors are bifunctional in that differential proteolytic processing can generate activator or repressor forms. This may occur in part through the discrete organization of downstream signaling factors within primary cilium.[30,31] There is a diversity of HH regulatory systems, such as those regulating

receptor expression, function, or ligand availability through the actions of genes such as Scube2 (reviewed in[32]). Another example is Smo-independent Shh signaling where Ptch1 can directly bind to cyclin B1, sequestering it at the cell surface. In such a case, the presence of Shh dissociates this binding and allows cyclin B1 to enter the nucleus.[33]

2.2.1 | Gli localization

At E10.5, *Gli1* and *Ptch1* are expressed in the peri-cloacal mesenchyme, with expression concentrated in mesenchyme surrounding the urethra.[22,34] For clarity, *Gli1* expression is not illustrated as evidence from knockout studies indicates that Gli1 does not directly influence GT development, however it is able to compensate for *Gli2* haploinsufficiency.[22] At E11.5 through to E13.5, mesenchymal *Gli1* expression expands around the UPE and is also found in GT ectoderm, a pattern complementary to that of *Shh*.[22,34] This is consistent with the view that Shh is acting as an epithelia-to-mesenchyme signaling factor during GT development.[35] By E15.5, *Gli1* RNA is observed extensively through the GT mesenchyme, concentrated in cells adjacent to the urethra and UPE, as well as subectodermal mesenchyme in particular at the ventral aspect of the GT.[25]

Gli2 is expressed in a similar location to Gli1 and Ptch1, bilaterally in the GT and mesenchyme at E10.5 to E11.5.[36] This pattern continues at E13.5 where subtle differences are apparent in the expression of *Gli2* compared with that of *Gli1* and *Ptch1*. Both *Gli1* and *Ptch1* are expressed extensively throughout GT mesenchyme, while *Gli2* occupies a more restricted location, concentrated at the lateral mesenchyme and immediately adjacent to the proximal urethral epithelium, but is lower in the distal GT.[36] *Gli3* is considered the primary repressor of HH actions and very few studies of its localization in the penis have been undertaken. Two studies of *Gli3* expression in the E13.5 and E14.5 GT reported expression within the proximo-lateral GT mesenchyme.[36,37] Another study at E10.5 to E14.5 in the context of global anorectal and urinary tract formation, found widespread *Gli3* expression in bladder mesenchyme that did not completely overlap with the location of *Gli1* and *Gli2*.[38]

An alternative way to localize *Gli* gene expression is to identify which cells are responding to Gli1 to Gli3. In one case, this was achieved by developing a *Gli*-LacZ reporter mouse model using Gli-responsive sequences derived from an upstream flanking region of the *Foxa2* gene.[22] Using this reporter model, HH activity (ie, cells responding to HH factors by activating any or all of *Gli1*-*Gli3*) is observed in the peri-cloacal mesenchyme at E10.5

that matches RNA localization data. Importantly, at E13.5 HH activity is absent from GT mesenchyme but concentrated in the UPE. Together with the localization of HH activity (see Section 2) and *Gli1* RNA in the UPE, these findings may have significant implications. They suggest that, rather than the currently held view that HH signaling is exclusively epithelial to mesenchyme, a component of HH responsiveness in the GT at E13.5 occurs in the UPE. Importantly, the authors of the *Gli*-reporter model indicate that it likely reflects the combined activity of Gli1 to Gli3, both positive and negative gene regulations, which may explain the spatial restriction of LacZ staining. While reporter lines may not faithfully recapitulate endogenous Gli activity, results from this Gli-responsive reporter model add weight to the idea that HH signaling axes are highly unconventional in the developing GT.

2.2.2 | Ptch1 localization

At E10 to E11.5 *Ptch1* is present within GT mesenchyme surrounding *Shh*-positive cloacal endoderm,[22,35,39,40] though the localization of these two markers does not perfectly co-localize. At E12.5, *Ptch1* localizes to the mesenchyme surrounding the urethra and UPE but with levels higher in the proximal mesenchyme and low in the dorsal and distal mesenchyme.[41] This expression pattern continues through E13.5, with a more complex pattern in the distal penis. For example, *Ptch1* is expressed in a subset of the UPE cells as well as extensively in the mesenchyme, in particular concentrated in mesenchyme immediately underlying penile urethra and ectoderm.[25] This is particularly striking when expression is viewed in transverse sections where both *Gli1* and *Shh* are expressed in the same region of the UPE.[22] The implications of this is that discrete regions of the UPE may be both producing HH factors and responding to them, as outlined above, a fact inconsistent with a traditional view of epithelium-to-mesenchyme HH signaling in the developing GT. Therefore, a better understanding of the localization of HH factors and their signaling mediators is critical to form a complete picture of their role in penis development.

3 | WNT FACTORS

The WNT family of secreted signaling factors execute diverse developmental functions and can be broadly classified as engaging canonical (β-catenin-mediated) and two types of noncanonical signaling: 1) The planar cell polarity pathway engaging ROCK and mediating actin

FIGURE 3 Expression and cellular localization of WNT ligands (A), pathway mediators and modulators (B,C). Expression patterns relate to structures identified in Figure 1. Horizontal bars denote co-localization within the urethral epithelium or preputial glands. Concentric circles denote co-localization in genital tubercle (GT) mesenchyme. Expression patterns relate to structures identified in Figure 1. β-Catenin and Lef1 localization reflects protein data. Extrapolation of published data is indicated by genes with "?"
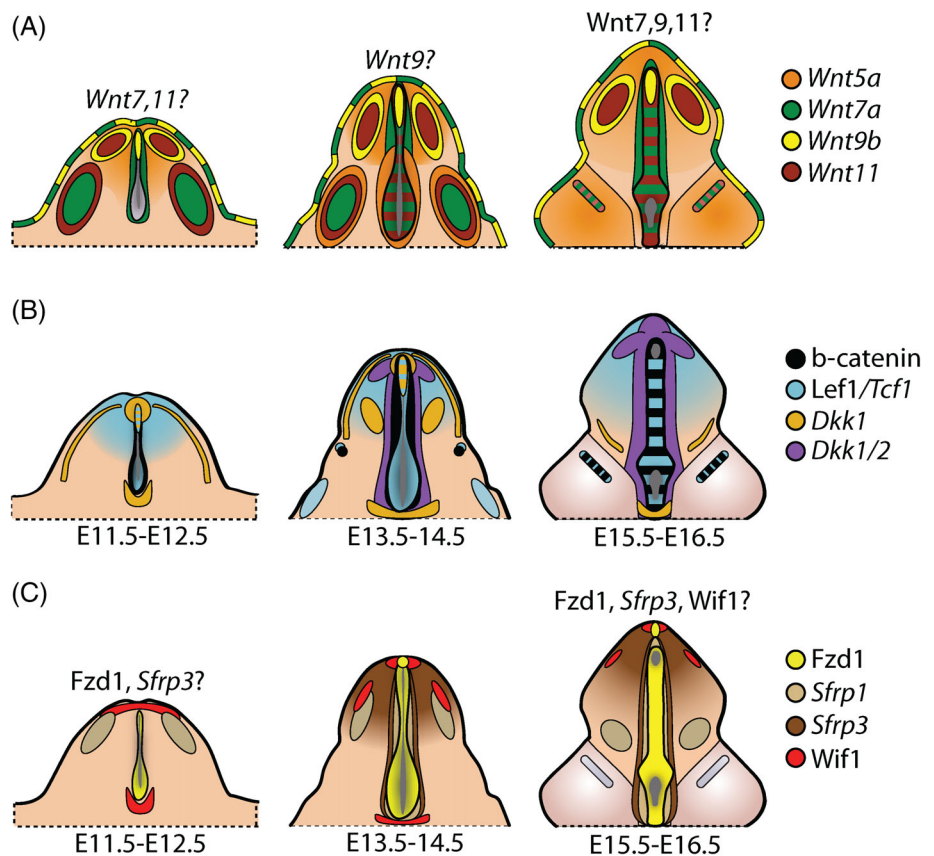
polymerization that allows asymmetrical cytoskeletal organization, and 2) The WNT/Ca^{2+} pathway that regulates intracellular calcium release and is involved in cell movement and adhesion. Wnt4, 5a, and 11 are able to signal via noncanonical pathways, though not all function exclusively via noncanonical systems.[42] The noncanonical class ligand, Wnt5a, was shown to be essential in early GT development, with knockout mice lacking a GT.[43–45] Furthermore, using tissue-specific inactivation models, the activation of WNT signaling in GT mesenchyme and ectoderm was also found to be important to maintain proliferation and tissue integrity, respectively.[46] Significant interaction between WNT signaling and the actions of the AR have also been demonstrated during the masculinization phase of GT development.[47]

3.1 | Wnt5a localization

Of the 19 WNT factors known, only a small number have been localized in the mouse GT, with *Wnt5a* being the most studied. Wnt5a is a component of the noncanonical pathway, and *Wnt5a* mutant mice have a complete failure of GT budding occurring early in development, with an inability of the URS endoderm to contact the cloacal ectoderm.[48] *Wnt5a* is expressed as early as E10.5 and by

E11.5 is concentrated at the distal tip with graded reduction of expression towards the proximal GT (Figure 3).[1,44–46] At E13.5 strong *Wnt5a* expression is observed in mesenchyme of the glans and preputial swellings, with weaker expression also observed at the posterior lateral edges of the cloaca, a pattern that persists until at least E14.5.[1,45]

3.2 | Localization of other WNT ligands

Many other WNT genes are known to be expressed during GT development (*Wnt2*, *2b*, *3*, *4*, *5b*, *6*, *7a*, *7b*, *9b*, *10b*, and *11*) but few have been studied on a spatiotemporal level. Therefore some factors are discussed below but not illustrated. For clarity, the localization of certain factors is an extrapolation where published data is missing at that developmental stage, and these instances are denoted in Figure 3.

At E12.5, *Wnt2* is detectable in the proximo-lateral region of the GT mesenchyme, while weak distal expression is also present. *Wnt3* expression concentrates at surface ectoderm and is also expressed at low levels in GT mesenchyme.[34,46] *Wnt4* is detectable in surface ectoderm and may also be expressed in urethral epithelium.[34] *Wnt6* is expressed exclusively in GT ectoderm at E14.5.[37]

Wnt7a is highly expressed in GT surface ectoderm, with lower levels in mesenchyme adjacent to UPE,[34] and at low levels in epithelium.[37] Some evidence of *Wnt7a* expression in proximal urethral epithelium has been reported.[49] Expression of *Wnt9b* is present in urethral epithelium as well as adjacent mesenchyme,[46] with a concentration at the GT ectoderm.[34] *Wnt11* expression is found in the proximal urethra and mesenchyme,[46] concentrating in subectodermal regions in the distal GT.[37]

3.3 | Response to WNT factors

Receptors of WNT ligands in the canonical pathway include the transmembrane Frizzled (Fzd) and Lrp5/6 proteins that upon activation result in engagement of intracellular partners such as Dishevelled and Axin, resulting in stabilization of cytoplasmic β-catenin.[50] β-Catenin subsequently transits to the nucleus where it associates with Tcf/Lef transcription factors to regulate WNT target genes. Modulation of WNT signaling occurs through the actions of several antagonists, such as secreted Frizzled-related proteins (sFRP) that includes WNT inhibitory factor 1 (Wif1) which bind and sequester WNT ligands, blocking their interaction with receptor complexes. Another antagonist of WNT signaling is the Dickkopf (Dkk) family proteins which bind and internalize Lrp5/6 proteins that are part of the WNT receptor complex. Therefore, it is proposed that sFRP members can antagonize both canonical and noncanonical signaling, while the DKK family inhibit only canonical signaling. Additional levels of WNT signaling modulation come from the E3 ubiquitin ligases Znrf3 and Rnf43 that can induce degradation of Frizzled proteins. Furthermore, the WNT activator R-spondin is important in the development of many other tissues such as gonads but whether it plays a role in modulating signaling in GT development has not been explored and serves as a valuable focus for future study, as *Rspo1* is detectable in mouse GT at E15.5 to 17.5.[47]

3.3.1 | β-Catenin localization

The primary mediator of canonical WNT signaling, β-catenin, is widely expressed in the developing GT and concentrates in urethral epithelium, GT ectoderm as well as the mesenchyme adjacent to the UPE (Figure 3). It is also expressed in the preputial glands at later stages of GT development (E15 onwards), with highest protein levels in epithelial cells.[34,46,47]

Given this broad pattern of expression it is not surprising that canonical WNT signaling is important in all three germ layers during GT development, as demonstrated in tissue-specific transgenic mouse models.[46] Using a TOPGAL reporter to determine canonical WNT signaling activity, activity was first detected at E10.5 in the cloaca. Distinct fields were detected at E13.5, with activity observed in the proximolateral and distal GT, concentrating in subectodermal mesenchyme and adjacent to UPE, respectively. By E14.5, canonical WNT activity expands to include a larger field adjacent to UPE, with proximal activity also observed in the midline of the GT.[46]

3.3.2 | Tcf/Lef localization

Lef1 is expressed throughout GT development but some conflict exists as to its precise localization. Some studies reported *Lef1* expression only in distal GT mesenchyme, preputial glands, and lateral subectodermal mesenchyme at E12.5 to E13.5,[34,46,49] while another study identified *Lef1* RNA and protein expression in the distal GT mesenchyme, as well as a subset of ectoderm and endodermal epithelium.[51] At E14.5 *Lef1* continues to be expressed in the GT ectoderm and adjacent mesenchyme, in particular the ventral region,[37] as well as in the distal GT and periurethral mesenchyme. At E16.5 Lef1 protein is expressed in peri-urethral mesenchyme, at sites of prospective corpora cavernosa formation and in the ventral prepuce mesenchyme. In the guinea pig, Lef1 protein was detected in GT mesenchyme at E17 but absent from urethral epithelium at this developmental stage.[52] *Tcf1* and *Tcf4* are both expressed in the distal urethral epithelium, with *Tcf1* additionally expressed in the ventral ectoderm as well as distal GT mesenchyme.[34,46]

3.3.3 | Dkk localization

Using a reporter mouse system, *Dkk1* activity is present in the cloacal membrane from E9.5 onwards though no precise localization data is available.[53] At E12 *Dkk1* mRNA is detectable in dorsal and ventral peri-cloacal mesenchyme.[54] From E11.5 to E13.5, reporter gene activity is found in the distal urethral epithelium as well as surrounding and subectodermal mesenchyme lateral to the ventral midline.[54] By E14.5 *Dkk1* reporter expression is high, active in distal urethral epithelium and adjacent mesenchyme but expression dramatically decreases by E16.5 and is undetectable at later stages.[53] Another analysis of *Dkk1* RNA levels demonstrated a similar pattern of expression, with clearly restricted *Dkk1* expression patterns, in which expression is reduced in the distal GT mesenchyme at E13.5 but persists in the ventral peri-

cloacal mesenchyme and lateral GT mesenchyme.[49,55] Furthermore, distal *Dkk1* expression marks the proximal edge of the Wnt5a expression domain.[49] Co-expression in this region may denote a "progress zone" in the distal GT, as suggested by the partial co-localization of the WNT activator, *Lef1*, in this region.[49]

At E11.5 *Dkk2* is expressed in regions that give rise to preputial swellings as well as dorsal and ventral peri-cloacal mesenchyme, at the proximo-lateral ventral GT mesenchyme. By 12.5 expression increases at the ventral aspect and extends towards the apical GT distolaterally, as well as distal subectodermal mesenchyme. This pattern of expression continues until at least E13.5.[56] At E14.5 the expression of *Dkk2* is similar to that of Dkk1.[37] The expression of *Dkk2* has also been assessed using a reporter mouse system and expression is localized to GT mesenchyme adjacent to urethral and UPE at E15.5, showing an androgen regulated sexually dimorphic expression pattern.[47] *Dkk3* is expressed in distal GT ectoderm and subectodermal mesenchyme at E14.5, as well as in the proximal GT mesenchyme.[37]

3.3.4 | Frizzled localization

From E14 to E17, increasing Fzd1 protein expression is apparent in GT ectoderm, urethral and UPE, with no expression apparent in GT mesenchyme.[57] Fzd6 protein is expressed in urethral epithelium and GT mesenchyme of the guinea pig, concentrating at regions associated with urethral closure.[52] The expression of other Fzd genes, *Fzd2*, *3*, *4*, *6*, *7*, and *8*, is detectable in the developing mouse GT[46] but only in a high throughput screen. At E14.5, *Fzd4* may be weakly expressed in urethral epithelium, *Fzd6* is strongly expressed in GT ectoderm and UE, *Fzd7* in GT mesenchyme and ectoderm. *Fzd10* is strongly expressed in GT ectoderm as well as distal subectodermal mesenchyme.[37]

3.3.5 | sFRP localization

Localization of these secreted inhibitors of WNT ligands has not been extensively studied but at E11.5 *Sfrp1* is expressed in GT mesenchyme. At E12.5 expression of *Sfrp1* is localized to GT mesenchyme, concentrated on the lateral side of preputial swellings. By E13.5 to E14.5, *Sfrp1* expression is observed in ventral peri-cloacal mesenchyme, and lateral mesenchyme of the ventral GT,[37] a finding supported by data showing weak expression in these regions at E15.5.[25] *Sfrp2* expression is observed throughout GT mesenchyme at E14.5, with expression highest in the proximal GT.[37] *Sfrp3* is expressed in mesenchyme adjacent to UE at E14.5.[37,49]

3.3.6 | Wif1 localization

An additional secreted inhibitor of noncanonical WNT signaling, Wif1, has a high affinity for Wnts 3a, 4, 5a, 7a, 9a, and 11,[58] and protein is weakly expressed in the apical URS endoderm and cloacal mesenchyme at E11.5. Expression increases by E12.5 when the URS is about to fuse with the coelomic mesenchyme. Wif1 protein expression is also observed at the distal GT endoderm and apical genital mesenchyme. Expression of *Wif1* decreases dramatically after formation of the urethral duct (E13.5),[49,59] though expression is still detectable at E14.5.[37]

4 | BONE MORPHOGENIC PROTEIN FACTORS

Bone morphogenic proteins (BMPs) are cytokines with established roles as morphogens in a diverse array of tissues. Many BMP factors share similarities with growth differentiation factors that are also part of the transforming growth factor beta (Tgfβ) superfamily. Only three BMP factors have been localized to the developing GT: *Bmp2*, *Bmp4*, and *Bmp7*. Bmp2 and Bmp4 belong to the same phylogenetic grouping and preferentially bind type I receptors and recruit type II receptors, while Bmp7 binds type II and recruits type I receptors.

4.1 | Bmp4 localization

Before initiation of GT outgrowth (E9.5), *Bmp4* is expressed in the site of future GT development on either side of the cloaca in UPE, with low expression in surrounding mesenchyme, before becoming restricted to mesenchymal cells by E11.5 (Figure 4).[1,20,21,60] Extensive *Bmp4* expression is also observed in proximal GT mesenchyme surrounding urethral epithelium and anterior to the cloaca.[1] By E12.5, *Bmp4* expression becomes restricted to bilateral dorsal and ventral mesenchyme adjacent to the distal urethral epithelium.[1,44,61] At this stage lateral swellings also express *Bmp4* and growth at the dorsal GT is associated with relocation toward the distal tip. This distal *Bmp4* expression persists at E14.5 and is present in the glans penis and preputial glands. Before E14.5 *Bmp4* expression is higher in the dorsal compared with ventral GT, after which it is equal in both regions.
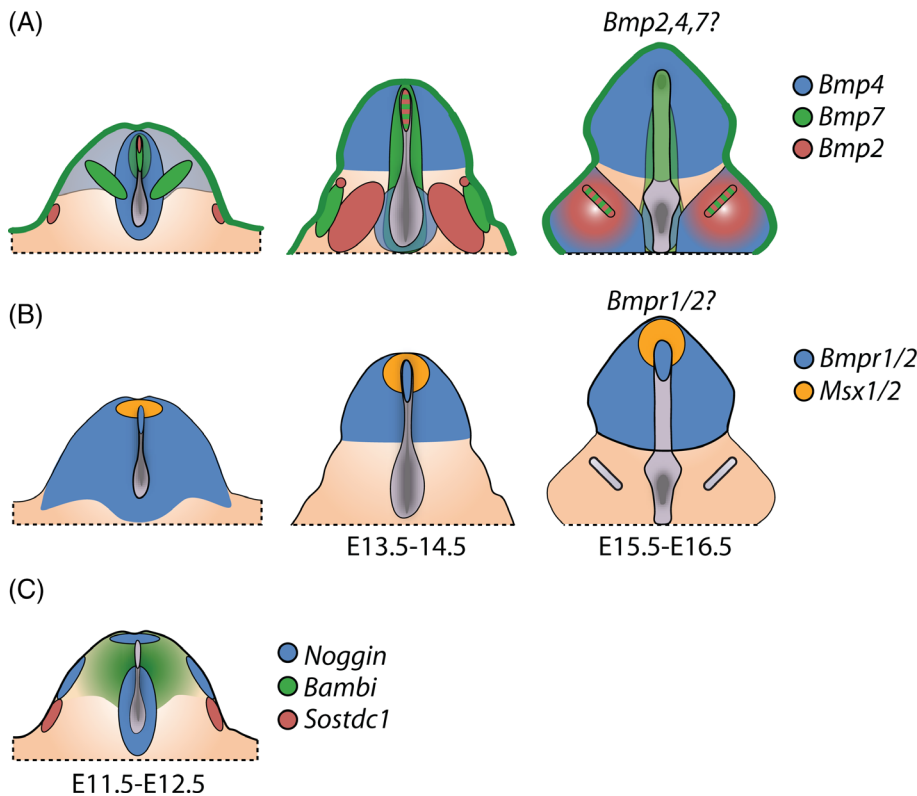
FIGURE 4 Expression and cellular localization of bone morphogenic protein ligands (A), receptors and downstream pathway factors (B,C). Expression patterns relate to structures identified in Figure 1. Bmp4 expression is lower at E11.5 to E12.5 (pale blue). Horizontal bars denote colocalization within the urethral epithelium or preputial glands. Extrapolation of published data is indicated by genes with "?"

4.2 | Bmp2 localization

Bmp2 expression is not observed before E12.5, at which time its expression is found in the distal dorsal and ventral surface of the GT. From E12.5 through E14.5 *Bmp2* is discretely expressed in the preputial swelling of the proximal GT.[1] Strong *Bmp2* expression is also observed in mesenchyme at the base of the GT. By E13.5 *Bmp2* is observed in the preputial glands, ventral swellings of the glans, proximal mesenchyme, and distal UPE in a thin sheet of epithelium luminal to epithelial cells expressing *Shh*.[20] *Bmp2* is also expressed in ventral swellings of the glans. Of relevance, a highly discrete expression of *Bmp2* is found in the ventral surface of the distal UPE at E13.5, a location consistent with the expression of *Fgf*8 at this time.[1]

4.3 | Bmp7 localization

In contrast to *Bmp4* and *Bmp2*, at E12.5 *Bmp7* is exclusively expressed in the urethral epithelium, with expression higher in the distal compared with proximal GT[40,44,62] and proximal lateral shelf mesenchyme.[62] Using a *Bmp7-LacZ* reporter mouse model, at E11.5 labeled cells exist in the URS, and mesenchyme of genital swellings.[63] At E12.5 labeled cells are present in UPE, adjacent mesenchyme and ventral ectoderm, with expression higher at distal regions. This pattern continues at

E13.5 with increasing expression in dorsal mesenchyme.[63]

4.4 | Response to BMP factors

BMPs can signal via canonical and noncanonical pathways. Canonically, BMPs bind cell surface receptors and form hetero-tetrameric complexes with serine/threonine kinase activity. Three type I and three type II2 Tgfβ receptors are able to bind BMPs (Bmp1A, 1B, and the type Ia activin receptor; Bmpr2, type IIA, and type IIB activin receptors). Therefore, BMPs may integrate with functions of the activin signaling pathway.

Conventionally, BMP ligands bind receptors and induce their phosphorylation, resulting in the phosphorylation of Smad1/5/8 that translocates to the nucleus together with Smad4 to regulate gene transcription. An intracellular modulator of this pathway is Smad6 that is able to ubiquitinate Bmpr1, inducing its degradation and inhibiting BMP signaling. Several Smad-independent, noncanonical BMP pathways exist such as those that activate the Mapk, Pi3K/Akt, or Rho-GTPase pathways. Several extracellular inhibitors of BMPs exist, including Noggin, Gremlin, and the pan Tgfβ inhibitor, Bambi.[64] The importance of Smad6 and many noncanonical BMP signaling pathways in GT development are still unexplored. The fact that regulation of BMP action

during tissue morphogenesis is tightly regulated at the posttranslational level[65] suggests that protein localization studies may be required to fully reveal important BMP signaling networks.

At E12.5, *Bmpr1a*, *Bmpr1b*, and *Bmpr2* (Figure 4B) are uniformly expressed throughout the GT mesenchyme and UPE at low levels,[44,62] becoming confined to developing glans and UPE by E13.5. Msx1 and Msx2 are targets of BMP signaling. *Msx1* is highest in mesenchyme adjacent to UPE at E11.5 and more broadly in the distal glans region at E12.5 through to E15.[62,66] In contrast, *Msx2* is expressed in the UPE and mesenchyme adjacent to the distal UPE at E12.5,[46] as well as in the distal GT ectoderm at E14.5.[37,46]

RNA for the BMP inhibitor, *noggin*, is expressed at low levels in mesenchyme surrounding the preputial UPE in the distal GT but not expressed in the UPE itself, a pattern that overlaps with the expression of *Bmp4*. In addition, *noggin* localizes to the ventral mesenchyme of the proximal GT.[44,62] The pan-TGFβ antagonist, *Bambi* is highly expressed in GT mesenchyme at E12.5,[67] with expression reportedly higher in distal vs proximal regions.[44]

5 | FIBROBLAST GROWTH FACTORS

Canonically, secreted fibroblast growth factors (FGFs) control cell proliferation, differentiation and survival through autocrine and paracrine signaling during tissue development, maintenance and repair. They also play a role in the endocrine regulation of metabolism. FGFs bind heparin sulfate proteoglycans that function to modulate diffusion through the extracellular matrix, regulating specificity and affinity of FGF signaling.[68]

Overall, mesenchymal FGF signaling is required for early GT outgrowth, while expression in the ectodermal is necessary for ectodermal differentiation, and the development of the urethral tube by regulating the interaction of ectodermal with urethral epithelium.[51] Critical downstream effectors of FGF signaling include genes of the Etv transcription factor family.

5.1 | FGF localization

Three FGFs have been localized in detail during mouse GT development, *Fgf8*, *Fgf9*, and *Fgf10*, which belong to separate phylogenetic groups. *Fgf8* is not expressed in genital primordia before the onset of budding (E10). After initiation of GT outgrowth (E10.5 onwards) *Fgf8*-expressing cells are restricted to the UPE and distal endoderm most closely associated with the surface ectoderm, and as GT outgrowth occurs this becomes restricted to the ventral distal urethral epithelium (Figure 5). By E14.5 *Fgf8* expression is not detectable.[1,21,48,69,70] *Fgf9* is also expressed in the distal urethral epithelium from E11.5 to 12.5 but occupies a broader cellular area compared with *Fgf8*.[69] It also concentrates at distal ectoderm and subectodermal mesenchyme at E14.5.[37] At *E11.5*, *Fgf10* is expressed in GT mesenchyme lateral to UPE, as well as subectodermal mesenchyme from E11.5-E14.5.[1,21,69,71] By E13.5, *Fgf10* is also expressed in the forming preputial swellings.[1,69]

5.2 | Response to FGF factors

Activation of cell surface FGF receptors results in tyrosine kinase activity feeding into RAS-MAPK, PI3K,
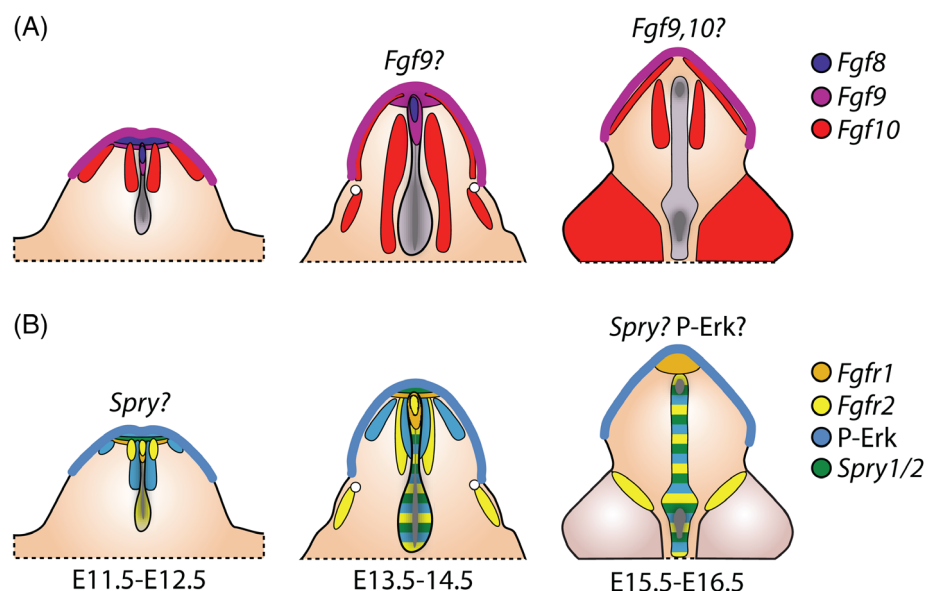

FIGURE 5 Expression and cellular localization of fibroblast growth factor ligands (A), receptors and associated factors (B). Expression patterns relate to structures identified in Figure 1. Horizontal bars denote co-localization within the urethral epithelium. Extrapolation of published data is indicated by genes with "?"

STAT, and PLC signaling pathways. Expression of *Fgfr1* and *Fgfr2* within the urethral epithelium appears to be important for urethral basement membrane formation, and mesenchymal FGF signaling is critical for activation of WNT and BMP signaling in all three tissue layers, regulating programmed cell death within the distal mesenchyme of the GT. In addition, *Fgfr1* and *Fgfr2* can be alternatively spliced to produce two isoforms that differ in their third extracellular Ig domain. These isoforms exhibit different ligand binding affinities and specificities and may be expressed in a tissue-specific manner. The *Fgfr2IIIb* isoform is known to be critical in penis formation, and knockout of this isoform results in defects in GT development resembling mice with a loss of FGF10.[69] FGF signaling can be inhibited by various factors, including the intracellular tyrosine kinase inhibitors belonging to the Sprouty family.

5.3 | FGF receptor localization

Fgfr1 is detectable in distal GT mesenchyme and urethral epithelium from E10.5 to 11.5, as well as the distal most dorsal mesenchyme from E12.5 to E13.5.[69] At E14.5, ventral mesenchyme also begins to express *Fgfr1*, with the highest expression continuing at the distal GT.[71] *Fgfr2*-expressing cells can be observed prior to the initiation of GT outgrowth (E10) and continue until at least E16.5, found in the UPE and urethral epithelium but absent from adjacent mesenchyme.[3,72] However, subectodermal expression was identified in another study.[21] By E13.5, *Fgfr2* expression is also observed in association with the lateral edges of forming preputial swellings, a pattern that continues through later stages of prepuce formation.[3] At E16.5, *Fgfr2* expression persists in UPE, with low levels observable in distal GT mesenchyme.[73] Fgf10 is a major ligand for Fgfr2, and *Fgfr2* is expressed in regions adjacent to *Fgf10*-expressing cells.[3] *Fgfr2* may be weakly expressed in mesenchyme abutting the UPE on the ventral surface.[69,71] In one study, *Fgfr3* was not detected in the mouse GT at E13.5.[69] However, the presence of *Fgfr3* has been reported in urethral epithelium, UPE, as well as ectoderm and distal mesenchyme of the GT in a high-throughput screen.[37] Furthermore, there are decreases in *Fgfr3* in male mice at E15.5 after treatment with an AR antagonist, flutamide, which together suggest *Fgfr3* expression is potentially relevant. This is particularly true in light of the fact that Fgf8 and Fgf9 have a higher affinity for Fgfr3 compared with Fgfr2 or Fgfr1, and Fgf9 has the unique ability to activate the IIIb variant of *Fgfr3*.[68]

The complexity of FGF signaling is highlighted when considering spatiotemporal changes in downstream targets, such as P-Erk. Through phosphorylated in response to several signaling pathways, P-Erk is thought to be primarily a marker of FGF signaling in the distal GT. P-Erk is detectable at E11.5 in distal GT mesenchyme surrounding the UPE, decreasing in this region at E12.5.[34,51] At E13.5 P-Erk is detectable in GT ectoderm and urethral epithelium and expression is maintained until at least E14.5.[51] A more recent Fgf/Erk reporter mouse model confirmed the concentration of FGF activity in mesenchyme adjacent to UPE.[74]

The Sprouty gene family (*Spry1-4*) act in complex ways to inhibit downstream FGF signaling effectors. It is thought their function is to fine-tune the response of cells to ligand.[75] At E14.5 the expression of *Spry1* and *Spry2* is largely restricted to the urethral epithelium, while low *Spry1* is also localized to distal GT mesenchyme on the dorsal surface.[71] Expression of *Spry* at other stages has not been investigated.

6 | ANDROGENS

Androgens are critical to the masculinization phase of GT development.[76–79] The AR functions as a ligand-activated transcription factor that is dependent on and modulated by co-factors at sites of DNA,[80] with significant direct and indirect interactions also known to occur. Early developmental programming effects of androgens influence the trajectory of GT development in humans and animals.[81] The AR interacts significantly with many of the signaling pathways discussed in this review, and mesenchymal AR signaling is critical for the masculinization phase.[47,82] A specific function for epithelial AR has not been found. Together, the central role of this hormone, and the emerging understanding of the complex and interwoven functions of sex steroid receptors, warrants its inclusion for analysis.

6.1 | AR localization

In the mouse, the testes begin producing androgens at E11.5 to 12.5.[83] *Ar* mRNA is detected at E11.5 in the mouse GT,[27] but no localization data is available. At E13.5, the male mouse GT displays strong cytoplasmic Ar protein localized in the urethral epithelium and mesenchyme in the GT midline (Figure 6). At E14, Ar is concentrated in the UPE, and weak expression is also present in the mesenchyme.[55] At this stage, the centrally located epithelial cells of the UPE have stronger Ar expression than that of the outer epithelial cells.[55] At E15.5, Ar is expressed in the surface ectoderm, the mesenchyme of the urethral fold, the UPE, the preputial
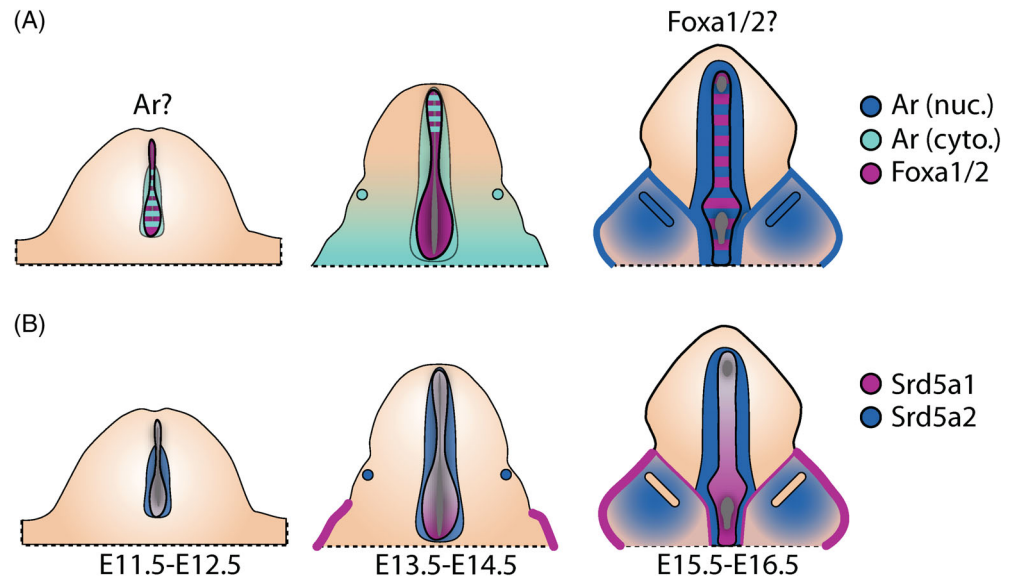
mesenchyme and glans mesenchyme (including the mesenchyme condensing to form the *os penis*).[27,47,62,84,85] At this stage in the proximal aspect of the GT, Ar is expressed in the mesenchyme adjacent to the UPE, but not at the more distal aspect.

In contrast to the GT at E13.5, Ar localization at E15.5 is almost entirely nuclear, suggesting activation of androgen signaling.[27] Ar protein was reported in the GT surface ectoderm adjacent to the distal urethra in one study,[62] as well as in condensing mesenchyme proximo-lateral to the urethral epithelium.

At E16, Ar is expressed in the mesenchyme adjacent to the urethral plate, as well as in UPE at higher levels in cells of the midline.[55] At E16.5 in the male mouse GT, Ar expression continues in the mesenchyme adjacent to UPE, the central cells of the urethral plate, as well as preputial glans and the corporal bodies.[82,84] At E18, Ar is primarily expressed in the urethral epithelium of the male mouse GT, the preputial glans and the preputial epithelium.[55] This expression pattern continues through to postnatal day 1.[27] In addition, Ar is expressed in the mesenchymal progenitors of the *os penis* in the rat.[86]

The distribution of Ar in the mouse GT is reflected in the male GT of other animal models and humans. Steroid autoradiography has revealed that androgen binds to urethral epithelial cells, mesenchymal cells adjacent to the urethra, preputial mesenchymal cells, *os penis*, corpus cavernosum and faintly in the surface ectoderm of the fetal rat GT.[87] This is also reflected in Ar localization in the embryonic guinea pig GT.[88]

In the human GT from gestational weeks 8 to 16, AR is localized in the mesenchyme of the urethral folds, corporal body, glans, preputial mesenchyme, and the UPE epithelium.[89,90] Interestingly, prominent localization of

AR in the UPE follows that of the urethral fold mesenchyme.[89,90] The relatively early expression of AR in the urethral fold mesenchyme suggests that androgens target mesenchymal cells to drive proliferation of the glans and prepuce, as well as signal cells of the UPE to proliferate and canalize.[89] Taken together, the localization of Ar in the mouse GT is largely consistent with that of humans and other animals.

6.2 | Foxa localization

Gene knockout studies demonstrate that only mesenchymal Ar is required for masculinization of the GT.[27] The transcription factors Foxa1 and Foxa2 have a profound role in septation of the cloaca and in urethral closure, which is reflected by their expression throughout the cloacal epithelium at E10.5 and the UPE at E11.5 but not in mesenchymal cells of the GT.[24] The restricted pattern of *Foxa1/2* expression continues at E14.5.[37] In other contexts, Foxa1 serves as a cofactor for the Ar[91,92] whereby it opens chromatin so that Ar can bind to low-affinity response elements which are otherwise inaccessible. Thus, Foxa1/2 may direct Ar in the UPE to specific response elements which are not accessible in mesenchymal cells, However this requires further investigation. Overall, the restricted expression of Foxa1/2 suggests that the target genes of Ar in the epithelium may differ from those in the mesenchyme. Interestingly, an immunolocalization study of Foxa1 in the human GT at 14 and 17 weeks showed that it is restricted to the urethral epithelium but is not expressed in UPE.[6] This raises the possibility that AR engages different co-factors even within epithelial cells subsets of the developing GT.

6.3 | Spatiotemporal activation of AR

Ar in the mouse GT at E13.5 is predominantly cytoplasmic, whereas at E15.5 it is nuclear.[27] Nuclear Ar is a surrogate marker of Ar activation, or at least the presence of sufficient ligand in the environment. Thus, Ar action may show spatial and temporal restrictions occurring beyond serum androgens that may include regional production of ligand to regulate spatiotemporal activation of the Ar. In the mid-region (on the proximo-distal axis) of the Guinea pig GT at E29, Ar localization in the urethral fold mesenchyme is primarily cytoplasmic. However, at the same timepoint in the proximal mesenchyme surrounding the enclosed urethra, Ar is primarily nuclear.[88] These findings suggest that proximo-distal activation of mesenchymal Ar during GT development may drive urethral closure.[7,88] Higher levels of nuclear Ar in the proximal mesenchyme of the embryonic human penis,[89] as well as the proximo-distal expression of 5α-reductase type 2 in the mouse GT (see later),[85] lends further support to this argument.

6.4 | 5α-Reductase localization

The Ar in the mammalian GT contributes to masculinization via the binding of testosterone, but it can also bind more potent androgens such as dihydrotestosterone (DHT).[93–96] DHT is produced from testosterone by 5α-reductase (Srd5a) enzymes encoded by *Srd5a1* (type 1) and *Srd5a2* (type 2).[96] Although 5αR deficiency in humans is associated with external genital defects,[97,98] *Srd5a2* or double *Srd5a1/Srd5a2* knockout mice undergo apparently normal virilization of external genitalia.[99] Therefore, species-specific dependencies on DHT may exist, or alternate pathways to activation of the AR that are more important in some species.

DHT is detected from E14.5 in the male mouse GT and levels increase dramatically at E15.5.[85] DHT levels are particularly high in the ventral-proximal aspect of the GT,[85] the region where urethral tube formation occurs at E16.5.[100] To the best of the authors' knowledge, the only data on *Srd5a1* expression in the mouse GT comes from a high throughput screen demonstrating that *Srd5a1* is expressed in urethral and UPE at E14.5.[37] Consistent with the expression pattern of DHT, *Srd5a2* mRNA is also present in the ventroproximal region of the GT at E14.5 and its expression spreads distally at E15.5, particularly in the ventral mesenchyme adjacent to the urethral plate where AR is also prominent.[85] However, at these stages *Srd5a2* mRNA was not detected in the UPE,[85] which is consistent with non-murine data discussed below.

The mRNA of *Srd5a genes* is also present in the embryonic rat GT.[101] *Srd5a1* mRNA is primarily concentrated in the male GT epithelium, including that of the UPE. On the other hand, *Srd5a2* mRNA is found mainly in the mesenchyme, particularly in the urethral folds.[101] We can translate the timing of *Srd5a* expression in the rat GT, which occurs at E17 but possibly earlier,[101] to that of the mouse by comparing the windows of androgen sensitivity between these animal models. The rat GT is sensitive to androgen exposure at E15.5 to 18.5,[102] while the mouse GT is sensitive to androgen exposure at E13.5 to E16.5.[27] Thus, the difference of ∼2 days in androgen sensitivity between these species and the cytoplasmic-nuclear shift in AR protein localization at E13.5 to 15.5 in the mouse GT (discussed earlier) explains the earlier timing of *Srd5a2* expression in the mouse GT which occurs by E14.5.

The above data are also reflected by *SRD5A2* expression in the human embryonic penis at weeks 16 to 20 of gestation, where it is concentrated in the mesenchyme adjacent to the urethra, particularly at the ventral portion of the developing urethra, and low in the urethral epithelium.[103] The localization of *SRD5A2* in the mesenchyme reflects the functional significance of AR signaling in this tissue. Human SRD5A2 has a greater affinity for testosterone,[96] thus the conversion of testosterone to DHT is likely to be higher in the mesenchyme, resulting in increased AR activation. Conversely, the localization of *Srd5a1* in the rodent UPE may lead to less DHT production than the mesenchyme, potentially reflecting a more subtle role of androgen signaling in this tissue. However, detailed analysis of the expression of *Srd5a1* in the mouse GT is required.

7 | CONCLUSION AND FUTURE PERSPECTIVES

External genital development relies on a multitude of signaling factors with discrete spatiotemporal activity that together represent a deeply integrated and interactive network. In mapping the dynamics of key signaling factors during early penis development it is clear that much is yet to be uncovered. This is particularly true for our understanding of HH signaling, where the current view of epithelial-to-mesenchyme signaling is insufficient to fully explain the expression of factors considered HH target genes. Secondly, evidence now exists supporting the potential for a spatial restriction in AR activation to occur, where target genes may be uniquely occupied by the Ar in discrete compartments of the developing GT.

Moving our understanding forward in this space requires "drilling down" to specific spatiotemporal

windows where expression patterns suggests key developmental events are occurring. Extensive RNA localization analysis and the use of sophisticated transgenic reporter models have revealed fundamental signaling networks at play. However, such experiments are limited and confounded by a variety of factors, including whether reporter models can faithfully recapitulate patterns of expression/activity and the fact that mRNA distribution does not always correlate with protein expression or activity. Overall, better defining protein localization patterns over space and time is required. This has the potential to uncover previously unappreciated interactions and signaling cross-talk during external genital development.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

AUTHOR CONTRIBUTIONS
Gerard Anthony Tarulli: Data curation (lead); investigation (lead); writing – original draft (lead); writing – review and editing (lead). **Samuel M Cripps:** Data curation (supporting); investigation (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Andrew J Pask:** Supervision (equal); writing – review and editing (supporting). **Marilyn Bernice Renfree:** Supervision (equal).

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