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Molecular signaling pathways that regulate prostate gland development

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Abstract

Prostate gland development is a complex process that involves coordination of multiple signaling pathways including endocrine, paracrine, autocrine, juxtacrine and transcription factors. To put this into proper context, the present manuscript will begin with a brief overview of the stages of prostate development and a summary of androgenic signaling in the developing prostate, which is essential for prostate formation. This will be followed by a detailed description of other transcription factors and secreted morphogens directly involved in prostate formation and branching morphogenesis. Except where otherwise indicated, results from rodent models will be presented since studies that examine molecular signaling in the developing human prostate gland are sparse at the present time.

Keywords

prostate development; androgen receptor; Hox genes; Nkx3.1; FoxA1; Notch; Fgf10; Shh; Bmp4; Bmp7; TgfB1; Wnt genes

Stages of prostatic development

In contrast to most male accessory sex glands, which develop embryologically from the Wolffian ducts (mesodermal), the prostate gland originates from the urogenital sinus (UGS) and is an endodermal structure. Although the developmental process is continuous, it can be categorized in five distinct stages involving determination, initiation or budding, branching morphogenesis, differentiation, and pubertal maturation (Fig. 1). Determination of the prostate occurs before clear morphological evidence of a developing structure and involves expression of molecular signals that commit a specific field of UGS cells to a prostatic cell fate. Phenotypic prostate development commences as UGS epithelial cells form outgrowths or buds that penetrate into the surrounding UGS mesenchyme in the ventral, dorsal, and lateral directions caudal to the bladder. In humans, prostate development occurs during the second and third trimester and is complete at the time of birth (Lowsley, 1912;Prins, 1993). This contrasts with the rodent prostate gland, which is rudimentary at birth and undergoes the majority of its development during the first 15 days of life. In the mouse, the initial outgrowth of epithelial buds occurs between fetal days 16.5–17.5 (f16.5–17.5) in a 19 day gestation strain (Sugimura et al., 1986), while in the rat it occurs at f18.5 in a 21 day gestation strain (Hayashi et al.,

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1991). At birth, the ventral, dorsal, and lateral rodent prostate lobes primarily consist of unbranched, solid elongating buds or ducts and subsequent outgrowth and patterning occur postnatally. During this time, proliferation of epithelial cells occurs primarily at the leading edge of the ducts (i.e. distal tips) (Prins et al., 1992). Branching morphogenesis begins when the elongating UGS epithelial buds contact the prostate mesenchymal pads that are peripheral to the periurethral smooth muscle. At that point, secondary, tertiary, and further branch points are established with continued proximal-to-distal outgrowth and complexity (Timms et al., 1994). Branching patterns are lobe-specific with ventral branching preceding that in the dorsolateral lobes by 3–4 days (Hayashi et al., 1991). Morphogenesis of the entire complex is completed between postnatal days 15 and 30. Final growth and maturation occur at puberty (days 25–40) when circulating androgens levels rise sharply.

Epithelial and mesenchymal cell differentiation is co-ordinated with branching morphogenesis and occurs in the proximal-to-distal direction (Prins and Birch, 1995; Hayward et al., 1996a, 1996b). Epithelial differentiation from progenitor cells into differentiated basal and luminal cells has been documented in the rat prostate with changing patterns of cytokeratins as well as alterations in androgen receptor (AR) expression, an early marker of epithelial cell differentiation (Prins and Birch, 1995; Hayward et al., 1996b). The process initiates between days 3 and 5 in the rat ventral prostate and ~ 2 days later in the dorsal and lateral lobes. Lumenization of the solid epithelial cords is concomitant with differentiation into basal and luminal cell layers and initiates in the proximal ducts around postnatal day 5, extending to the distal tips by ~ day 12. Between days 10 and 15, functional differentiation commences, as defined by the synthesis of secretory products by differentiated luminal epithelial cells (Prins and Birch, 1995). Concomitant with epithelial differentiation, the prostatic mesenchyme undergoes differentiation postnatally. As UGS epithelial ducts penetrate into the prostate mesenchymal pads, mesenchymal cells condensate around the tip and form a distinctive pattern along the length of the basement membrane. Between days 3 and 5, cells adjacent to the ducts form a periductal layer of smooth muscle cells while interductal cells differentiate into mature fibroblasts (Prins and Birch, 1995; Hayward et al., 1996a). Lying between the basement membrane and the periductal smooth muscle is an extremely thin single cell layer of differentiated fibroblasts. As the proximal ducts branch and grow, the periductal cell layer tapers in the distal direction forming a single layer of smooth muscle cells at the distal tips of the mature prostate (Chang et al., 1999b). As the prostate undergoes branching morphogenesis, a branched vascular bed forms in parallel with neovascularization forming within the prostatic stromal elements and capillary beds extending to the ductal basement membrane (Shabsigh et al., 1999).

Hormonal regulation of prostate development

The determination and initiation of prostatic development in the human and rodent fetus is entirely dependent upon androgens produced by the fetal testes. Surgical or chemical castration (i.e. anti-androgen administration) of rodents during critical periods of fetal life results in inhibition of prostate development (Price, 1936; Jost, 1953; Price and Williams-Ashman, 1961; Cunha, 1973; Lasnitzki and Mizuno, 1977). However, the extent of inhibition depends on the timing of androgen ablation relative to bud initiation. To study this in detail, Cunha employed organ culture of murine UGS explants from male mice which typically initiate prostate budding at f17 *in vivo* (Cunha, 1973). UGS retrieved from f12–13 mice, before fetal testes production of testosterone at f14, did not produce prostatic buds when cultured for 6–8 days in the absence of androgens. However, UGS explants removed on f14 or f15, i.e. after testosterone production began *in vivo*, and cultured for 6 days without androgens produced buds in 15% and 53% of the tissues, respectively. By f16, 100% of male UGS explants grew prostatic buds when cultured further in the absence of androgens. Using a similar study design with male rats that normally initiate prostate budding at f18.5, Lasnitzki and Mizuno, (1977)

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observed comparable results. Explants of male rat UGS removed between f14.5 and f16.5 failed to bud when cultured in the absence of androgen, while UGS removed on f17.5–18.5, that were exposed *in vivo* to endogenous testosterone, developed prostate buds when cultured without androgens, albeit at half the normal number. The rat explant budding response to androgens *in vitro* was dose-dependent, and dihydrotestosterone (DHT) showed greater inductive capacity than testosterone. Furthermore, a single day exposure of the f16.5 rat UGS explant to testosterone followed by culture in the absence of androgens was sufficient to drive bud formation, albeit it at a lower number, and continued exposure to testosterone was required to achieve maximal bud number (Lasnitzki and Mizuno, 1977; Takeda et al., 1986). Together these findings indicate that while androgens are essential for prostate determination and for maximal bud number and length during initiation, budding can continue to a large degree in the absence of testosterone due to irreversible commitment of the tissue. Rodent neonatal prostate explant cultures have further shown that while branching morphogenesis can occur in the absence of exogenous androgens, maximal organ growth with full branching as well as complete cellular cytodifferentiation are only realized with the addition of exogenous testosterone (Lipschutz et al., 1997).

In the 1970s, it was determined that the primary androgen responsible for prostatic development is DHT, the reduced metabolite of testosterone (Wilson and Gloyna, 1970). DHT is formed intracellularly in the prostate epithelium by 5α-reductase and has been shown to have higher affinity for the AR as compared with the parent compound, testosterone (Fang et al., 1969). Human males with 5α-reductase deficiency syndrome have complete absence of prostate morphogenesis with normal development of the seminal vesicles and vas deferens which empty into a blind vagina (Sitteri and Wilson, 1974). Similarly, treatment of pregnant female rats with a 5α-reductase inhibitor from f14–22 obliterated the formation of prostatic buds, an effect that could be reversed with concomitant administration of DHT (Wilson and Lasnitzki, 1971). However, more recent studies challenge the absolute requirement of DHT for bud induction after the determination stage because testosterone and non-reducible synthetic androgens were capable of inducing equivalent bud numbers as DHT (Foster and Cunha, 1999).

Androgen action is mediated through interaction with nuclear AR which are members of a superfamily of transcription factors (Liao and Fang, 1969; Committee, 1999). Evidence for the absolute necessity of AR for prostate development comes from the observation of prostatic absence in mice or humans with complete dysfunctional AR (Bardin et al., 1973; Brown, 1995). As shown in Fig. 2, AR are highly expressed in the UGS mesenchyme before and during prostate morphogenesis whereas epithelial AR expression is induced after budding and branching morphogenesis has begun (Shannon and Cunha, 1983; Takeda et al., 1985; Husmann et al., 1991; Prins and Birch, 1995). Classical tissue recombinant studies by Cunha demonstrated that AR in the mesenchyme, and not epithelial AR, are responsible for prostatic morphogenesis (Cunha and Chung, 1981; Cunha et al., 1987). When wild-type murine UGS mesenchyme was recombined with AR-deficient murine UGS epithelium and grafted under the renal capsule, the AR-deficient epithelium underwent androgen-dependent ductal morphogenesis, epithelial proliferation, and columnar cytodifferentiation forming glandular epithelium that resembled normal prostate. On the contrary, when AR-deficient UGS mesenchyme was recombined with wild-type UGS epithelium, vaginal-like differentiation occurred. Although further analysis revealed that epithelial AR are required for expression of secretory proteins in mouse (Donjacour and Cunha, 1993) and rat prostates (Prins and Birch, 1995), epithelial proliferation and cytodifferentiation appear to be largely driven by paracrine factors under mesenchymal AR control. Mesenchymal cell differentiation into periductal smooth muscle in the prostate also requires a signal from the epithelium (Cunha et al., 1992). Because we have shown that AR induction in prostate epithelium begins as early as postnatal days 1–2 (before cytodifferentiation of the epithelium and mesenchyme) (Prins and Birch, 1995), it is possible that androgen-driven epithelial signals contribute to morphogenesis of the

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prostate by affecting the differentiation of adjacent mesenchymal cells. Recent evidence with AR inactivation restricted to murine prostate epithelial cells confirms the above model and also provides evidence that epithelial AR regulates basal cell proliferation (Simanainen et al., 2007). It is noteworthy that AR expression does not vary along the proximal–distal axis of the developing and adult prostate (Prins et al., 1992; Prins and Birch, 1995), thus differential gene expression along this axis is likely driven by factors other than androgens.

There is also clear evidence for a role of other steroids including estrogens and retinoids during prostate development and the readers are referred to recent reviews (Prins et al., 2001; Huang et al., 2004; Prins and Korach, 2008). Studies from our laboratory have identified specific receptors for these steroids during rat prostate morphogenesis, which vary in a time and cell-specific manner (Prins and Birch, 1997; Prins et al., 1998, 2002; Pu et al., 2003). While these transcription factors are not essential for prostate development, we propose that they modulate expression of specific genes that are involved in differentiated function and homeostasis. A schematic summarizing steroid action through specific receptors during prostatic development is shown in Fig. 2.

Developmental genes

Appendicular patterning (proximal-to-distal outgrowth), as seen during continuous branching morphogenesis of glandular structures, is dictated by time-specific and region/cell-specific expression of master regulatory genes that are evolutionarily conserved throughout the animal kingdom. So common are signal pathways across species and between organs of a single species that it is envisioned there is a conserved “morphogenetic code” or common set of rules that is used repeatedly in different combinations to effect formation of separate organs (Hogan, 1999). Although common morphoregulatory genes are expressed by all branched structures, the critical difference is that spatial and temporal combinations of these as well as organ-specific genes give rise to unique structures. Precise coordination of these events implies tight feedback interactions and, for the prostate, androgenic regulation at some level. In this review, we will consider two major categories of morphoregulatory genes involved in prostate development: (1) Nuclear transcription factors that include common and organ-specific homeobox genes and (2) secreted signaling ligands encoded by a small number of conserved multigene families including *Hedgehogs, Wnts, Fgfs,* and *Bmps/Tgfβ/activin* (Hogan, 1999). These latter positive and negative regulatory molecules communicate paracrine and autocrine signals between epithelial and mesenchymal cells via their cognate receptors. Importantly, while specific genes may drive cell determination, proliferation, differentiation, or spatial patterning, the interpretation of new signals will always be determined by a cell’s history. In recent years, a marked number of studies in rodent models has permitted formation of a “prostatic morphogenetic code” (Fig. 3). Based on work from our laboratory, we have schematized the temporal expression pattern of several of these key genes over the different stages of rat prostate development (Fig. 1) and these results as well as studies from multiple laboratories will be highlighted below.

There are several critical points that must be borne in mind regarding prostate development. The adult prostate gland is a heterogeneous ductal structure with defined proximal, central, and distal regions (Lee et al., 1990). Similarly, during morphogenesis of the prostate, expression of developmental genes, and secretion of paracrine factors is heterogeneous across regions and cell types along the proximal–distal axis. This positional specification must be incorporated into models that describe molecular regulation of prostate development. Frequently, regional expression is most complex at the distal tip and sites of branchpoints where differential and reciprocal signaling is essential for morphogenetic changes. We have recently termed this region the “distal signaling center” similar in nature to distal regions in the limb and lungs (Pu et al., 2003). Regional expression of developmental genes by a subpopulation

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of cells also results in gradients of secreted morphogens. Complexity is added to this model when interpretation of the morphogen by receiving cells is non-linear due to differing sensitivity thresholds (e.g. presence/absence of cognate receptors). Another level of complexity arises when positive and negative morphogenetic signals as well as their secreted inhibitors overlap in specific regions. Finally, while specific studies typically focus on the nature and role of individual morphoregulatory genes, it is important to appreciate the signaling networks that arise due to cross-regulation in gene expression, a topic that will be discussed at the end of this review.

HOMEOBOX GENES AND TRANSCRIPTION FACTORS

Axis positioning and tissue determination involve expression of specific members of the homeobox gene superfamily (Gehring, 1994). These master regulatory genes encode transcription factors that contain a highly conserved ~ 60 amino acid peptide segment, the DNA-binding Ahomeodomain, which recognizes specific regulatory regions of target genes. Specific homeobox genes have been identified within developing prostate tissue and are thought to, in part, account for prostate determination, budding and morphogenesis. These include members of the *Hox* gene family (Warot et al., 1997) and the *NK* gene family (Bieberich et al., 1996; Bhatia-Gaur et al., 1999).

Hox genes

The largest and most extensively studied members of the homeobox superfamily are the *Hox* genes, which determine patterning in body regions from *Drosophila* to humans. In mammals, gene duplication has led to four *Hox* clusters (A, B, C, and D) on separate chromosomes encoding a total of 39 *Hox* genes (Krumlauf, 1994). Similar genes in the separate clusters are considered paralogs and are largely, although not always, redundant. Expression of these genes, from the 3′ to 5′ end of each cluster, follows a strict pattern of spatial and temporal colinearity during embryogenesis. The 3′ genes designate anterior regions while the 5′ genes encode posterior regions. A generalized model for regional tissue specification is that nested, partially overlapping expression domains of several genes in a *Hox* cluster determine segment identity. As a rule, the most 5′*Hox* genes expressed in a given tissue have specification dominance over the more anterior *Hox* genes that are co-expressed in that tissue.

As the prostate is one of the most posterior organs in the male, the most posterior genes of the *Hox* clusters are involved in prostate gland identity. *Hox*a13 and *Hox*d13 have similar expression profiles and patterns in the developing rodent prostates and are believed to have functional redundancy (Podlasek et al., 1997, 1999b). Studies with null mutant mice have shown essential roles for *Hox*a13 in prostate growth and *Hox*d13 in prostate growth and branching with compound mutants exhibiting severely hypoplastic prostate rudiments despite normal testis (Podlasek et al., 1997, 1999b; Warot et al., 1997). Bushman and colleagues characterized the expression of *Hoxa*-13 and *Hoxd*-13 in the mouse prostate and observed that levels are highest during fetal life and decline postnatally (Oefelein et al., 1996; Podlasek et al., 1999b). While expression is strongest in the mesenchyme, epithelial expression is also found at lower levels during fetal and neonatal life. Expression patterns for *Hoxa*13 and *Hoxd*13 differ in the rat prostate where levels are lower during the budding and morphogenesis stages and rise to higher expression levels in the adult prostate (Huang et al., 2007). The significance of these differences is unclear.

In contrast to the A and D paralogs, *Hox*b13 localizes exclusively to epithelial cells in the murine and rat prostate (Sreenath et al., 1999; Economides and Capecchi, 2003; Huang et al., 2007). Furthermore, levels rapidly rise during the postnatal period as the epithelium differentiates with expression localized to central duct and distal tip epithelial cells (Huang et al., 2007). A clear increasing anterior-to-posterior expression gradient is observed with highest

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levels in the rat ventral lobe, declining expression in the lateral and dorsal lobes, and minimal detection in the anterior prostate (coagulating gland). It is notable that while *Hox*a13 and *Hox*d13 are expressed in the seminal vesicles, *Hox*b13 is restricted to UGS-derived reproductive tract structures, which suggests that *Hox*b13 is important in prostate identity (Huang et al., 2007). Studies with *Hox*b13 null mutant mice revealed an essential role in epithelial cell differentiation, because loss of secretory gene production and cell polarity were observed in the ventral lobe (Economides and Capecchi, 2003). This was supported by recent studies from our laboratory in which lentiviral vectors expressing *Hox*b13 in undifferentiated rat prostate cells were capable of driving differentiation to a luminal cell phenotype (Huang et al., 2007). Of particular interest, *HOX*B13 is expressed in normal adult human prostates and in all specimens of prostate cancer where levels are frequently elevated. Based on this ubiquitous *HOX*B13 expression, it has been suggested that the rodent ventral lobe, typically regarded as having no human homolog, is in fact most representative of the human prostate with regards to *Hox* gene expression (Edwards et al., 2005).

When examining posterior *Hox* gene expression in the male accessory glands including the separate rat prostate lobes, we observed that each structure has a unique *Hox* gene profile, which we propose contributes to the separate lobe branching patterns and functional identity (Huang et al., 2007). This includes the more anterior *Hox*a 9, *Hox*a 10, and *Hox*a 11 genes which are also expressed in the developing rat prostate, although at levels 10-fold lower than the *Hox*13 genes. [Note: To date, *Hox*c13 has not been found in the prostate gland (Takahashi et al., 2004).] Organ culture studies using newborn rat prostate lobes revealed that the posterior *Hox* genes expressed during prostate development, including *Hox*a13, *Hox*d13, and *Hox*b13, are up-regulated by testosterone (Huang et al., 2007). Interestingly, this was specific to the ventral lobe because lateral lobe *Hox* genes were not affected by androgens. Furthermore, androgens had limited effects on *Hox* gene expression in the adult prostate where they only up-regulated *Hox*b13 levels. Together, these findings suggest that androgenic regulation of *Hox* genes may contribute to prostate gland morphogenesis and maintenance of epithelial differentiated status.

Studies on human prostate *HOX* gene expression have been confined to adult tissues and cells which show expression of all *HOX*13 paralogs as well as several anterior *HOX* genes (Miller et al., 2003; Jung et al., 2004b; Takahashi et al., 2004). While there are no reports on the expression or roles for *HOX* genes during human prostate development, studies on human prostate cancer have identified the potential involvement of *HOX* gene dysregulation in human prostate cancers (Waltregny et al., 2002; Miller et al., 2003; Jung et al., 2004a; Edwards et al., 2005). Based on these reports, it has been suggested that normal *HOX* expression is necessary for homeostasis of the human gland.

*Nkx*3.1

A novel member of the *NK* homeobox gene family, *Nkx*3.1, the mammalian homolog of *Drosophila NK*-3 (bagpipe), was identified in 1996, and its expression in the male reproductive tract was restricted to UGS-derived prostate and bulbourethral gland epithelium (Bieberich et al., 1996; Schiavolino et al., 1997). Importantly, this gene is expressed in the fetal mouse UGS epithelium at bud sites before bud formation suggesting a role for *Nkx*3.1 in prostate determination (Bieberich et al., 1996; Bhatia-Gaur et al., 1999). Expression of *Nkx*3.1 continues during ductal outgrowth and branching morphogenesis and is highest at the distal regions of the elongating and branching structures. In the rat prostate lobes, we observed a sharp peak in *Nkx*3.1 expression between days 6 and 15 as the epithelium undergoes cytodifferentiation with a marked decline to relatively lower steady-state levels thereafter (Prins et al., 2006). Epithelial *Nkx*3.1 expression is maintained throughout life and is believed to be important for epithelial homeostasis. Null mutant *Nkx*3.1$^{-/-}$ mice exhibit defective branching patterns, perturbed

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functional differentiation and adult onset of prostatic intraepithelial neoplasia (PIN) indicating roles for *Nkx*3.1 in prostate branching morphogenesis and differentiation (Bhatia-Gaur et al., 1999; Schneider et al., 2000; Tanaka et al., 2000; Kim et al., 2002). The importance of *Nkx*3.1 in maintaining epithelial homeostasis is strongly supported by multiple studies in animal models and humans, which show that *Nkx*3.1 acts as a tumor suppressor and that loss of expression is involved in prostate carcinogenesis and progression (Bowen et al., 2000; Shen and Abate-Shen, 2003; Bethel et al., 2006; Guan et al., 2007).

*Nkx*3.1 expression in the adult mouse prostate and the human prostate LNCaP cell line is directly up-regulated by androgens at the transcriptional level (Bieberich et al., 1996; Prescott et al., 1998). We recently demonstrated that androgens strongly and rapidly increase *Nkx*3.1 expression in the developing rat prostate lobes, which provides another pathway whereby androgens influence prostate development (Pu et al., 2007). However, because epithelial AR expression is absent in the fetal UGS epithelial cells when *Nkx*3.1 is initially expressed, it is unlikely that prostate development is initiated through direct androgen action on *Nkx*3.1 gene transcription. Importantly, *Nkx*3.1 expression during prostate formation has been shown to be strictly dependent on epithelial *Shh* expression (Schneider et al., 2000). Multiple *cis*-regulatory elements that mediate distinct expression domains of *Nkx*3.1 have been identified and key elements important for prostatic expression are contained in a distal 5Kb region located > 7Kb downstream from the coding sequence (Chen et al., 2005). Further deletion analysis is required to identify transcription factors that act through this 5Kb region to regulate prostatic expression during early development.

Fox A1 and A2

Forkhead box genes (*Fox*), formerly known as hepatocyte nuclear factor or *HNF* genes, encode a superfamily of winged-helix transcription factors from *Drosophila* to mammals (Kaestner et al., 2000). Multiple *Fox* genes have been identified that are specific to endodermal-derived structures and several are involved in organ development (Clevidence et al., 1993). Of these, *Fox*A1 (formerly *Hnf*3) localizes to the developing prostate epithelium where it plays an important role in ductal morphogenesis and epithelial cell maturation (Kopachik et al., 1998). *Fox*A1 expression is observed in rat and mouse f18 UGS epithelial buds and levels increase with prostatic development and are maintained throughout adult life (Kopachik et al., 1998; Gao et al., 2005). Sustained expression of *Fox*A1 in the rodent and human prostate is required for probasin and PSA expression, respectively, through direct interactions with both *Fox*A *cis*-regulatory elements and AR on gene promoters (Gao et al., 2005). In contrast, *Fox*A2 is only expressed in prostate epithelial cells at the mesenchymal interface during the early budding stage and rapidly declines thereafter (Mirosevich et al., 2004). Null mutant *Fox*A1$^{-/-}$ mice are neonatal lethal, and Matusik and colleagues determined the prostate phenotype using renal capsule organ rescue and tissue recombination (Gao et al., 2005). At birth and postnatal day 1, prostate rudiments at the budding stage were identical to wild-type prostates indicating that *Fox*A1 loss does not affect prostate bud initiation. After renal grafting, prostate growth was reduced, lumen formation was incomplete, epithelial cells were disorganized, their differentiation was arrested at the intermediate stage, and they failed to express secretory gene products. Although *Fox*A1 expression is restricted to epithelial cells, periductal smooth muscle layers were expanded in size in *Fox*A1$^{-/-}$ rescued prostates, perhaps due to persistent expression of paracrine-acting *Shh* by the developmentally arrested basal-type epithelial cells. It is noteworthy that expression of several mesenchymal expressed paracrine factors including *Fgf*10, *Fgf*7, and *Bmp*4 were markedly increased in *Fox*A1$^{-/-}$ rescued prostates indicating indirect regulation of these genes by this epithelial cell transcription factor. Importantly, AR expression was not affected by loss of *Fox*A1. In all, these detailed studies demonstrate an essential role for *Fox*A1 in prostate epithelial cell differentiation and continued function in both AR-independent and AR-dependent manners.

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Notch1/delta/jagged

The *Notch* signaling pathway is a highly conserved cell-cell signaling system involved in cell fate specification and patterning in developing tissues (Bolos et al., 2007). It consists of a single-pass transmembrane *Notch* receptor glycoprotein that interacts with *Jagged/Delta* membrane proteins on adjacent cells to initiate activation. Activation involves proteolytic cleavage of *Notch*, releasing the intracellular domain, which translocates to the nucleus where it interacts with transcription factors and regulates gene expression including *Hes*1, a known downstream target. Nuclear localization of the cytoplasmic domain of *Notch*1 as well as *Hes*1 expression is observed in mouse UGS epithelium and in early prostatic buds on f18 indicating its activation at that early stage (Grishina et al., 2005). *Delta-like ligand*1 (*Dll*1) is expressed in epithelial cell clusters adjacent to mesenchyme where buds emerge while *jagged*1 expression localizes to UGS epithelium and proximal mesenchyme at that time (Grishina et al., 2005). Furthermore, *Maniac Fringe*, which glycosolates *Notch*1 and potentiates its interaction with *Dll*1, also localizes to the epithelium of the initial prostatic buds. Together, these localization patterns place an active *Notch*1 signaling pathway at the sight of bud initiation in the developing prostate suggesting a potential role in that process.

Gao and colleagues have characterized the role of *Notch* signaling in postnatal murine prostate development. *Notch*1 is highly expressed in the mouse prostate epithelial compartment at the time of birth, remains high throughout morphogenesis and declines to low expression in adults (Shou et al., 2001). While *Notch*1 is initially expressed by all progenitor cells, upon cytodifferentiation *Notch*1 localizes to only basal epithelial cells (Wang et al., 2004). Inhibition of *Notch* cleavage using secretase inhibitors in a neonatal rat ventral prostate organ culture system markedly reduced branching morphogenesis and interfered with epithelial differentiation (Wang et al., 2006b). After six days of organ culture, the majority of epithelial cells co-expressed basal (CK14) and luminal (CK8) cell cytokeratins rather than distinct cell populations as seen in control cultures. Furthermore, these intermediate cells were highly proliferative. Because null mutant *Notch*1$^{-/-}$ mice are embryonic lethal, mice homologous for loxP-flanked *Notch*1 and positive for interferon-inducing Mx-Cre transgene in the prostate epithelium were used to examine phenotypes over time (Wang et al., 2006b). After development was completed (d15–25), deletion of *Notch*1 was induced and three weeks later, ventral prostates exhibited reduced secretions, enhanced epithelial proliferation, increased epithelial infoldings with occasional bridging and clusters of predifferentiation epithelial cells co-expressing CK8 and CK14. Hyperplastic phenotypes were observed as the animals aged. Together, these findings provide strong support that *Notch* signaling inhibits expansion of prostatic progenitor cells and facilitates epithelial differentiation during development and that continued pathway activation plays a role in maintaining homeostasis.

To determine gene pathways affected by *Notch*1 signaling, prostate-specific *Notch*1 was deleted by crossing loxP-*Notch*1 mice with *Nkx*3.1+/−Cre mice and prostate gene expression was examined by microarrays (Wang et al., 2006b). Networks containing c-Fos and c-Jun were the most affected pathway supporting a critical role for *Notch*1 in cell specification and differentiation. Interestingly, despite known interactions in other developing structures, genes involved in *Shh* and *Wnt*signaling pathways were not affected by *Notch*1 deletion in the prostate.

SECRETED SIGNALING MOLECULES

In addition to developmental determination by homeobox genes and other transcription factors, branching morphogenesis is driven by a complex interplay between epithelial and mesenchymal cells through secretion of paracrine and autocrine factors. While many secreted epithelial–mesenchymal signals have been characterized, a small number of highly conserved signaling molecules have been found to be critical during embryogenesis (Hogan, 1999). In

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particular, combinations of *Hedgehogs*, *Wnts*, *Fgfs*, and *Bmps/Tgfβ/Activins* to a large extent control soft tissue development. These positive and negative regulatory molecules are spatially and temporally regulated and communicate signals between cells via their cognate receptors. Below we review members of these key families that are known to be involved in prostate gland development.

Sonic hedgehog (*Shh*)

Sonic hedgehog (*Shh*) is a member of the conserved *Hedgehog* family, which also includes *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*) and is expressed in developing tissues from *Drosophila* to mammals. *Shh* is a secreted glycoprotein produced by epithelial cells at the mesenchymal interface in developing structures where it is involved in determination of cell fate, proliferation and embryonic patterning (see review by Ingham and McMahon, 2001). This secreted morphogen binds to membrane-bound *patched* (*ptc*) receptors on adjacent mesenchymal cells and establishes epithelial-mesenchymal cell cross-talk. Liganding of *ptc* by *Shh* relieves its inhibition on *smoothened* (*smo*) resulting in activation of *Gli* transcription factors, the downstream effectors. In vertebrates, there are 3 known *Gli* transcripts; *gli*1, *gli*2 and *gli*3, which have both redundant and unique actions. Importantly, *GLI*1 and *gli*2 are transcriptional activators, while *gli*3 is believed to be a transcriptional repressor (Walterhouse et al., 1999; Meyer and Roelink, 2003), which permits tight regulation of *Shh* actions. Both short-range and long-range actions of *Shh* have been described which differ as a function of concentration gradients (Gritli-Linde et al., 2001). *Shh* is considered to be a master regulatory morphogen because it regulates the expression of other secreted morphogens and homeobox genes in several structures including the prostate (Goyette et al., 2000; Schneider et al., 2000; Haraguchi et al., 2001; Perriton et al., 2002; Chuang and McMahon, 2003; Pu et al., 2004). It also induces *ptc* and *gli*1 expression thus establishing an autoregulatory loop (Marigo and Babin, 1996).

Shh is expressed in prostate epithelial cells at the earliest stages of prostate bud induction in the rodent prostate gland and rapidly declines over the next several days as morphogenesis is completed (Podlasek et al., 1999a; Lamm et al., 2002; Berman et al., 2004; Pu et al., 2004). As with other organs, *Shh* is expressed in a spatially defined manner. During the initial budding phase, *Shh* has a broad epithelial expression along the ductal length, which rapidly transitions into a distal tip pattern as the ducts elongate and branch (Fig. 5). Of note, expression patterns are heterogeneous at the distal signaling center with foci of high *Shh* expression at specific sites, which may permit highly localized actions in those cells resulting in differential growth and branchpoint formation (Pu et al., 2004). In developing mouse and rat prostates, *ptc* expression localizes to the condensed mesenchymal cells adjacent to the elongating epithelial ducts with strongest expression surrounding the distal tips (Fig. 5) (Lamm et al., 2002; Pu et al., 2004). A weaker *ptc* signal is also found within the epithelial cells in the distal but not central or proximal regions of the branching ducts, which provides an opportunity for autocrine *Shh* action in the distal epithelium. *Gli*1, *gli*2, and *gli*3 are all expressed in mesenchymal cells at the distal signaling center (Lamm et al., 2002; Pu et al., 2004) with some noteworthy differences. While *gli*1 and *gli*2, the transcription activators, are also expressed in periductal mesenchyme along the ductal length, *gli*3, the transcription repressor, is restricted to the distal tips (Pu et al., 2004). Additionally, *gli*1 and *gli*3, but not *gli*2, are expressed in distal tip epithelial cells immediately adjacent to mesenchyme. Finally, a potential *Shh* signaling modulator, *Scube*1, was recently identified as highly expressed in UGM and distal-tip prostate mesenchyme during morphogenesis (Vanpoucke et al., 2008). In total, this complex pattern of transcription activators and repressors may permit differential *Shh* actions at specific sites in both epithelial and mesenchymal cells during prostate development with the highest *Shh* signals transmitted at the distal signaling center.

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The human fetal prostate expresses *SHH* with a strong increase in expression between fetal weeks 10 and 13, the time when active morphogenesis is underway (Barnett et al., 2002). Recent analysis has shown the full component of all *SHH* signaling pathway genes in the human fetal prostate including *SMO*, *PTC*1 and *GLI*1 as well as *DHH*, which supports an active role for this pathway in human prostate development (Zhu et al., 2007). In addition to the peak between weeks 10 and 16, there was a subsequent decline followed by a second expression peak at week 28 corresponding with elevated proliferation, which indicates a potential active role for *SHH* during third trimester prostate growth and differentiation.

Specific roles for *Shh* during prostate development have been examined in rodent models by a number of laboratories using a variety of approaches. Studies using anti-*Shh* antibodies (Podlasek et al., 1999a) and cyclopamine to block *Shh* signaling showed that a functional *Shh* pathway was required for bud initiation and ductal elongation placing *Shh* as a critical inducer for prostate formation. However, subsequent studies using UGS tissues from null mutant $Shh^{-/-}$ mice were able to demonstrate bud initiation in organ culture (Freestone et al., 2003; Berman et al., 2004) and differentiated prostate formation when grafted in whole or as recombinants of $Shh^{-/-}$ epithelium with wild-type UGS mesenchyme under the renal capsule (Berman et al., 2004). These studies led to the conclusion that *Shh* is not required for prostate bud induction or elongation. This was more recently challenged by additional examination of the $Shh^{-/-}$ mouse prostate that showed compensatory expression of *Ihh*, which is not normally expressed in the prostate (Doles et al., 2006). Further, this *Ihh* was capable of activating the *hedgehog* signaling pathway in $Shh^{-/-}$ prostates thus indicating that prostatic budding, elongation and differentiation in $Shh^{-/-}$ prostates are possible due to continued *hedgehog* pathway signaling. Additional studies with $Gli^{-/-}$ mice revealed the requirement of *Gli*2 for normal budding as well as considerable functional redundancy between these transcription factors (Shaw and Bushman, 2007). In total, the weight of these recent studies leaves the original antibody and inhibitor experiments more reliable in predicting an essential role for *hedgehog* signaling for prostate initiation and development, although a more definitive assessment will be required for an absolute determination.

Beyond the issue of *Shh*'s role in prostate budding, other studies have demonstrated a role for *Shh* in maintenance of progenitor cells within the prostate for normal ductal patterning (Berman et al., 2004) and for epithelial proliferation, differentiation and branching (Freestone et al., 2003; Pu et al., 2004). Excess *Shh* added to an organ culture system resulted in reduced ductal growth and branching (Freestone et al., 2003; Wang et al., 2003). However, this was shown to be a function of *Fgf*10 down-regulation and *Bmp*4 up-regulation throughout the prostate resulting in growth restraint (Pu et al., 2004). In contrast, localized *Shh* delivery with microbeads at the distal tips revealed localized *Fgf*10 down-regulation concomitant with localized growth inhibition. Based on these findings, we propose that focal expression of *Shh* within the distal signaling center results in localized gradients of growth inhibitory and stimulatory factors that combined, permit differential growth and branching during the branching morphogenesis stage (Fig. 4). This will be discussed further in the section on signaling networks.

While evidence has shown that androgens are capable of up-regulating prostatic *Shh* expression (Podlasek et al., 1999a; Freestone et al., 2003; Pu et al., 2004), similar *Shh* levels in male and female f17 UGS tissues and continued expression of *Shh* following AR blockade cast doubt on its absolute necessity (Freestone et al., 2003; Pu et al., 2007). Furthermore, *Shh* is expressed in late fetal UGS epithelium and budding prostate ducts when epithelial AR expression is limited or absent. Blockade of *Fgf*10 signaling in short-term rat prostate organ culture was able to block androgen stimulation of *Shh* expression indicating that androgen regulation of prostate *Shh* expression during early development is indirectly mediated through the mesenchyme (Pu

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et al., 2007). Currently, the factors or signals that induce and drive *Shh* expression in the UGS epithelium during early prostate gland initiation remain unresolved.

Fibroblast growth factor-10

*Fgf*10 is a member of the fibroblast growth factor (*Fgf*) family of secreted morphogens which consists of 23 known members (Ornitz and Itoh, 2001; Raman et al., 2003). *Fgf*s have a high affinity for heparin and glycosaminoglycans (GAGs) which position them for interaction with membrane associated, tyrosine kinase *Fgf* receptors (*Fgf*R) on target cells (Uematsu et al., 2000). Developmental studies have shown a critical role for *Fgf*-10 in initiation and directional outgrowth of buds as well as ductal branching in many branched structures including the prostate gland (Bellusci et al., 1997; Thomson and Cunha, 1999). In the prostate, as in other branched structures, *Fgf*10 expression is spatially restricted to the distal aspects of the glands where it is believed to function as a chemoattractant for elongating ducts and an inducer of ductal branching through stimulation of epithelial cell proliferation (Lu et al., 1999; Thomson and Cunha, 1999; Donjacour et al., 2003). As previously shown (Huang et al., 2005), the expression pattern of *Fgf*10 in the mesenchymal pad is broad during early stages of ductal budding and elongation and subsequently condenses tightly around the elongating ducts with strongest expression at the distal tips during active branching morphogenesis (Fig. 5). Identical *Fgf*10 patterning is observed in the separate rat prostate lobes. There are four *Fgf* receptors (*Fgf*R) with multiple splice variants that have varying affinities for the *Fgf* ligands, which adds considerable complexity during development. The splice variant of *Fgf*R2, *Fgf*R2*iii*b, is the specific transmembrane receptor for *Fgf*10 as well as for *Fgf*7 (Finch et al., 1995). It is expressed by prostatic epithelial cells thus establishing an important stromal-epithelial cell paracrine pathway during development. We observed a spatially restricted pattern for *Fgf*R2*iii*b in the distal tips of elongating rat prostate ducts at day 1 (Fig. 5) with continued distal expression during branching morphogenesis that results in restricted epithelial cell proliferation at these distal sites (Huang et al., 2005).

Studies with null mutant *Fgf*10$^{-/-}$ mice established an essential role for *Fgf*10 in prostate initiation and branching morphogenesis, because prostates were rudimentary with limited bud numbers, and growth was severely restricted (Donjacour et al., 2003). Renal grafts of *Fgf*10$^{-/-}$ prostatic rudiments in intact wild-type hosts showed little growth with limited differentiation. Importantly, while *Fgf*10 plus testosterone could partially restore prostatic growth of *Fgf*10$^{-/-}$ rudiments, *Fgf*10 alone was ineffective suggesting that *Fgf*10 is essential but not sufficient for prostate bud development and that *Fgf*10 must interact with other testosteroneinduced genes for prostate formation. Short-term culture of mesenchyme-free, epithelial ducts isolated from newborn rat ventral prostates revealed that while testosterone alone was incapable of initiating ductal branching, this could be induced by *Fgf*10 alone, which demonstrates that *Fgf*10 is sufficient for branch point formation (Huang et al., 2005). Furthermore, *Fgf*-10 induced branching was blocked by a *Mek*1/2 inhibitor, showing that *Fgf*10/*Fgf*R2*iii*b acts through the *ras/raf/Mek/Erk*1/2-signaling pathway in the developing prostate. This was recently confirmed in organ cultures of f18 murine UGS which showed that the *Mek*/*Erk*1/2 pathway was essential for *Fgf*10 stimulated bud induction and elongation in the presence of testosterone (Kuslak and Marker, 2007). Tissue and cell-specific requirement for *Fgf*R2 in murine prostate morphogenesis was also demonstrated using a Cre-LoxP system to delete *Fgf*R2 in UGS epithelium (Lin et al., 2007). Interestingly, the ventral lobe was more sensitive to epithelial *Fgf*R2 deletion because it was absent in *Fgf*R2^{cn} mice while small dorsal and lateral lobes developed. In those regions, ducts were underdeveloped with impaired branching and epithelial aberrations indicating that the *Fgf*R2 pathway is also necessary for terminal differentiation. Furthermore, androgen dependency for prostatic homeostasis was disturbed in the absence of epithelial *Fgf*R2, which supports a role for *Fgf*R2 in mediating androgenic action in the prostate gland.

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*Fgf*10 was initially described as a prostatic andromedin defined as a paracrine-acting, androgen-regulated, secreted mesenchymal factor that drives proliferation or differentiation of the epithelium (Lu et al., 1999). This was challenged in other studies that failed to show robust androgen regulation of *Fgf*10 gene expression (Thomson and Cunha, 1999; Thomson, 2001). However, more recent experiments using shorter-term cultures of developing prostates (Pu et al., 2007) and earlier time points (Kuslak and Marker, 2007) have shown that *Fgf*10 expression in the newborn rat ventral and lateral lobes and the fetal mouse UGS mesenchyme is indeed up-regulated by exogenous androgens. We have also shown that *Fgf*R2*iii*b is up-regulated in the ventral prostate by testosterone which can further amplify *Fgf*10-mediated action as a function of androgen levels in the developing tissue. Using an *Fgf*R2 antagonist and a *Mek* inhibitor, *Fgf*10 signaling was shown to be essential for testosterone stimulation of epithelial *Shh* and *Hox*b13 expression in the ventral prostate thus establishing that *Fgf*10 functions as an androgen-regulated paracrine factor that influences epithelial cell gene expression of other morphoregulatory genes (Pu et al., 2007).

Most branched structures expressing *Fgf*10, including the prostate, also express endogenous regulators of *Fgf* action to maintain tight regulation of its proliferative signals. This includes the *Sprouty* proteins that modulate receptor tyrosine kinases including epidermal growth factors (*Egf*s) and *Fgf*s. While the role of *Sprouty* proteins has not been examined for the developing prostate gland to date, it deserves mention that *Sprouty* 1, 2 and 4 are expressed in the human adult prostate and levels are down-regulated in prostate cancer (Wang et al., 2006a). Of interest, a novel variant of *Sprouty*1 that represents a fetal isoform is also observed in prostate tumour cells and tissues (Fritzsche et al., 2006) suggesting that dysregulation of developmental *Sprouty* genes may contribute to abnormal growth with disease. In addition to down-regulation of *Fgf*10 expression by *Shh* as described above, stromal*Tgf*β1 has also been shown to directly suppress *Fgf*10 expression at the proximal promoter in the developing rat prostate (Tomlinson et al., 2004b). These regulatory networks are further highlighted in the last section.

Bmps/Tgf β/activins

Bone morphogenetic proteins (*Bmp*s) are members of the *Tgf*β gene superfamily and, in general act as inhibitors of proliferation during development (Hogan, 1996). Secreted *Bmp*s initiate cell signaling by binding transmembrane Type II receptors (*Bmp*RII or *Act*RII), which complex with Type I receptors (*Alk*3 and *Alk*6) and activate intracellular pathways involving Smads 1, 3 and 5. In the mouse and rat prostate, *Bmp*-4 is broadly expressed in the UGM and prostate mesenchymal pads before and during bud initiation and levels decline postnatally with expression localized to periductal mesenchyme along the length of the elongating and branching ducts (Lamm et al., 2001; Prins et al., 2006). *Bmp*R1 are expressed by both mesenchymal and epithelial cells in mouse prostate indicating that *Bmp* actions may be mediated on both cell types during development (Lamm et al., 2001). While targeted disruption of *Bmp*-4 is embryonic lethal, Bushman and colleagues found that *Bmp*4$^{+/-}$ heterozygotes possess an increased number of branching tips in the murine ventral prostate indicating that it functions as a prostatic growth inhibitor (Lamm et al., 2001). This conclusion was further supported by organ culture studies with exogenous *Bmp*4, which prevented ductal budding and outgrowths. Based upon its localization pattern, actions in organ culture systems, increased growth in *Bmp*4$^{+/-}$ prostates and rapid decline in expression as morphogenesis proceeds, it is believed that *Bmp*4 restricts ductal outgrowths and that clearing of its expression is required for bud initiation. Continued *Bmp*4 expression along the ductal length is thought to play an active role in branching morphogenesis by limiting epithelial cell proliferation at restricted sites as modeled in Fig. 4 (Lamm et al., 2001; Pu et al., 2004; Huang et al., 2005; Prins et al., 2006). Because *Bmp*4 expression is regulated by other prostatic growth factors including up-regulation by *Shh* and down-regulation by *Fgf*10, we propose this results in *Bmp*4 expression gradients at distinct sites that contributes to ductal branching (Pu et al., 2004; Huang et al.,

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2005). In contrast to secreted growth stimulators, we found that androgens decrease the expression of *Bmp*4 in the developing prostate and propose that repression of the growth repressors contributes to androgenic regulation of prostate development (Pu et al., 2007).

*Bmp*7 is another *Bmp* family member expressed in the prostate gland that plays an inhibitory role in prostate development (Grishina et al., 2005). Expression of *Bmp*7 has been localized to UGM before bud initiation in the mouse prostate and to epithelial cells during postnatal life (Grishina et al., 2005). In the rat prostate, we observe increasing expression of epithelial *Bmp*7 between days 1 and 5 with localization restricted to the distal signaling center in the separate lobes (Huang et al., 2005). *Bmp*7 ligands to *Bmp*RII and Type I receptors *Alk*2 and *Alk*6 and activates Smad1. Interestingly, while *Alk*2, 3 and 6 are expressed in mouse f18 UGS epithelial cells, *Alk*6 alone is found in newborn proximal mesenchyme while *Alk*2 and *Alk*3 are present in the distal mesenchyme which may provide differential responses at these distinct sites (Grishina et al., 2005). To determine a role for *Bmp*7 during development, null mutant $Bmp7^{-/-}$ mice were analyzed and their prostates exhibited a twofold increase in branching. Furthermore, addition of recombinant *Bmp*7 to organ cultures inhibited morphogenesis which together indicates that similar to *Bmp*4, *Bmp*7 functions to restrict prostate growth during development (Grishina et al., 2005). However, due to expression in distinctly different cell compartments, the mechanisms of growth inhibition are likely to differ. Of particular interest, *Notch*1 signaling was derepressed in *Bmp*7 null prostates resulting in widespread *Notch* activity throughout the epithelium. It is proposed that *Bmp*7 may restrict ductal branching during prostate development by limiting epithelial domains with *Notch*1 activity (Grishina et al., 2005).

Like many secreted growth regulators, actions of *Bmp*4 and, to a lesser degree, *Bmp*7 are modulated by a secreted endogenous inhibitor termed *noggin*. *Noggin* functions by binding to available *Bmp* ligands in the extracellular regions thus blocking their interactions with transmembrane receptors. A recent study by Bushman and colleagues demonstrated the critical importance of *noggin* during prostate development using null mutant $noggin^{-/-}$ mice, which showed complete absence of the ventral mesenchymal pad and loss of ventral prostate budding with restricted budding in the dorsolateral regions (Cook et al., 2007). This find reinforces the concept that unopposed *Bmp* action in the UGM will inhibit prostate formation. Further, organ culture studies revealed that *Bmp*4 inhibited proliferation of p63+epithelial cells at the distal tips while *noggin* addition blocked this action. The authors propose that mesenchymal-expressed *noggin* interacts with secreted *Bmp*4/7 to create a gradient of *Bmp* signaling along the ductal axis that restricts and stimulates outgrowth at specific sites.

*Tgf*β1 has also been shown to have a growth inhibitory role during prostate gland development. Both *Tgf*βRI and *Tgf*βRII are found in developing prostate stromal and epithelial cells thus permitting *Tgf*β action in both cell populations (Chang et al., 1999a). While *Tgf*β2 and *Tgf*β3 protein localize to rat prostate epithelium upon differentiation, active *Tgf*β1 localizes to the postnatal periductal mesenchymal cells as they differentiate into smooth muscle cells (Chang et al., 1999a). Similarly, in mice, high levels of *Tgf*β1 mRNA were observed in mesenchyme surrounding areas of active epithelial duct formation (Timme et al., 1994). Organ culture studies with newborn mouse (Tanji et al., 1994) and rat (Itoh et al., 1998; Tomlinson et al., 2004a) prostates demonstrated that exogenous *Tgf*β1 inhibited testosterone-induced growth and branching morphogenesis. This may be mediated in part through induction of epithelial $p21^{cip1/waf1}$, a known downstream gene of prostatic *Tgf*β1, which drives epithelial cells into a terminal differentiation pathway, effectively limiting their proliferation (Chang et al., 1999a). It is noteworthy that *Tgf*β1 actions varied along the proximal–distal axis with suppression of epithelial and stromal cell proliferation in the proximal ducts yet stimulation of epithelial cell proliferation at the younger and less differentiated distal tips (Tomlinson et al., 2004a). The growth inhibitory actions of *Tgf*β1 may also be mediated, in part, by *Tgf*β1-induced

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redistribution of nuclear AR to the cytoplasm in prostate smooth muscle cells effectively suppressing androgen action in those cells (Gerdes et al., 1998). In addition, *Tgf*β1 was shown to repress prostatic *Fgf*10 expression which may further contribute to its growth inhibitory effects (Tomlinson et al., 2004a).

Activins, also members of the *Tgf*β gene superfamily, have been shown to influence prostate gland development. *Activin*s are comprised of homo- and heterodimers of β_A and β_B subunits, which form *activin*s A, AB and B. They have been shown to play important roles in development of multiple structures including the mammary glands (Welt and Crowley, 1998). Studies by Risbridger and colleagues demonstrated a specific role for *activin*s in rat prostate gland development (Cancilla et al., 2001). *Activin* β_A localized to mesenchymal cells surrounding the distal tips of branching ducts, while *activin* β_B was found in some mesenchymal and fibroblastic stromal cells but not in smooth muscle. As the epithelium differentiated into luminal cells, *activin* β_B was strongly expressed. Specific receptors ActRIA and ActRIIA were found throughout the epithelium of developing glands implicating them as direct targets. Addition of exogenous *activin* A inhibited ductal branching and elongation in newborn rat ventral lobes by limiting epithelial cell proliferation at the distal tips. It also suppressed stromal differentiation of smooth muscle cells towards the distal duct regions. Actions of *activin*s in tissues are counteracted by *activin*-binding follistatin proteins. Follistatin was expressed throughout the epithelium of developing prostates and maintained into the mature glands. In newborn prostate explants grown in the absence of testosterone, addition of follistatin increased growth and branching. Together these findings support a balanced interaction between *activin*s and follistatins in regulating ductal growth and branching with concentrated action at the distal tips (Cancilla et al., 2001).

Wnt genes and signaling regulators

The *Wnt* genes encode a large, highly conserved family of secreted glycoproteins that play important roles in controlling tissue patterning, cell fate and proliferation within a broad range of embryonic contexts (Cadigan and Nusse, 1997; Nelson and Nusse, 2004). *Wnt* genes are the mammalian homologues of the *Drosophila* polarity gene, *wingless*. The 19 mammalian *Wnt* proteins identified to date associate with ECM proteogylcans and bind to *frizzled* (*Fzd*) cell surface receptors thus mediating cross-talk between cells (Cadigan and Nusse, 1997). Vertebrate *Wnt*s have been divided into two functional groups by reference to their downstream signaling pathways. In short, the canonical *Wnt*s signal through nuclear β-catenin/TCF-LEF while the noncanonical *Wnt*s function through alternate pathways that include Ca1/PKC or RhoA/JNK (Bejsovec, 2005). While both pathways involve initial liganding to *Fzd* receptors, the canonical pathway includes recruitment of a coreceptor, LRP5/6 on the cell membrane, while the non-canonical pathway does not involve this molecule. In addition to *Wnt* ligands, receptors and downstream signaling molecules, the *Wnt* network also includes a number of extracellular secreted regulators that antagonize *Wnt* actions. Secreted frizzled-related proteins 1–5 (*sFrp*s) have a cysteine-rich domain similar to *Fzd* receptor ligand-binding domain and dampen *Wnt* actions by competitive binding for available *Wnt*s. *Wnt* inhibitory factors (*Wif* 1 and 2) also bind to secreted *Wnt*s and block their ability to interact with *Fzd* receptors on the cell membrane (Hsieh et al., 1999). The *dickkopf* (*Dkk*) proteins 1–4 bind to the canonical coreceptor LRP 5/6 and interfere with canonical *Wnt* signaling specifically (Mao et al., 2001). The large number of secreted inhibitory molecules stresses the critical importance of tight control of *Wnt* signaling to effect normal development and maintain tissue homeostasis.

Despite a large number of studies that have demonstrated a role for aberrant *Wnt* signaling during prostate carcinogenesis and progression (Chesire and Isaacs, 2003; Yardy and Brewster, 2005), there is little published work on the role(s) of *Wnt* genes during prostate development. The numerous functions of *Wnt* signaling in animal development include crucial morphogenic

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roles of many, if not most, organs and thus it is expected that this includes the prostate gland. Constitutive expression of stable β-catenin in prostate epithelium of transgenic mice resulted in epithelial hyperplasia and squamous metaplasia by 8 weeks of age with transdifferentiation to epidermal-like cell lineages suggesting that canonical *Wnt* signaling plays a key role in cell determination, differentiation and proliferation in the prostate (Bierie et al., 2003). A role for β-catenin is also suggested for normal prostate epithelial proliferation because nuclear β-catenin increases in proliferating prostate epithelium of castrated rat prostates following androgen replacement (Chesire et al., 2002). SAGE libraries of adult and developing mouse prostates, UGE and UGM were screened and revealed strong expression of several *Wnt* gene family members during early development, which was confirmed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for *Wnt* 4, *Wnt*11, *Fzd*1, *Fzd*7, LRP, *Lef*1 and s*Frp*2 (Zhang et al., 2006).

We have screened for expression of *Wnt* genes and signaling components in the developing rat ventral prostate using DNA arrays and a PCR array and have identified expression of several *Wnt*s, *Fzd*s, *Dsh*s, most intracellular signaling molecules involved in canonical and non-canonical *Wnt* signaling as well as extracellular regulators (unpublished findings). As shown in Fig. 6A, six *Wnt* genes were expressed with high signal intensity in Day 3 ventral lobes including three canonical *Wnt*s (*Wnt*2, *Wnt*2b and *Wnt*7b), three non-canonical *Wnt*s (*Wnt*4, *Wnt*5a and *Wnt*11), *Fzd*2 and 4 and *Dsh*1. Temporal *Wnt* gene expression profiles were gathered by qRT-PCR and all, except *Wnt*7b, showed high expression at birth with levels declining during and after the completion of morphogenesis (Fig. 1, unpublished findings). In contrast, *Wnt*7b expression was relatively low during early development and expression rapidly increased upon functional cytodifferentiation. Along with spatially restricted expression, these dynamic temporal expression profiles suggest important roles for these morphogens during prostate gland development. Detailed studies with *Wnt*5a demonstrated that this noncanonical *Wnt* is a growth and branching repressor during morphogenesis (Huang et al., 2006). Of particular interest, we observed with explant cultures that androgens repress *Wnt*5a expression in the ventral prostate (Pu et al., 2007), again supporting our proposal that androgens repress the growth repressor genes to drive prostate gland development.

Expression and functions for the secreted *Wnt* regulators has also been examined in the developing mouse and rat prostate glands. *SFrp*1 (Joesting et al., 2005) and *sFrp*2 (Zhang et al., 2006) were found to be highly expressed in developing mouse prostates using SAGE libraries and Affimetrix DNA arrays, respectfully, which was confirmed by RT-PCR. For both genes, signal was found in the early UGM and prostate mesenchyme, but with bud development, signal was concentrated in the ductal epithelium. Addition of recombinant *sFrp*1 to rat ventral prostate explant cultures resulted in increased growth over 5 days relative to BSA-treated controls. Similarly, we observed that *Dkk*1 protein added to newborn rat ventral lobes stimulated growth and branching over a four-day period (Fig. 6B). Because *Dkk*1 antagonism is specific to canonical signaling, these findings suggest that canonical *Wnt* signaling may play an inhibitory role with regards to epithelial cell proliferation during development.

Cross-talk between developmental genes

As we have mentioned throughout this review, it is clear there are complex signaling networks in the developing prostate gland that involve cross-regulation of morphoregulatory gene expression. We propose that these gene regulatory networks organize normal prostate development through a temporal series of reciprocal signals and feedback loops that tightly regulate proliferation, differentiation, ductal outgrowth and branchpoint formation. This is schematized in Fig. 7 where we highlight known interactions of developmental genes in the fetal and newborn prostate gland. Androgen action during prostate development includes

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stimulation or repression of several genes and this action may be potentiated through a resultant cascade of cross-regulatory networks that work together to drive prostate gland development. Mesenchymal *Fgf*10, acting via epithelial *Fgf*R2*iii*b, directly up-regulates epithelial *Shh* expression resulting in up-regulation of mesenchymal *ptc* and *gli*s which down-regulate mesenchymal *Fgf*10 expression, thus establishing a negative feedback loop to provide tight control of branching (Pu et al., 2004;Huang et al., 2005). *Fgf*10 also down-regulates expression of *Bmp*4, an established restrictor of growth and branching in the prostate gland (Huang et al., 2005). Because *Fgf*10 and *Bmp*4 have opposing actions with regards to prostatic ductal outgrowth, localized down-regulation of *Bmp*4 expression by *Fgf*10 may contribute to *Fgf*10's stimulatory effects. Furthermore, because *Shh* up-regulates mesenchymal *Bmp*4 expression at focal sites in prostatic ductal tips (Pu et al., 2004), down-regulation by *Fgf*10 will contribute to the reciprocal regulation necessary to sculpture the prostatic form. Similar upregulation of mesenchymal *Wnt*5a by *Shh* may further contribute to focal growth at the ductal tips (Huang et al., 2006). Both mesenchymal *Fgf*10 and epithelial *Shh* stimulate expression of the epithelial homeobox genes, *Hox*b13 and *Nkx*3.1, that drive epithelial differentiation. *Shh* also has reciprocal stimulatory action with the early epithelial transcription factor *Fox*A2, which itself is repressed by *Fox*A1 as ducts elongate (Gao et al., 2005). *Fox*A1 stimulates *Nkx*3.1 expression thus in addition to its own role in promoting epithelial differentiation, *Fox*A1 maintains differentiation by networking with *Nkx*3.1 (Gao et al., 2005). *Fgf*10 increases epithelial *Bmp*7 expression that in turn blocks epithelial *Notch*1 expression (Grishina et al., 2005), which may serve to enhance proliferation and suppress premature differentiation during the early growth phase. Mesenchymal *Tgf*β1, which becomes functional as periductal mesenchyme differentiates into smooth muscle (Chang et al., 1999a), down-regulates *Fgf*10 expression (Tomlinson et al., 2004a), which will serve to brake prostatic growth as development is completed.

Undoubtedly, there are other yet uncharacterized morphoregulatory genes with additional interactions that together contribute to the growth, branching and differentiation of the prostate gland during development. We look forward to learning of these actions in the coming years and predict this will eventually lead to a thorough understanding of the prostate developmental processes. In addition to providing a more complete developmental picture, this information will be of tremendous value towards understanding dysgenesis in growth and differentiation that occurs in benign prostatic hyperplasia and prostate cancer upon aging.

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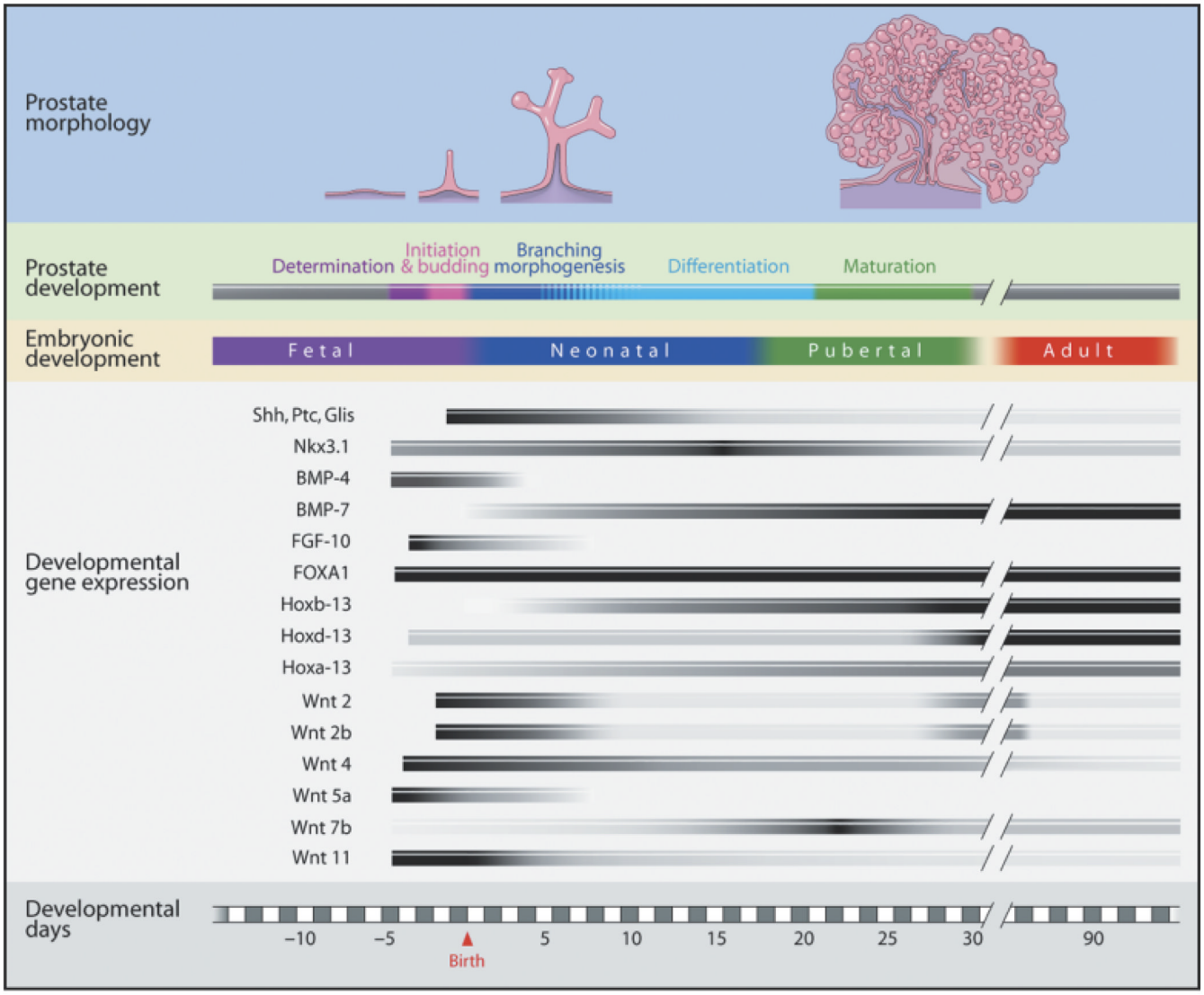

Fig. 1.

Rat prostate developmental stages, timeline and morphoregulatory gene expression. The days of fetal and postnatal life are shown at the bottom. Ventral prostate morphology and developmental stages (top) are sequentially aligned to the corresponding days that they appear. Note that cellular differentiation occurs during the later days of branching morphogenesis as indicated by blue-striped lines. Temporal patterns of morphoregulatory genes expression are shown in black-gray-white bars representing relative levels of gene expression as determined by quantitative real-time reverse-transcriptase polymerase chain reaction.

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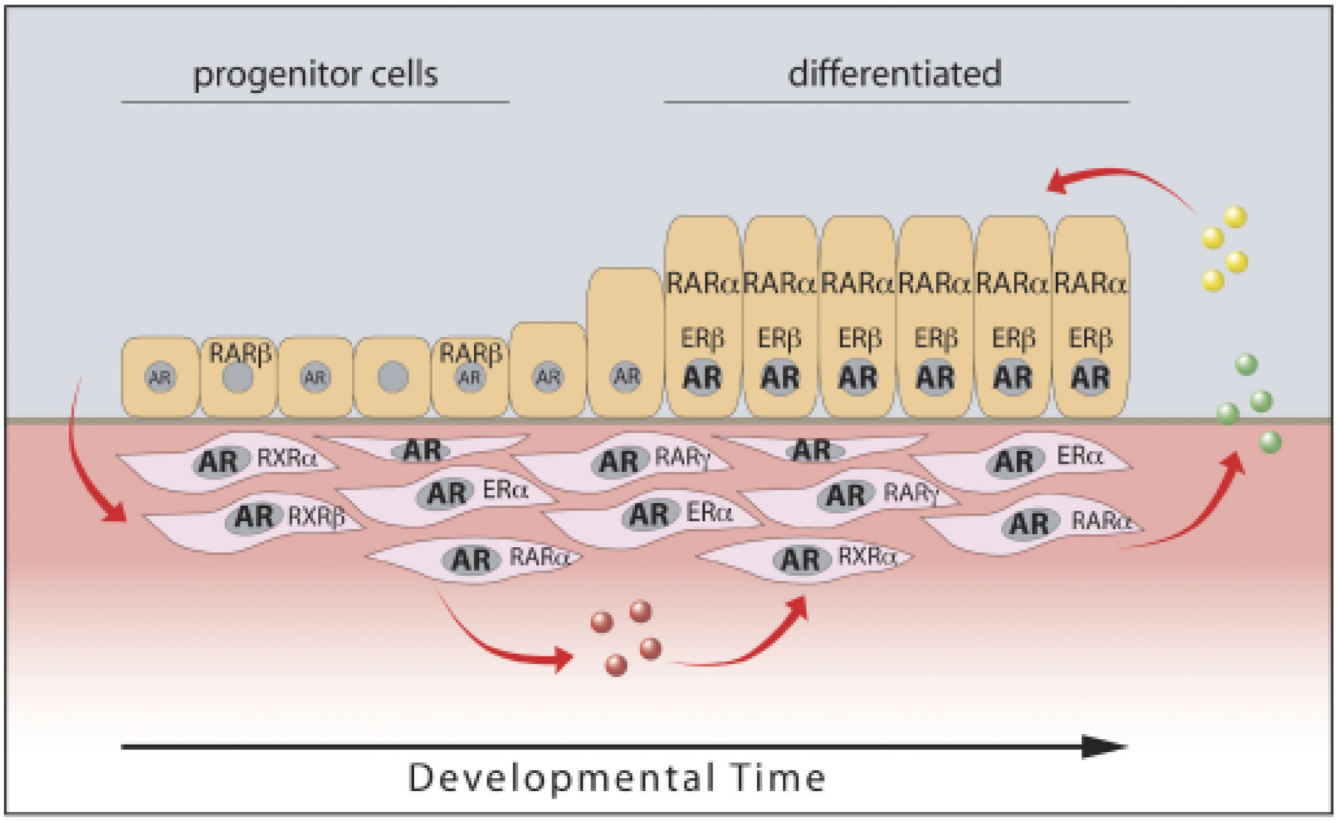

Fig. 2.

Steroid receptor localization and expression over time during rat prostate development. During early perinatal stages, androgen receptor (AR) is highly expressed in mesenchymal cells while epithelial progenitor cells have no or limited AR. In response to circulating androgens (converted to dihydrotestosterone intracellularly), mesenchymal cells produce autocrine (pink circles) and paracrine (green and yellow circles) growth and differentiation factors, which drive epithelial cell proliferation and differentiation. As epithelial cells differentiate, AR is induced to high expression levels. Androgen driven secreted factors from the epithelium are proposed to provide reciprocal signals to the mesenchyme to promote differentiation to smooth muscle cells. Other steroid receptors expressed in a temporal and cell-specific manner include mesenchymal estrogen receptor α (ERα, during early stages), epithelial ERβ (upon differentiation), basal cell retinoic acid receptor β (RARβ), RARα (upon functional differentiation), and periductal mesenchymal RARα, RARγ, RXRα and RXRβ.

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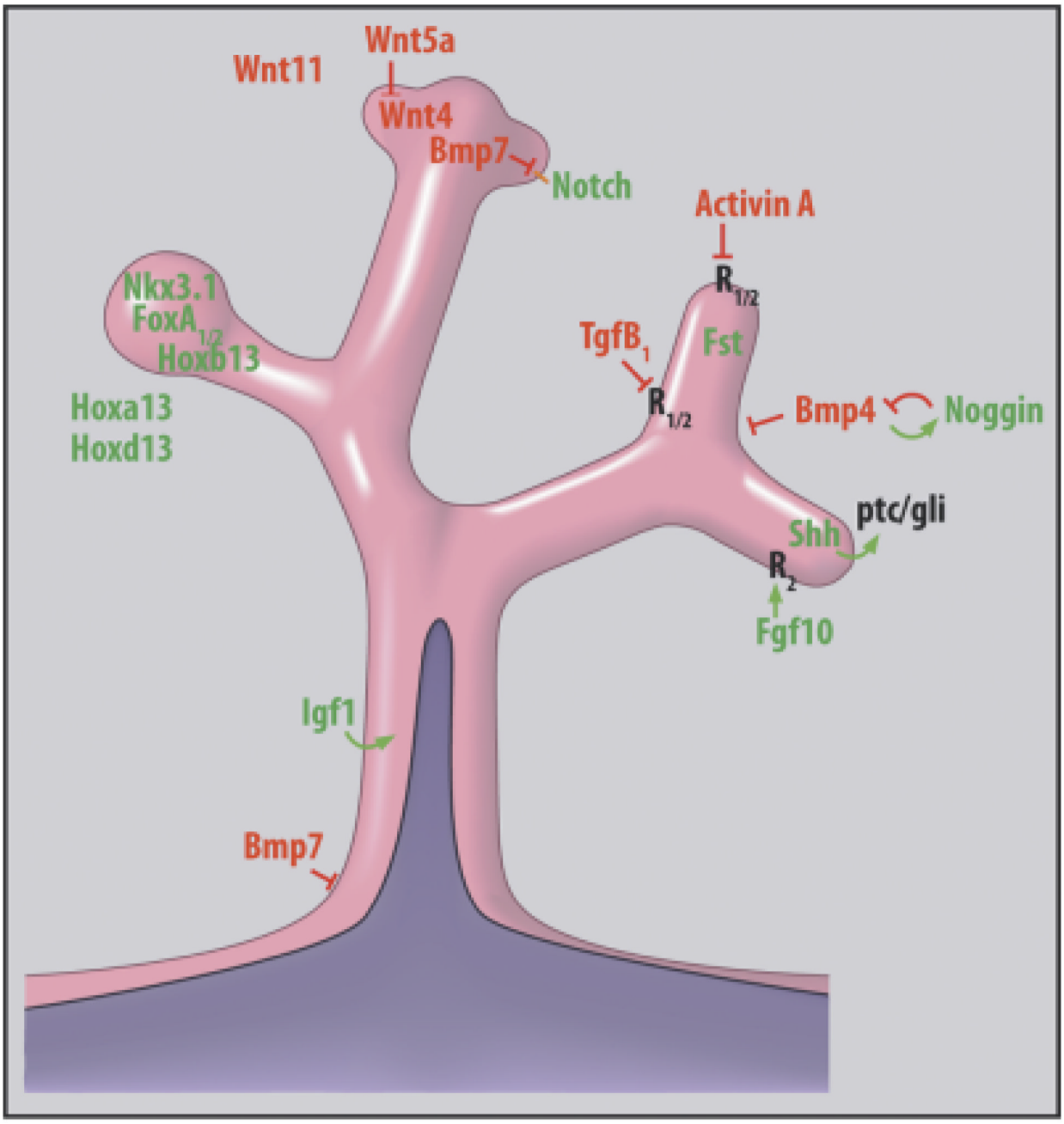

Fig. 3.

A schematized model of an elongating and branching prostate duct showing the localization of multiple transcription factors, secreted morphogens and their respective receptors to form the prostatic morphogenetic code. Factors in green denote stimulatory molecules while factors in red represent inhibitory molecules. Arrows show paracrine stimulatory pathways emanating from the epithelium or mesenchyme in the direction of the secreted morphogen. T-bars denote inhibition as a function of the secreted morphogen. The schematic represents work from the author's laboratory as well as multiple investigations cited throughout the manuscript.

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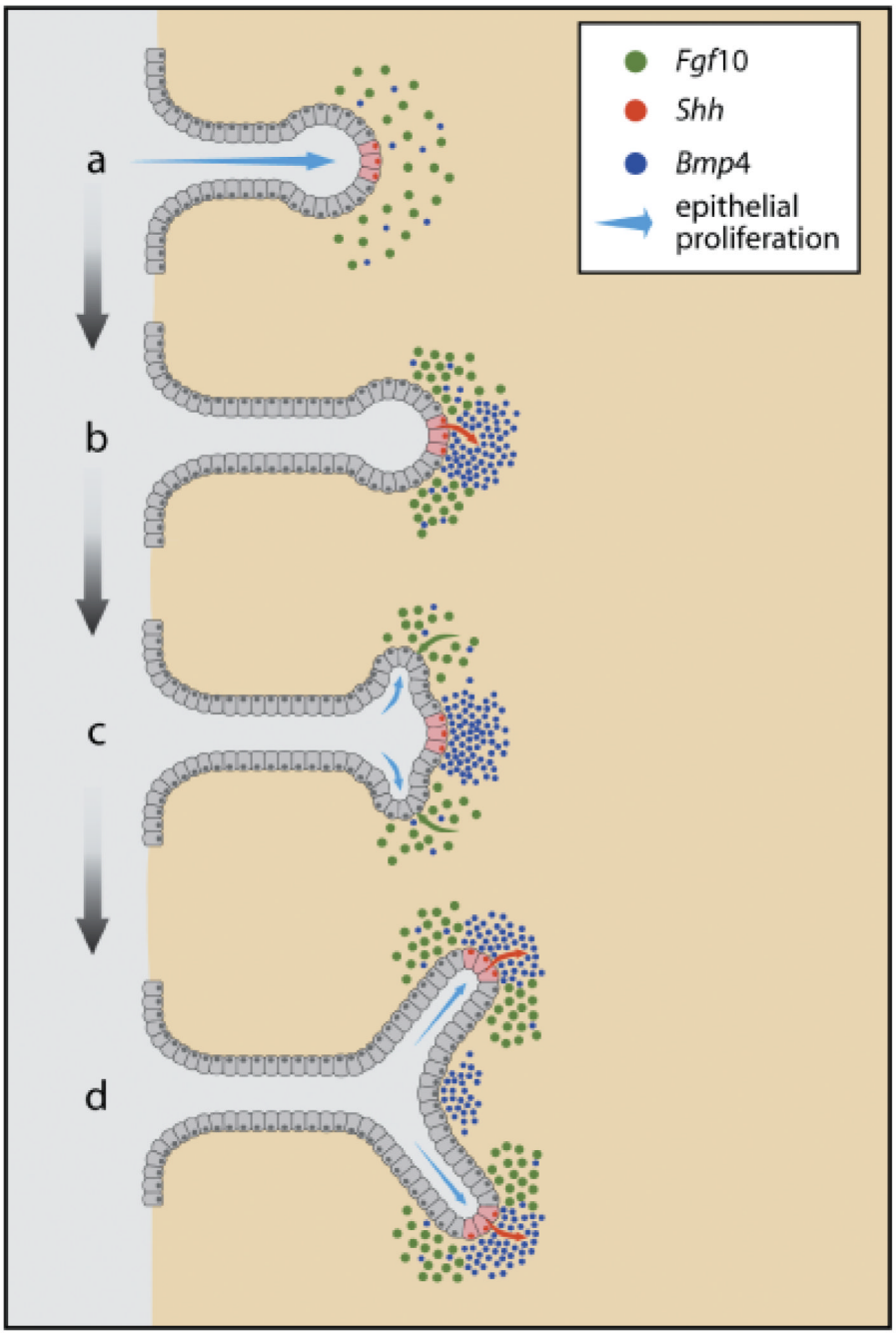

Fig. 4.

A proposed model for dichotomous branching of the developing rat prostate ducts as controlled by localized expression and cross-talk of secreted morphoregulatory factors. The distal duct tips express *Shh* in discreet focal areas (for simplicity, only one shown in red) while the distal mesenchyme expresses *Fgf*10 (green dots) and *Bmp*4 (blue dots). As these cells make contact with each other (b), the secreted *Shh* (red arrow) activates *ptc* on mesenchymal cells, locally down-regulates *Fgf*10 (loss of green dots) and up-regulates *Bmp*4 (blue dots) expression. The focal downregulation of *Fgf*10 results in lateral subdomains of higher *Fgf*10 expression adjacent to the *Shh* foci, which in turn, down-regulates *Bmp*4 in that region and activates (green arrow) higher epithelial proliferation via epithelial *Fgf*R2*iii*b (c). The disparate epithelial

proliferation rates in the lateral domains results in the sprouting of two buds on each side of the *Shh* foci (blue arrows) which initiates a branchpoint (d). Further, the elevated *Fgf*10 in the lateral domains up-regulates *Shh* and *ptc* expression (d), which allows repetition of the above steps and results in complex branching patterns (Based on results published by Huang et al., 2005).

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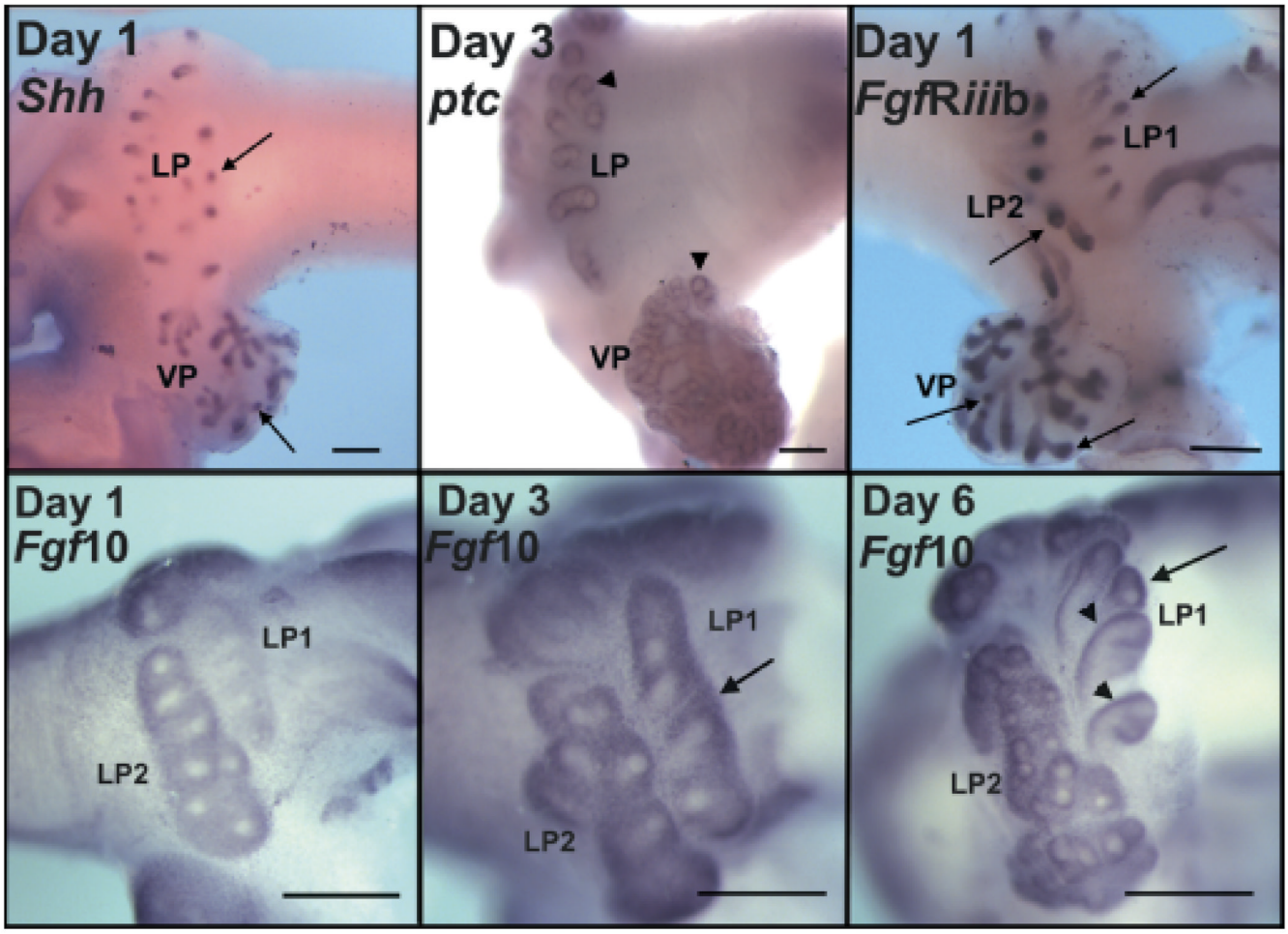

Fig. 5.

Whole mount *in situ* hybridization of *Shh*, *ptc*, *Fgf*R2*iii*b and *Fgf*10 expression during rat prostate development. Days and gene are labeled on each image. Top row shows *Shh* (left), *ptc* (center) and *Fgf*R2*iii*b (right) at postnatal days 1 or 3. LP denotes lateral prostate with LP1 and LP2 representing two separate ductal regions and VP denotes the ventral lobe. Arrows point to *Shh* and *Fgf*R2*iii*b expression within the epithelium at the distal tips while arrowheads highlight periductal mesenchymal *ptc* expression at the distal tips. Bottom row shows *Fgf*10 mRNA in the rat lateral prostate lobe at the ductal elongation stage (day 1 and 3) and at the start of branching morphogenesis (day 6). Arrows point to regions of strongest *Fgf*10 expression in the distal tip mesenchyme at days 3 and 6. Arrowheads show condensed periductal *Fgf*10 expression along the ductal length. Bar = 200 μm. (See Huang et al., 2005 for details)

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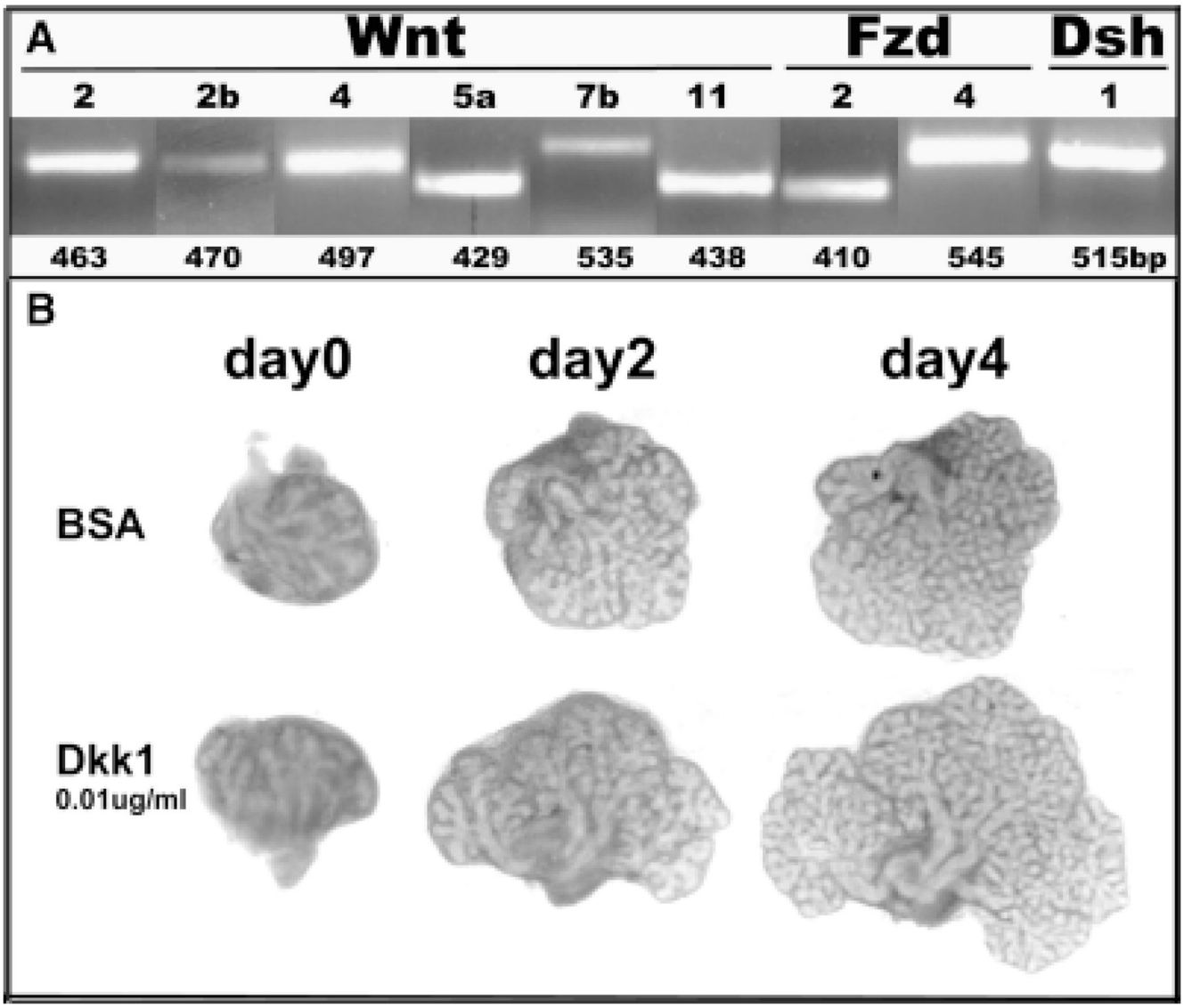

Fig. 6.

Wnt gene expression and action during rat prostate development. **A**: reverse-transcriptase polymerase chain reaction revealed expression of *Wnt*s 2, 2b, 4, 5a, 7b, and 11 as well as *Fzd* 2 and 4 and *Dsh* 1 in the day 3 rat ventral prostate. **B**: Contralateral rat ventral prostate lobes were collected on the day of birth and cultured in the presence of 10nM testosterone with bovine serum albumin or 0.01 μg/ml *Dkk*1 for 4 days. This was repeated in 4 separate prostates. The data shows a growth promoting effect of canonical *Wnt* inhibition by *Dkk*1 protein.

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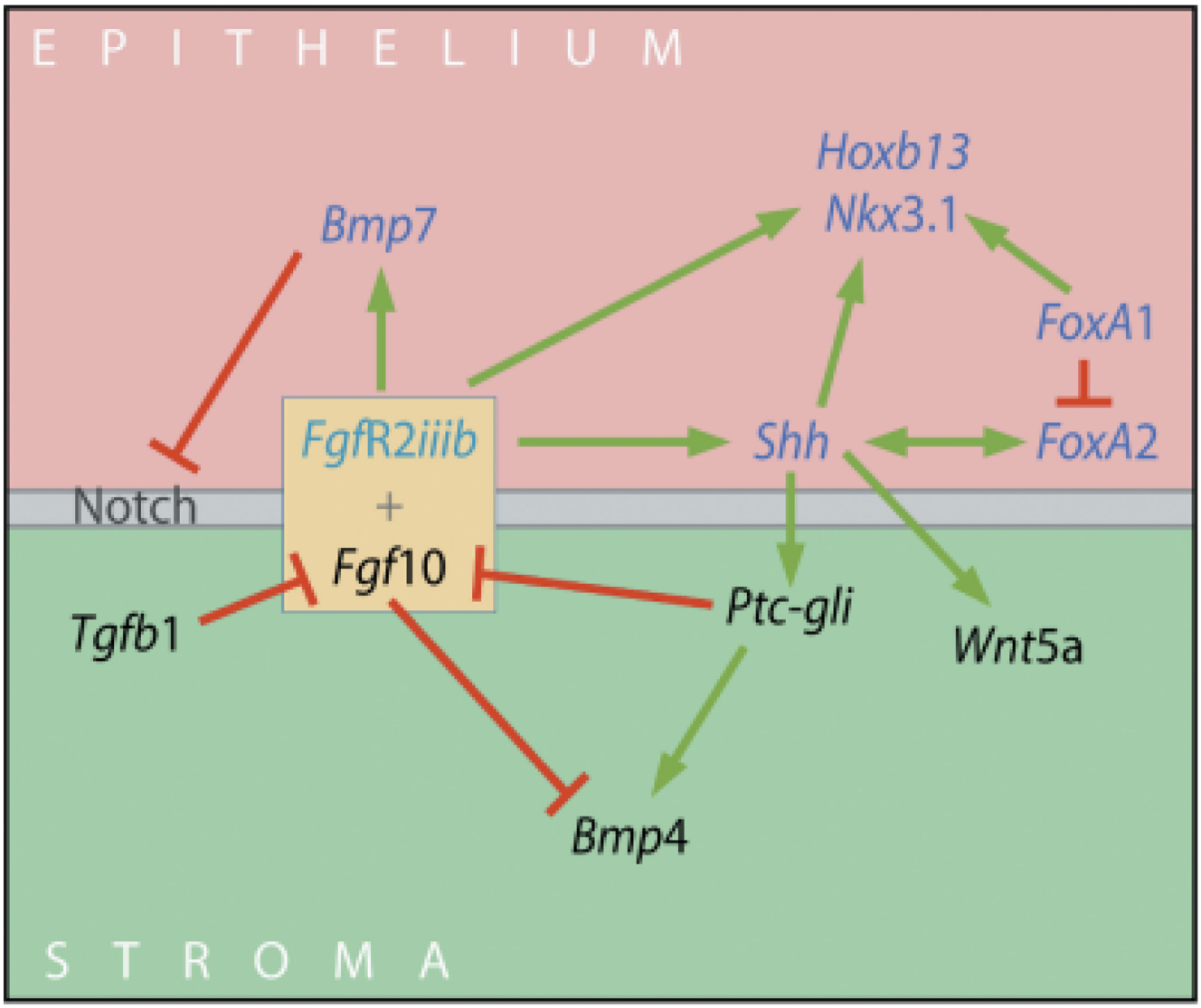

Fig. 7.

A schematic representation of regulatory networks between secreted morphogens and transcription factors in the epithelial and mesenchymal cells at the distal signaling center of the developing prostate gland. *Fgf*10 (mesenchymal) and *Fgf*R2*iii*b (epithelial) upregulate (green arrows) epithelial expression of *Shh* and *Bmp*7 involved in branching morphogenesis as well as *Hox*b13 and *Nkx*3.1 involved in epithelial differentiation. *Shh* up-regulates *ptc* and *gli* in adjacent mesenchymal cells which down-regulates (red lines) *Fgf*10 expression thus establishing a negative feedback loop for controlled growth. *Shh-ptc-gli* also up-regulates the growth inhibitory *Wnt*5a and *Bmp*4 molecules in the mesenchyme while *Fgf*10 down-regulates their expression which further serves to tightly control localized tissue growth. *Fox*A1 stimulates expression of *Nkx*3.1 and inhibits *Fox*A2 expression which has reciprocal up-regulation with *Shh*. *Fgf*10/*Fgf*R2*iii*b up-regulates *Notch* expression, which drives ductal growth while *Bmp*7 down-regulates *Notch* and suppresses regional growth. *Tgf*β1 suppresses *Fgf*10 expression, which may serve as a brake for growth as development nears completion.