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Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis

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Abstract

Previous studies have demonstrated that the Hedgehog (Hh) signaling pathway plays a critical role in the development and patterning of many endodermally derived tissues. We have investigated the role of *Sonic hedgehog* (*Shh*) in formation of the prostate gland by examining the urogenital phenotype of *Shh* mutant fetuses. Consistent with earlier work reporting an essential role for *Shh* in prostate induction, we have found that *Shh* mutant fetuses display abnormal urogenital development and fail to form prostate buds. Unexpectedly, however, we have discovered that this prostate defect could be rescued by three different methods: renal grafting, explant culture in the presence of androgens, and administration of dihydrotestosterone (DHT) to pregnant mice, indicating that the prostate defect in *Shh* mutants is due to insufficient levels of androgens. Furthermore, we find that the inhibition of Hh pathway signaling by treatment with cyclopamine does not block prostate formation in explant culture, but instead produces morphological defects consistent with a role for Hh signaling in ductal patterning. Taken together, our studies indicate that the initial organogenesis of the prostate proceeds independently of Shh, but that Shh or other Hh ligands may play a role in subsequent events that pattern the prostate.

Keywords: Hedgehog; Endoderm; Organogenesis; Ductal morphogenesis; Tissue recombination; Androgens

Introduction

Organogenesis of gut-derived tissues in vertebrates is mediated by epithelial–mesenchymal interactions that induce regionalized expression of tissue-specific transcription factors, which in turn regulate organ patterning and differentiation (reviewed in Grapin-Botton and Melton, 2000; Roberts, 2000). To date, however, most studies have focused on patterning and differentiation of foregut- and midgut-derived tissues, while relatively little is known about organogenesis of hindgut derivatives, such as the prostate gland.

The formation of the mammalian prostate occurs through epithelial budding from the urogenital sinus, which is derived from an extension of the caudal hindgut that arises during midgestation. In the mouse, prostate organogenesis occurs toward the end of gestation, at approximately 17.5 days post coitum (dpc), when prostatic epithelial buds emerge from the urogenital sinus under the influence of unknown inductive signals from the mesenchyme. During subsequent ductal morphogenesis, occurring in the first 3 weeks of postnatal development, the prostatic epithelial buds elongate and canalize to form ducts, which branch extensively into the

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surrounding mesenchyme (Sugimura et al., 1986; Timms et al., 1994). At maturity, the rodent prostate gland is a multilobular tissue, consisting of anterior, dorsolateral, and ventral lobes, which are arranged circumferentially around the bladder and display characteristic patterns of ductal branching, histological features, and secretory protein production (Hayashi et al., 1991; Sugimura et al., 1986). The identities of these lobes are likely to be specified at early stages of prostate formation, based at least in part on the position of the emerging epithelial buds. Notably, the emerging prostatic epithelial buds are marked by expression of the *Nkx3.1* homeobox gene, which is dependent on epithelial–mesenchymal interactions during prostate formation, and represents the earliest known marker for the prostatic epithelium (Bhatia-Gaur et al., 1999; Sciavolino et al., 1997).

The prostate gland also represents a model system for studying the generation of sexual dimorphism, as its formation, growth, and function are continually dependent on androgen receptor signaling (reviewed in Cunha, 1994; Cunha et al., 1987; Hayward et al., 1997). Notably, functional androgen receptors are in the urogenital sinus mesenchyme during embryogenesis, whereas they are found in both the mesenchyme and epithelium postnatally. Consistent with this observation, tissue recombination studies using mutant mice have demonstrated that androgen receptors are initially required in the mesenchyme to produce signals for prostate induction and growth, and only later in the epithelium for the secretory function of differentiated cell types.

Recent studies have shown that the Shh signaling pathway plays a key role in mediating epithelial–mesenchymal interactions during formation of many endodermal tissues. For example, Shh expressed by ventral foregut endoderm activates Hh pathway targets in adjacent primitive lung mesenchyme through binding to and inactivating its transporter-like receptor Patched (Ptc). Such binding alleviates inhibition by Ptc of the seven-transmembrane protein Smoothened (Smo), allowing activation of Hh target genes through the Gli family of latent transcription factors. One such target is *Ptc* itself, whose high-level expression illustrates Hh pathway activity that is required in developing lung mesenchyme to support branching morphogenesis of adjacent airway endoderm (Bellusci et al., 1997; Litingtung et al., 1998).

In the case of the prostate gland, previous studies have proposed that *Shh* maybe required not only for branching morphogenesis, but also for the initial formation of prostatic buds. Based on neutralizing antibody treatment of grafts and pharmacological inhibition of Shh signaling in organ culture assays (Lamm et al., 2002; Podlasek et al., 1999), this potential role for Shh in the early outgrowth of prostatic epithelial buds (prostate induction) is distinct from roles described in other developing organs. For example, lung buds form normally in mice with deficient Hh signaling, but fail to undergo subsequent branching morphogenesis (Hogan et al., 1997; Litingtung et al., 1998; Motoyama et al., 1998). Likewise, hair follicle formation is initially normal in mice lacking Shh function, but subsequent morphogenesis is severely impaired (Chiang et al., 1999; St-Jacques et al., 1998).

Consequently, we have investigated the role of Shh in prostate formation through phenotypic analysis of mutant mice. Although *Shh* mutant embryos fail to form prostate glands, we find that the urogenital sinus tissues from these mutants can form Nkx3.1-expressing prostatic tissue in tissue recombination and organ culture assays. Furthermore, our results obtained by treatment of prostatic organ cultures with Shh protein or with the Hh pathway antagonist cyclopamine indicate that Hh signaling is not required for prostate formation, but may instead play a role in prostatic ductal morphogenesis.

Methods

Mouse strains and genotyping

Shh mutant mice were maintained in a CD1 outbred strain background and genotyped as described (Chiang et al., 1996). XY fetuses were identified using PCR primers directed against *Sry*: 5′GAGAGCATGGAGGGCCAT 3′and 5′CCACTCCTCTGTGACACT 3′(Bowles et al., 1999). The $Nkx3.1^{tm2(lacZ)Mms}$ allele will be described in further detail in a separate study (Y.-P. Hu, M. Reynon, N.D., S.M. Price, C.A.-S., M.M.S., in preparation). $Nkx3.1^{tm2(lacZ)Mms}$ heterozygous embryos were obtained by mating of $Nkx3.1^{tm2(lacZ)Mms}$ homozygous males with C57Bl/6 females.

Tissue recombination

Tissue recombinations and tissue grafts were performed essentially as described (Cunha, 1994; Cunha and Donjacour, 1987). In brief, wild-type urogenital sinuses were removed at 16.5 dpc, and dissected away from urethra, ductus deferens, and ureters; for *Shh* mutants, urogenital regions containing portions of bladder and hindgut were used without extensive dissection. Epithelial and mesenchymal components were separated by treatment with 1% trypsin at 4°C for 90 min, followed by mechanical dissociation. Tissue recombinations were constructed by combination of dissociated mesenchyme and epithelium on 0.4% agar plates, followed by incubation with DMEM/10% fetal bovine serum overnight at 37°. Successful recombinations were surgically implanted under the kidney capsule of male *nude* mice the next day, and were harvested following 4 weeks of growth.

Explant culture

For organ culture, wild-type and *Shh* mutant urogenital regions were collected as described above, but without

trypsinization. Organ culture of urogenital sinus rudiments was performed essentially as described (Lopes et al., 1996). In brief, explants were cultured in a serum-free medium containing Ham's F12/DMEM H-16 (50:50), 1 g/l glucose, 2 mM glutamine, nonessential amino acids, HEPES buffer, 10 μg/ml insulin, 10 μg/ml transferrin (Sigma) 50 μg/ml gentamycin, 50 U/ml penicillin, 50 μg/ml streptomycin; testosterone (4-Androsten-17βeta-ol-3-one) was dissolved in DMSO and used at concentration of 10^{-7} M. Explants were cultured for 7 days at 37°C with 5% CO_2 on Millicell CM 0.4 Micron filters (Millipore, Bedford, MA), with media changed every 48 h.

Shh-N terminal peptide (R&D Systems) was dissolved in PBS/0.1% BSA and used at 30 nM concentration. Cyclopamine purified from *Veratrum* extract and its inactive analogs tomatidine (Sigma) and solanidine (ICN, Costa Mesa, CA) were dissolved in MeOH (cyclopamine and tomatidine) or EtOH (solanidine), and used at a concentration of 5 μM.

For morphometric analysis, prostatic ducts were digitally traced on photomicrographs of β-galactosidase-stained explants using Adobe Illustrator (Adobe Systems), and two-dimensional areas were calculated using CADTools (Hot Door, Inc.) plug-in software. Statistical analysis was performed using Graphpad Prism (GraphPad Software, Inc.).

In situ hybridization, immunohistochemistry, and β-galactosidase staining

Radioactive in situ hybridization on paraffin sections was performed according to Berman et al. (1995), using ^{33}P-labelled riboprobes reverse-transcribed from a 1-kb *Eco*RI fragment of the *Nkx3.1* cDNA (Sciavolino et al., 1997). After autoradiography, slides were stained with hematoxylin and eosin and photographed under dark-field and bright-field illumination. Dark-field images were false-colored green and overlayed onto corresponding bright-field images using Adobe Photoshop (Adobe Systems). Nonradioactive in situ hybridization to cryosections was performed as described (Sciavolino et al., 1997). For β-galactosidase staining, explants were fixed in 2% paraformaldehyde and stained as described (Ben-Arie et al., 2000). Immunohistochemical staining was performed according to Kim et al. (2002), using an antimouse Nkx3.1 polyclonal antiserum (Kim et al., 2002), or anti-p63 monoclonal antibody (Santa Cruz).

In utero virilization

Timed pregnant females from crosses between mice heterozygous for the mutant *Shh* allele (Chiang et al., 1996) were injected subcutaneously with triolein (ICN) in 20% EtOH vehicle, or with vehicle containing 50 mg/kg dihydrotestosterone (Steraloids, Newport, RI) from 10.5–17.5 dpc of gestation. Fetuses were harvested on 18.5 dpc and processed for routine histology.

Real-time RT-PCR analysis

For mRNA quantitation, first-strand cDNA was prepared from organ cultures using Trizol reagent and SuperScript reverse transcriptase (Invitrogen). Real-time PCR amplification of first-strand cDNA was performed using a Mx4000 quantitative PCR instrument (Stratagene) with LUX™ fluorogenic primers (Invitrogen), using the following primer pairs: for *Ptc*, unlabeled 5′GGCCTTCGCTGTGGGATTA 3′ and FAM (6-carboxy-fluorescein)-labeled 5′ CAACGCCA-CAGCTCCTCCACGTTG 3′; for β-actin, unlabeled 5′GGTTGGCCTTAGGGTTCAGG 3′and JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxy-fluorescein)-labeled 5′CACGCCACCTTCTACAATGAGCTGCGTG 3′. Results for *Ptc* levels were normalized to β-actin levels.

Results

Urogenital phenotype of Shh mutant mice

Previous studies have shown that components of the Hh signaling pathway are expressed in the developing urogenital system at stages before prostate formation (Lamm et al., 2002; Podlasek et al., 1999). We have now found that *Shh* is expressed in the urogenital sinus (UGS) epithelium at 16.5 dpc, in the basal layer immediately adjacent to the urogenital mesenchyme (Fig. 1A); after prostatic buds emerge, *Shh* is localized to the nascent epithelial buds (Lamm et al., 2002; data not shown). In contrast, *Ptc* is expressed in the urogenital mesenchyme adjoining the epithelium (Fig. 1B), indicating that the Hh pathway is active in signaling from the epithelium to the mesenchyme. Other downstream targets of the Hh pathway are expressed in the urogenital mesenchyme at 16.5 dpc, including *BMP4* and *HIP*, which encodes a soluble inhibitor of Hh signaling (Chuang and McMahon, 1999) (Figs. 1C,D).

To evaluate whether *Shh* is indeed required for prostate formation, we examined the urogenital phenotype of male *Shh* mutant fetuses that survived to 18.5 dpc. These fetuses display severe cyclopia and limb defects, and were identified as genotypically male by PCR using primers directed against the Y-linked sex-determining gene *Sry*. We found that XY *Shh* mutant fetuses have severe morphological defects in the urogenital sinus, including failure of prostatic budding by 18.5 dpc; we also confirmed the anorectal defects previously described for *Shh* mutants (Kimmel et al., 2000; Mo et al., 2001). Histological sections of XY *Shh* mutants through the urogenital sinus revealed profound disorganization of the UGS and a complete absence of ductal budding immediately caudal to the bladder neck, indicating a lack of prostate formation (n = 7) (Fig. 2). Seminal vesicle differentiation was also undetectable in *Shh* mutant male embryos, suggesting a broader requirement for Shh in male phenotypic sexual differentiation. To confirm the

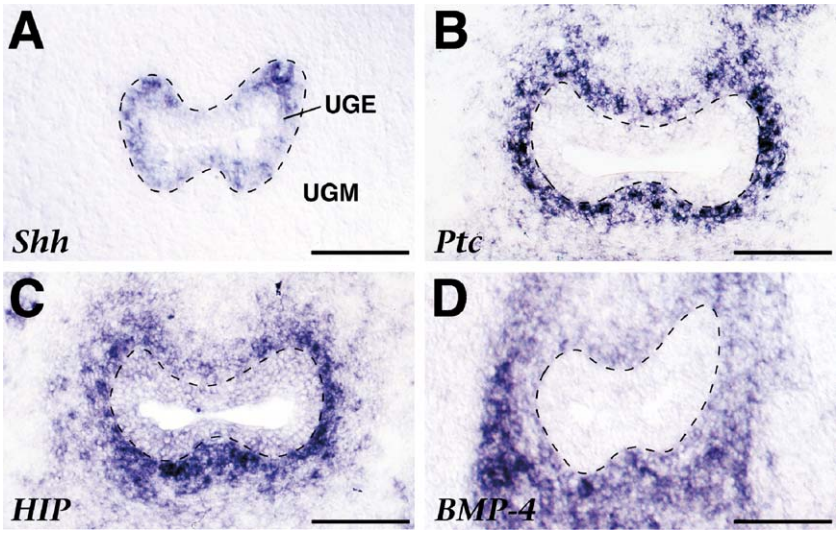

Fig. 1. Expression of *Shh* pathway components in 16.5 dpc urogenital sinus. In situ hybridization detection of *Shh* (A), *Ptc* (B), and the downstream targets *BMP4* (C) and *HIP* (D) in transverse sections caudal to the bladder, dorsal side up. Dashed lines indicate boundary between the urogenital epithelium (UGE) and urogenital mesenchyme (UGM). Note that *Shh* expression is restricted to the urogenital epithelium, that *Ptc* and *HIP* are expressed primarily in urogenital mesenchyme, and that *BMP4* is exclusively expressed in mesenchyme. Scale bars correspond to 100 μm.

absence of prostatic buds, we performed in situ hybridization to detect expression of *Nkx3.1*, which represents the earliest known marker of prostatic ductal budding. No evidence of *Nkx3.1* expression was detected in XY *Shh* mutants, consistent with the absence of prostatic ductal budding (data not shown).

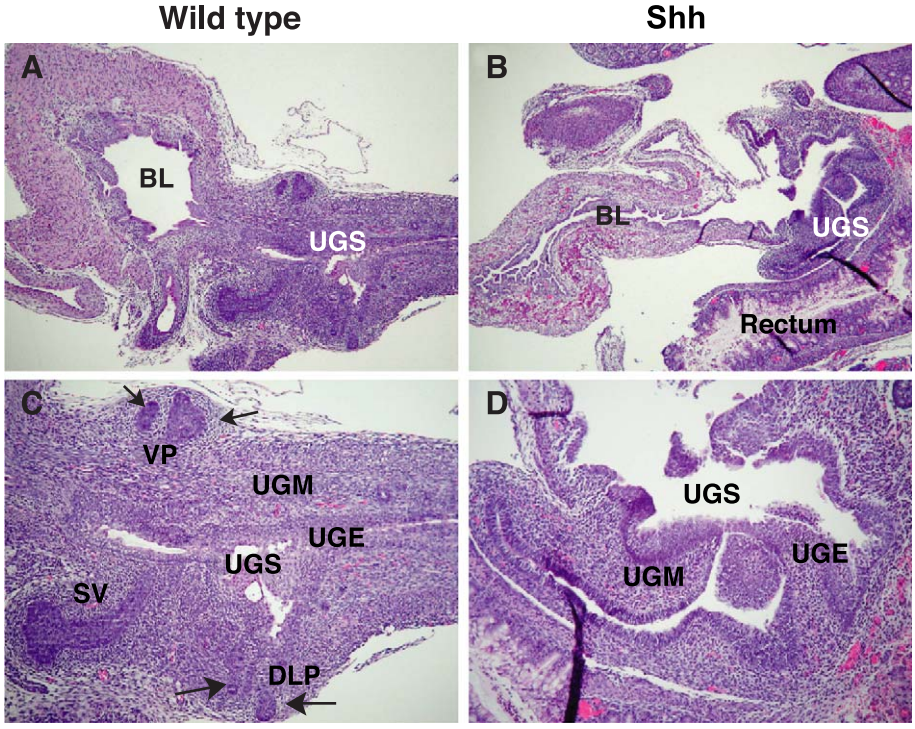

Fig. 2. Failure of male sexual differentiation in 18.5 dpc *Shh* mutant embryos. Photomicrographs of hematoxylin and eosin (H&E)-stained sagittal sections show urogenital sinus (UGS) caudal to the bladder (BL) in both wild-type (A,C) and *Shh* mutant (B,D) males. Epithelial buds (arrows in C) evaginate from urogenital sinus epithelium (UGE) into urogenital sinus mesenchyme (UGM) in wild-type males, giving rise to nascent ventral (VP) and dorsolateral (DLP) prostate (C). Prostate buds are absent in *Shh* mutant males (D). Also note prominent seminal vesicle (SV) development in wild-type males (C), which is absent in *Shh* mutants (D). Abbreviations: BL, bladder; DLP, dorsolateral prostate; SV, seminal vesicle; UGE, urogenital epithelium; UGM, urogenital mesenchyme; UGS, urogenital sinus; VP, ventral prostate.

Rescue of prostate formation in kidney grafts of tissue recombinants

The absence of prostate budding in *Shh* mutants could be interpreted as being consistent with the previously proposed role for *Shh* in prostate induction (Lamm et al., 2002; Podlasek et al., 1999). However, given the severe urogenital defects in *Shh* mutants, we considered the alternative hypothesis that the absence of prostate formation might represent a secondary consequence of earlier morphogenetic defects. To test this possibility, we performed grafts of urogenital sinus tissue under the kidney capsule of *nude* male mice to examine whether prostate differentiation could proceed in a heterologous context.

We examined prostate differentiation from urogenital tissue of *Shh* mutants at 16.5 dpc (both XY and XX) using a tissue recombination assay, in which epithelial and mesenchymal components are enzymatically dissociated and recombined with wild-type tissues, followed by renal grafting (Cunha, 1994; Cunha and Donjacour, 1987). Using wild-type tissues, the combination of urogenital epithelium with urogenital mesenchyme will normally cause robust prostatic differentiation, as assessed by histological appearance and by expression of *Nkx3.1*, whereas urogenital epithelium or mesenchyme alone will fail to form prostate (Bhatia-Gaur et al., 1999; Cunha et al., 1987). Since *Shh* is normally expressed in the urogenital epithelium, we focused on tissue recombinations of *Shh* urogenital sinus epithelium (UGE) combined with wild-type mesenchyme (UGM). We found that these tissue recombinants would readily differentiate into prostatic tissue following 4 weeks of growth in *nude* male hosts, and were largely indistinguishable from wild-type controls (Figs. 3A,B). At the histological level, the characteristic histology of all three prostatic lobes (anterior, dorsolateral, ventral) could be observed (Table 1), suggesting that the rescue of prostatic differentiation was not lobe-specific. To confirm the prostatic identity of these tissues, we performed immunohistochemical staining using an Nkx3.1 antiserum, which demonstrated that luminal epithelial cells with prostatic morphology in the tissue recombinants expressed Nkx3.1 (Figs. 3D,E). Basal cells were also present in their expected locations in these tissue recombinants, as shown by immunohistochemical detection of the basal cell marker p63 (Figs. 3G,H).

Since prostate differentiation was rescued by tissue recombination, we next examined whether grafting of intact urogenital sinus ("tissue rescue") would be sufficient to result in prostatic differentation. Since the urogenital sinus of *Shh* mutants is morphologically abnormal, and the prostatic region of the urogenital sinus cannot be precisely defined, a relatively large portion of tissue was used for

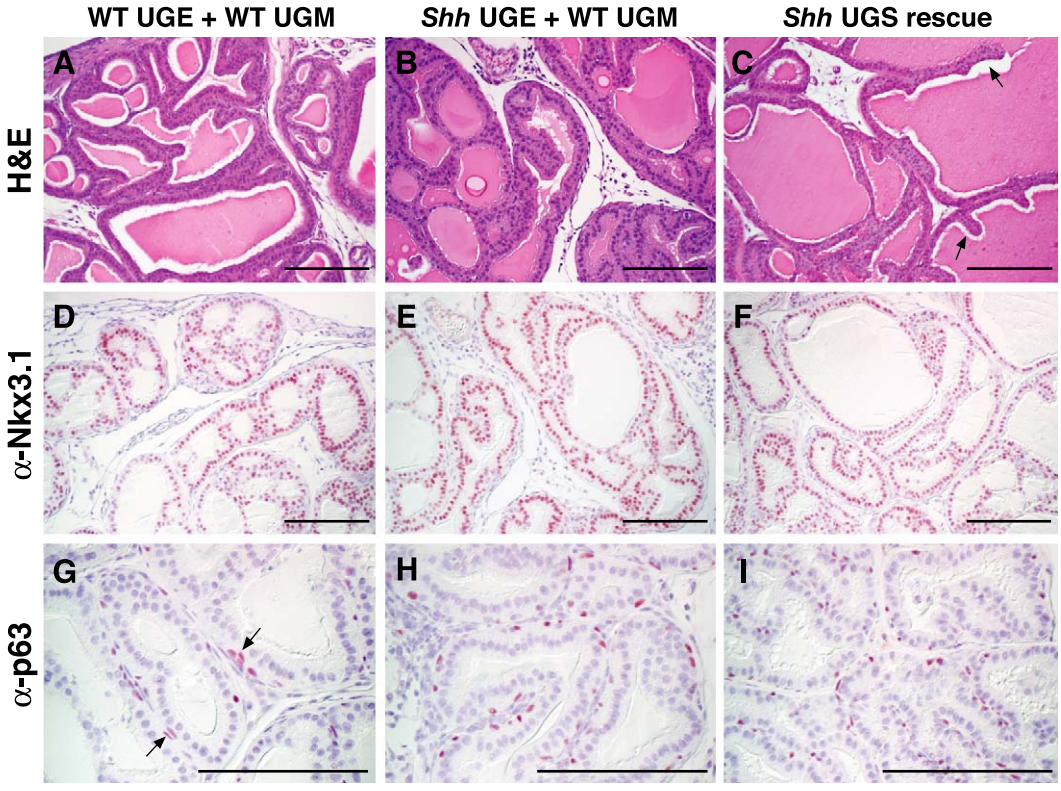

Fig. 3. Rescue of prostatic differentiation in *Shh* urogenital sinus grafts and tissue recombinants. (A–C) H&E staining of wild-type graft (A), *Shh* UGE + WT UGM tissue recombinant (B), and *Shh* mutant graft (C). (D–F) Nkx3.1 immunostaining of wild-type (D), *Shh* UGE + WT UGM tissue recombinant (E), and *Shh* graft (F). (G–I) p63 immunostaining of wild-type (G), *Shh* UGE + WT UGM tissue recombinant (H), and *Shh* graft (I). Scale bars correspond to 100 μm.

Table 1
Summary of tissue recombination and graft data

	n	AP[a]	DLP[a]	VP[a]	Nkx3.1 immunostaining
WT controls	*n* = 2	2/2	2/2	2/2	2/2
Shh UGE + WT UGM	*n* = 8	5/8	4/8	4/8	4/8
Shh UGS	*n* = 5	5/5	5/5	3/5	5/5

[a] Lobe identity was inferred from the histological appearance of prostatic tissues.

these grafts, usually including regions of the bladder and caudal hindgut. As a consequence, histological analysis of the resulting grafts revealed differentiation of other tissue types from *Shh* mutants, including gut and seminal vesicle (data not shown). However, as with the tissue recombinants, grafting of *Shh* mutant urogenital sinus resulted in regions of robust prostate differentiation, as assessed by histology and immunohistochemical detection of Nkx3.1 and p63 (Table 1; Figs. 3C,F,I).

Androgen-mediated rescue of prostate formation in Shh mutant explants and embryos

Given our unexpected observation of prostatic differentiation in *Shh* tissue recombinants and rescues, we next investigated whether prostatic differentiation was dependent on the specific environmental conditions within a kidney graft. To examine this issue, we utilized a prostate organ culture assay that utilizes chemically defined media supplemented with testosterone (Methods; Lopes et al., 1996). Using wild-type urogenital sinus from 16.5 dpc embryos, copious prostatic budding could be readily observed following 1 week of culture; importantly, this budding required the presence of androgens, since no budding was observed in their absence. We performed parallel cultures of *Shh* homozygous mutant and phenotypically wild-type littermate urogenital regions at 16.5 dpc, and found that both sets of explants displayed ample budding after 1 week of culture (*n* = 15) (Figs. 4A,B). Since the *Shh* urogenital regions used

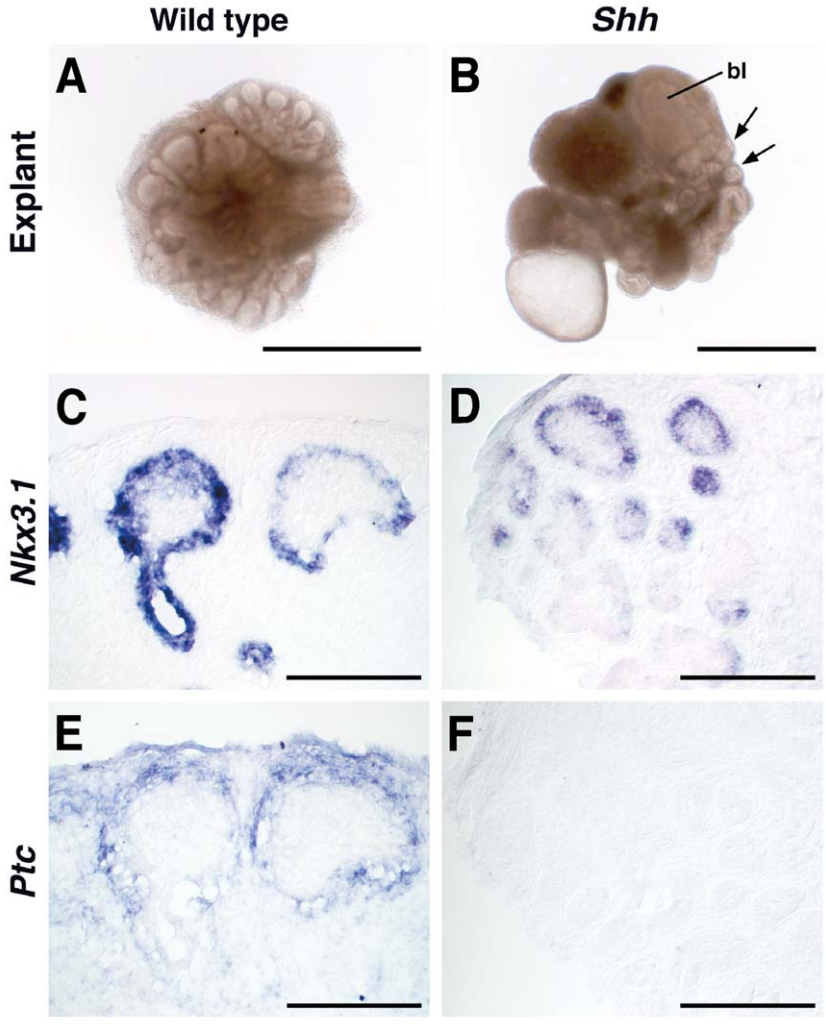

Fig. 4. Formation of prostatic tissue in *Shh* urogenital sinus explants in organ culture. (A,B) Whole-mount images of *Shh* urogenital sinus explants explanted at 16.5 dpc followed by 7 days organ culture. (C,D) In situ hybridization detection of *Nkx3.1* in sections of wild-type (C) and *Shh* mutant (D) explants. (E,F) In situ hybridization detection of *Ptc* in sections of wild-type (E) and *Shh* mutant (F) explants. Scale bars correspond to 1 mm (A,B) or to 100 μm (C–F).

for explant culture were highly abnormal, and contained regions that might correspond to seminal vesicle and ampullary gland outgrowths, we confirmed that prostatic budding was present in these explants by detection of *Nkx3.1* expression using in situ hybridization ($n = 5$) (Figs. 4C,D). Importantly, in situ hybridization of adjacent sections with a probe for *Ptc* demonstrated that *Ptc* expression could be detected in mesenchyme adjoining *Nkx3.1*-expressing prostatic epithelium in wild-type urogenital explants, but not in mesenchyme from *Shh* mutant explants (Figs. 4E,F). This result indicates that Hh pathway activity is absent in *Shh* mutant urogenital sinus during prostatic differentiation, and that compensatory expression of other *hedgehog* genes is not responsible for the rescue of prostatic differentiation in explants.

Given the ability to rescue prostatic differentiation by explant culture of *Shh* mutant urogenital sinus, we considered the possibility that endogenous androgen levels were insufficient for prostate development in *Shh* mutant fetuses in vivo, but that exogenous high androgen levels in explants and tissue recombinants could rescue this defect. To test this possibility, we elevated fetal androgen levels by administering DHT to pregnant mothers carrying progeny from a *Shh* heterozygous intercross. We found that DHT administration from 10.5 to 17.5 dpc could rescue budding from the urogenital sinus of fetuses examined at 18.5 dpc (Fig. 5). The expression of *Nkx3.1* as detected by in situ hybridization confirmed the prostatic identity of these buds (Fig. 5A).

Analysis of Hh pathway requirements in urogenital sinus explants

The ability to rescue prostatic budding from *Shh* mutant urogenital sinuses using three different approaches indicates that *Shh* is not directly required for prostate induction. To address whether *Shh* might have a subsequent role in prostatic ductal morphogenesis, we used a novel urogenital sinus explant organ culture assay that specifically visualizes prostatic duct outgrowth. For this purpose, we utilized urogenital sinus isolated from heterozygotes for *Nkx3.1*$^{tm2(lacZ)Mms}$, which contains a *lacZ* knock-in into the *Nkx3.1* locus and expresses β-galactosidase in prostate epithelium (Y.-P. Hu, M. Reynon, N.D., S.M. Price, C.A.-S., M.M.S., in preparation). Expression of β-galactosidase in these *Nkx3.1*$^{tm2(lacZ)Mms}$/+ mice recapitulates the pattern of Nkx3.1 expression in the epithelium of prostatic buds and prostate ducts, as well as the bulbourethral gland and urethral glands; no expression is found in other urogenital tissues. Although the *Nkx3.1*$^{tm2(lacZ)Mms}$ allele

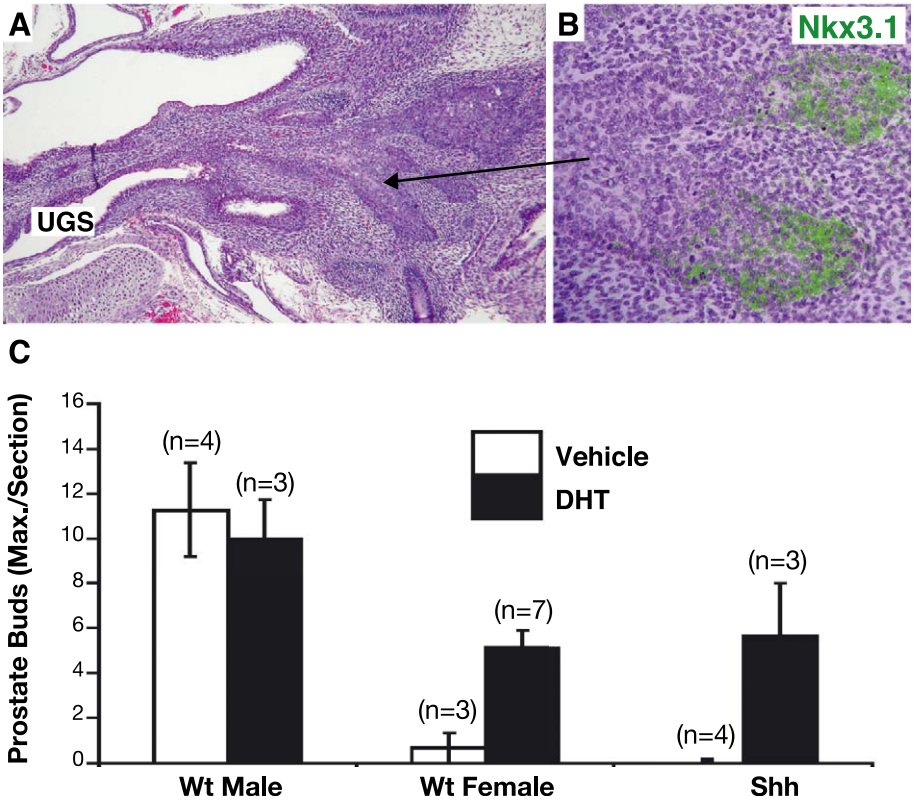

Fig. 5. Formation of prostatic ductal buds in *Shh* mutant fetuses exposed to DHT in utero. (A) Photomicrograph of H&E-stained section showing epithelial buds evaginating from androgenized *Shh* mutant UGS at 18.5 dpc. (B) In situ hybridization detection of *Nkx3.1* expression (green) performed on adjacent section demonstrates prostatic identity of the buds. (C) Graphical summary of prostatic bud-number in sections from wild-type and *Shh* mutant fetuses from mothers treated with DHT.

is null for *Nkx3.1*, there are no known phenotypic effects for *Nkx3.1* heterozygotes during prostatic budding or ductal morphogenesis, although there is a moderate reduction in ductal tip number in *Nkx3.1* homozygotes (Bhatia-Gaur et al., 1999).

Using *Nkx3.1*$^{tm2(lacZ)Mms}$ heterozygous fetuses as a source of 16.5 dpc urogenital sinus, we performed organ cultures in the presence or absence of Shh N-terminal peptide or the Hh pathway antagonist cyclopamine (Cooper et al., 1998). Following 1 week of culture, the formation and outgrowth of prostatic ducts could be easily observed and quantitated by staining for β-galactosidase activity; notably, seminal vesicle and ampullary gland ducts arising in these explants do not express *Nkx3.1* and remain unstained. We found that altering levels of Hh pathway activity dramatically affected ductal morphology without significantly affecting prostate budding. Thus, explants subjected to Hh pathway activation by treatment with Shh N-terminal peptide (n = 20) developed more slender ducts compared to untreated controls (n = 13) (Figs. 6A,B,E,F). In contrast, Hh pathway blockade with 5 μM cyclopamine (n = 14) had the opposite effect, yielding explants with abnormally blunt and crowded ducts (Figs. 6C,G); control explants treated with the inactive analogs tomatidine (n = 6) or solanidine (n = 4) showed wild-type morphology (Figs. 6D,H; data not shown). These observations were reinforced by histological analyses showing that explants from each treatment condition had poorly canalized ducts, suggesting that the morphological differences were not due to alterations in lumenal volumes (Figs. 6I–L). However, although explants cultured under the different conditions had similar numbers of ductal tips (Fig. 7A), morphometric analyses showed that Shh-treated explants had significantly reduced epithelial area per duct, whereas epithelial area per duct was markedly increased in cyclopamine-treated explants (Fig. 7B) (n = 4 explants analyzed per treatment group). Notably, these morphological differences alter the proportion of prostatic epithelial cells in proximity to the surrounding mesenchyme, suggesting Hh pathway activity may regulate the extent of epithelial–mesenchymal interactions in prostate morphogenesis.

To demonstrate that cyclopamine treatment effectively suppresses Hh pathway activity under our assay conditions, we measured expression levels of the Hh target gene *Ptc*. Real-time PCR assays demonstrated a 3–4× fold decrease in *Ptc* transcript levels relative to β-actin in explants treated with 5 μm cyclopamine versus untreated control explants (Fig. 7C). This result indicates that prostate budding can proceed despite Hh pathway inhibition, and suggests that the altered ductal morphology observed following cyclopamine treatment derives from inhibition of Hh pathway activity, not from nonspecific toxic effects.

The opposing effects of Shh and cyclopamine treatment on prostatic ductal bud morphology suggest that Hh signaling has an endogenous role in patterning during outgrowth of nascent prostatic buds. However, since treatment with cyclopamine does not inhibit the initial formation of pros-

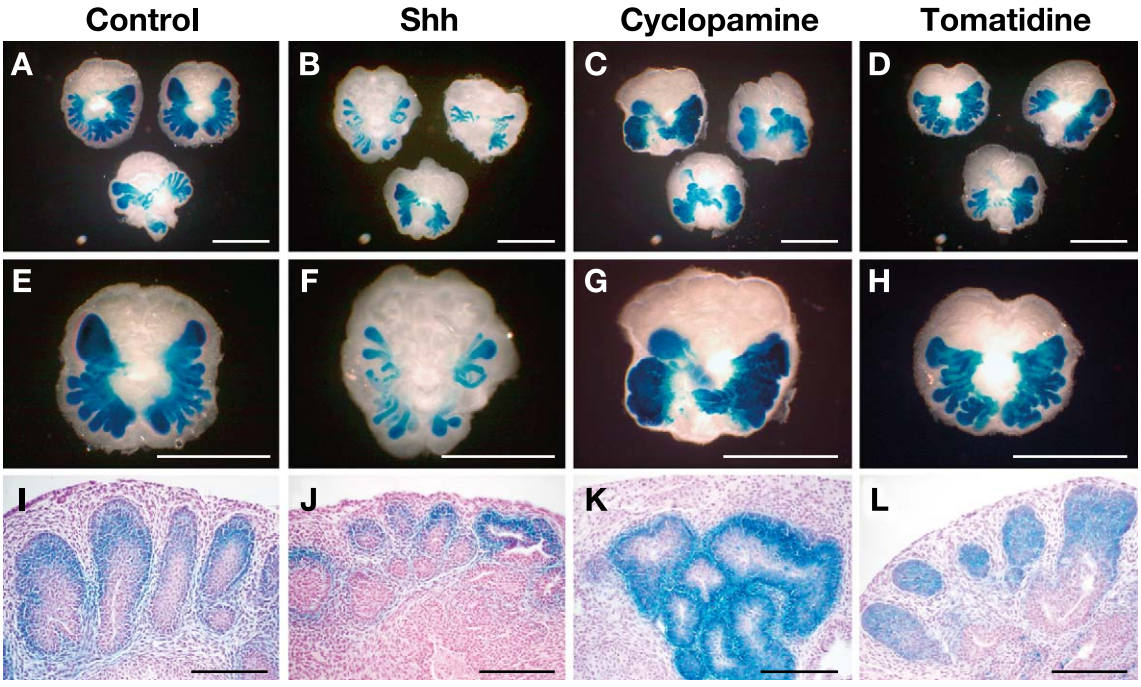

Fig. 6. Morphology of ductal buds in urogenital sinus organ cultures. (A–H) β-galactosidase staining of *Nkx3.1*$^{tm2(lacZ)Mms}$/+ urogenital sinus tissue explanted at 16.5 dpc and cultured for 7 days, without additions (A,E), in the presence of Shh N-terminal peptide (B,F), in the presence of 5 μM cyclopamine (C,G), and in the presence of 5 μM tomatidine (E,H). (I–L) Sections of β-galactosidase stained urogenital sinus explants, counterstained with hematoxylin. Scale bars correspond to 1 mm (A–H) or to 100 μM (I–L).

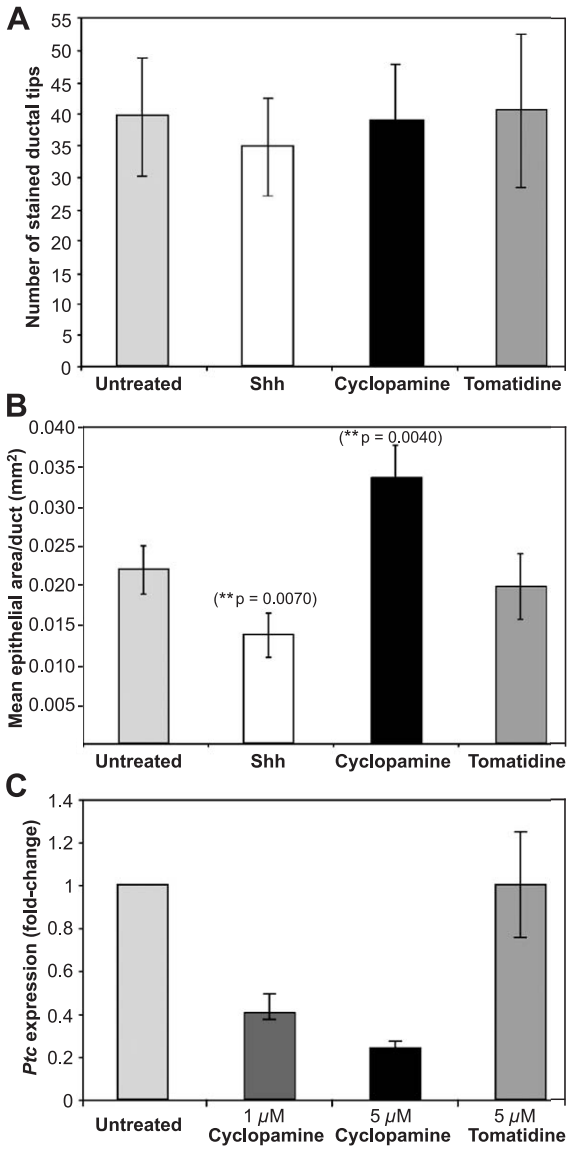

Fig. 7. Quantitation of ductal tip numbers, average duct area, and *Ptc* expression in *Nkx3.1*$^{tm2(lacZ)Mms}$/+ urogenital sinus organ cultures. Error bars correspond to one standard deviation. (A) Number of β-galactosidase-stained ductal tips in urogenital sinus explants. (B) Mean two-dimensional area of β-galactosidase stained ducts, normalized to number of ductal tips per explant. (C) Real-time RT-PCR quantitation of *Ptc* expression levels in urogenital sinus explants under indicated conditions, normalized to β-actin levels. Data represent the average of two independent experiments, performed in duplicate.

tatic buds, Hh activity is unlikely to be required for prostate induction in culture. Combined with our findings on the phenotype of *Shh* mutants, we conclude that *Shh* is not required for prostate induction in vivo.

Discussion

In the course of our investigation of the molecular basis for prostate organogenesis, we have reevaluated the role of Shh signaling in prostate development. Our overall conclusion is that the failure of prostate formation in *Shh* mutant mouse embryos reflects an insufficiency in androgen levels, rather than a direct role for Shh signaling in prostate formation. More generally, this analysis illustrates the broader lesson that the phenotypic absence of a structure in a mutant does not necessarily imply a direct role for the wild-type gene product in its formation.

We have provided several lines of evidence that prostate induction (budding) does not require *Shh*, and most likely does not require Hh pathway activity. Although XY *Shh* mutant fetuses lack prostate tissue, we have demonstrated that tissue recombination and grafting can completely rescue this defect. Moreover, prostatic budding from *Shh* mutant UGS can also occur in organ culture in defined media in the presence of androgens, indicating that the renal environment is not essential for prostatic bud rescue. Furthermore, administration of DHT to *Shh* mutant fetuses in utero can rescue the defect in formation of prostatic ductal buds. In combination, these observations suggest that the failure of prostatic budding in *Shh* mutants is due to androgen insufficiency; however, the small size of mutant fetuses at 16.5 dpc has precluded our ability to measure androgen levels in serum (N.D. and M.M.S., unpublished observations). Finally, inhibition of Hh pathway activity by culture with cyclopamine does not abrogate prostatic epithelial budding in wild-type urogenital explants, but does result in altered duct morphology. Taken together, our data indicate that *Shh* is not required either for formation of prostatic buds, or their initial outgrowth.

Instead, our results using the explant organ culture system suggest that Hh signaling is likely to play an important role in patterning of prostatic ducts once outgrowth commences. Our interpretation of the role of *Shh* in prostate formation is consistent with the known roles of the Hh pathway in other tissues, such as lung and hair follicles, and supports the proposed role of the Shh target BMP4 in limiting prostatic ductal branching (Lamm et al., 2001). Although we did not observe a significant effect of Shh or cyclopamine on ductal tip numbers, we observed dramatic and opposing effects of these treatments on prostatic duct morphology, suggesting an endogenous role for Hh signaling in regulating the epithelial–mesenchymal interface. Notably, the slender ducts observed in Shh-treated explants have an increased percentage of epithelial cells in proximity to mesenchyme, while the fatter ducts in cyclopamine-treated explants have a decreased percentage of epithelium adjacent to mesenchyme. Since epithelial–mesenchymal interactions regulate most aspects of prostate development, including organ size and architecture (Cunha et al., 1987), it seems likely that aberrantly patterned epithelial ducts with diminished exposure to mesenchymal signals may fail to provide an appropriate substrate for normal branching morphogenesis.

Our findings suggest alternative interpretations of studies published by Podlasek et al. (1999) in earlier analyses of

Shh activity in prostate formation. Their previous work showed that addition of an Affi-Gel bead soaked in Shh neutralizing antibody could inhibit prostate formation from 15.5 dpc urogenital sinus grown in renal grafts, whereas we observed essentially normal prostate morphology in grafts grown from *Shh* mutant urogenital sinus. Furthermore, Lamm et al. (2002) reported that organ culture of 14.5 dpc urogenital sinus in the presence of 10 μM cyclopamine resulted in a 71% decrease in ductal tip number, whereas we did not observe a significant change in ductal tip number in explants cultured with 5 μM cyclopamine or with Shh N-terminal peptide. It is conceivable that this discrepancy is due to different sources and concentrations of cyclopamine, or to differences in culture systems that could reveal an effect of cyclopamine on ductal branching, rather than inhibition of initial duct formation. In addition, we note that our organ culture data are unlikely to be due to potential toxicity of cyclopamine, since we observed apparently opposite effects on prostate ductal morphology after addition of Shh peptide. Finally, our results suggest that the abrogation of prostate morphogenesis in urogenital sinus engrafted with a source of Hh neutralizing antibody (Podlasek et al., 1999) may not reflect an inability to form prostate tissue, but may instead indicate reduced viability of the malformed organs that form under the influence of Hh pathway blockade.

Our results are consistent with a recent study demonstrating that addition of Shh to explant cultures of postnatal rat ventral prostate leads to inhibition of ductal branching, whereas cyclopamine treatment results in enlarged ductal tips (Wang et al., 2003). Unlike our assay using embryonic mouse urogenital sinus, this study used postnatal explants that had already initiated prostate formation and ductal morphogenesis, and were cultured in the absence of exogenous testosterone, which is required for normal prostate growth in vivo. In addition, Freestone et al. (in press) have performed similar organ culture experiments in the presence of testosterone, and have also observed altered morphology of rat ventral prostate ductal tips following cyclopamine treatment. We note that the lack of detectable abnormalities in the ductal morphology of *Shh* mutant prostates grown in tissue recombinants or grafts in *nude* mice suggests either that the requirement for Hh signaling in ductal morphogenesis is specific to in vitro culture systems, or that the kidney and/or maturing prostate epithelium can complement defective Shh signaling, perhaps through expression of other Hh ligands. While additional studies will be necessary to distinguish between these possibilities, our results clearly indicate that Shh is not required for prostate induction.

The androgen insufficiency of *Shh* XY mutant embryos presumably reflects a direct or indirect role for Hh signaling in the differentiation and/or function of testicular Leydig cells, the primary site of androgen biosynthesis. One possibility is that Shh is indirectly required for Leydig cell differentiation due to its essential role in the pituitary–gonadal axis during fetal development, since *Shh* is necessary for formation of the pituitary gland (Treier et al., 2001). Analyses of *hypogonadal* mutant mice, which are deficient for gonadotropin-releasing hormone (GnRH), suggest that pituitary gonadotrophs are not required for fetal development of Leydig cells (O'Shaughnessy et al., 1998); in contrast, *Nkx2.1* mutant mice lack the pituitary gland and have altered Leydig cell morphology and reduced testosterone levels (Pakarinen et al., 2002). A specific role for Hh signaling in Leydig cell differentiation has been shown by the findings that *Desert hedgehog* (*Dhh*) XY mutants have profound defects in fetal Leydig cell differentiation (Clark et al., 2000; Yao et al., 2002), as do fetal testes treated with cyclopamine in organ culture (Yao and Capel, 2002). Other potential explanations for the *Shh* XY androgen-deficient phenotype include possible defects in export of testosterone from the fetal testis or its delivery to the urogenital sinus mesenchyme. Finally, *Shh* may be required for normal testes development, which is supported by the observation that *Shh* mutant fetuses display defective urogenital ridge development and have rudimentary testes at 16.5 dpc (B. Capel, personal communication; D.M.B., N.D., and M.M.S., unpublished observations).

Our findings also imply that expression of *Nkx3.1* in the prostate is not dependent on *Shh* function, since the prostatic epithelium formed in *Shh* mutant grafts and explants display abundant *Nkx3.1* expression. Previous studies had suggested that *Nkx3.1* was downstream of *Shh*, based on the absence of *Nkx3.1* expression in *Shh* urogenital sinus (Schneider et al., 2000); however, this observation is consistent with the absence of prostate formation in *Shh* mutants, and with the androgen-dependence of *Nkx3.1* expression (Bieberich et al., 1996; He et al., 1997; Prescott et al., 1998; Sciavolino et al., 1997). In contrast, *Nkx3.1* appears to be downstream of *Shh* in the developing sclerotome during midgestation embryogenesis, since *Nkx3.1* expression in the ventromedial region of nascent somites is dependent on the presence of Shh signaling from the notochord (Kos et al., 1998).

The lack of a direct regulatory relationship between Hh signaling and *Nkx3.1* in normal prostate development is consistent with their opposite roles in prostate cancer progression, since Hedgehog pathway activity appears to be elevated in human prostate carcinomas (Dahmane et al., 2001; S.S.K., D.M.B., and P.A.B., in preparation; W. Bushman, personal communication), whereas Nkx3.1 protein expression is decreased or absent in most human carcinomas and in mouse models (Bowen et al., 2000; Kim et al., 2002). Recently, we demonstrated a requirement for Hh pathway activity in malignant growth of a variety of tumors, and a relationship between levels of Hh pathway activity and the rate of tumor growth (Berman et al., 2002, 2003; Watkins et al., 2003). In prostate cancer, the rate of tumor growth and progression is strongly correlated to Gleason grade, which evaluates branching patterns histologically. It is therefore possible that further studies of Hh signaling in prostate branching will lead to a better understanding of prostate carcinogenesis and tumor progression.

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