Hedgehog signaling in prostate epithelial–mesenchymal growth regulation

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Abstract

The prostate gland plays an important role in male reproduction, and is also an organ prone to diseases such as benign prostatic hyperplasia (BPH) and prostate cancer. The prostate consists of ducts with an inner layer of epithelium surrounded by stroma. Reciprocal signaling between these two cell compartments is instrumental to normal prostatic development, homeostasis, regeneration, as well as tumor formation. Hedgehog (HH) signaling is a master regulator in numerous developmental processes. In many organs, HH plays a key role in epithelial–mesenchymal signaling that regulates organ growth and tissue differentiation, and abnormal HH signaling has been implicated in the progression of various epithelial carcinomas. In this review, we focus on recent studies exploring the multipotency of endogenous postnatal and adult epithelial and stromal stem cells and studies addressing the role of HH in prostate development and cancer. We discuss the implications of the results for a new understanding of prostate development and disease. Insight into the cellular and molecular mechanisms underlying epithelial–mesenchymal growth regulation should provide a basis for devising innovative therapies to combat diseases of the prostate.

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Introduction

Hedgehog (HH) signaling plays an important role in development, homeostasis, and cancer. In the prostate, HH signaling modulates ductal morphogenesis through complex epithelial–mesenchymal interactions during development. In the adult prostate, HH is active in the stroma and HH–responding cells show properties of stromal stem cells. In prostate cancer, HH mainly signals through a paracrine mode, although uncertainty exists as to whether autocrine HH signaling occurs in some tumor cells. The potential roles of HH signaling in prostate development and cancer have been previously summarized by the Bushman group and others (Shaw and Bushman, 2007; Vezina and Bushman, 2007; Gonnissen et al., 2013). Here we review new findings on the multipotency of endogenous postnatal and adult epithelial and stromal stem cells, stage-dependent roles of HH signaling in prostate development, and the possible role of HH signaling in prostate cancer, including its potential involvement in castration-resistant prostate cancer.

Epithelial differentiation in prostate development

The prostate develops from the endodermal urogenital sinus (UGS) and the surrounding urogenital sinus mesenchyme (UGM). Prostate development is initiated by androgen acting on the mesenchyme to induce reciprocal paracrine mesenchyme–epithelial interactions (Cunha and Lung, 1978; Cunha et al., 1986). In mouse, the prostatic ducts start to form after embryonic day 17 (E17) as solid epithelial buds formed from the UGE that invade the surrounding UGM. Extensive epithelial branching occurs after birth as nascent prostatic ducts elongate, branch, and arborize to form elaborate networks; simultaneously, the buds canalize in a proximal to distal direction along the developing ducts (Sugimura et al., 1986a). Importantly, specification of the prostate epithelium requires androgen signaling in the mesenchyme, whereas androgen signaling in the epithelium is responsible for differentiation of the epithelium to enable production of prostatic secretions (Cunha and Lung, 1978; Cunha et al., 1987).

The prostate is a heterogeneous gland with regionally distinct features. The human prostate can be divided anatomically into central, transition, and peripheral zones (Fig. 1). Interestingly, carcinoma arises mainly in the peripheral zone, whereas benign prostatic hyperplasia develops mainly in the transition zone (McNeal, 1988; De Marzo et al., 1998). Unlike the human prostate that is a compact gland, the mouse prostate includes four paired lobes situated circumferentially around the urethra: anterior (AP),
dorsal (DP), lateral (LP), and ventral (VP) prostate (Fig. 1). The DP and LP are sometimes collectively referred to as the dorsolateral lobes of the prostate (DLP). At birth, each lobe of the VP consists of 1–3 main ducts with secondary and tertiary branches, whereas the more complex DLP initially has 9–12 unbranched proximal main ducts on each side (Sugimura et al., 1986a). Ductal branching is completed by 60–90 days after birth with more than 70% of the ducts being formed during the first 15 days (Sugimura et al., 1986a). Although the proximal part of the LP is adjacent to the DP, the distal part of the LP grows closer to the VP, which may explain the similarity of distal ductal architecture between LP and VP if the environment is a critical determinant (Sugimura et al., 1986a).

Interestingly, chemically induced carcinoma occurs mainly in the epithelial compartments (Letellier et al., 2007; Trompetter et al., 2008). Using p63Cre/+ ; R26YFP/+ mice to fate map p63+ cells, they found that YFP+ cells contributed to all three epithelial lineages of the adult prostate, indicating p63+ cells are multipotent during the embryonic stage. To study the fate of basal epithelial cells during postnatal development, Ousset et al. (2012) used basal cell specific K5CreER/+ ; R26YFP/+ mice and treated them with tamoxifen at P1 to label CK5+ basal cells. After 4 weeks of chase, they identified YFP+ basal, luminal, and neuroendocrine cells, indicating that CK5+ cells at P1 are multipotent. To verify the result, they used a different mouse line to label basal cells (CK14-rtTA; TetO-Cre; R26YFP) and found that K14+ basal cells also exhibit multipotency at P1. Furthermore, by titrating down doxycycline to allow clonal analysis, they found that while some YFP+ clones contained both basal and luminal cells, other clones contained only basal cells or luminal cells, demonstrating that at P1 some CK14+ cells are already committed to being unipotent basal or luminal progenitors. Interestingly, in 6-week-old mice intermediate cells which expressed both CK5 and CK8 were only found in clones containing both basal and luminal cells 4 weeks after doxycycline administration, suggesting intermediate cells likely represent a transitional stage between multipotent cells and differentiated cells. Using CK8CreER; R26YFP mice to label CK8+ luminal cells at P1, analysis of YFP+ cells in 4-week-old mice revealed that YFP+ cells only contributed to CK8+ luminal cells, indicating CK8+ luminal cells at P1 are unipotent and can only generate luminal cells. In summary, prostate epithelial cells are heterogeneous and become lineage-restricted during development.

An important question regarding the ontogeny of the epithelial cell lineage is whether basal cells are required for the formation of luminal cells; in other words, whether multipotent epithelial cells undergo a strict linear differentiation from basal cells to luminal cells. The p53 homolog p63 is expressed in the basal cells of many epithelial organs, including the prostate, and is required for the development of numerous epithelia (Signoretti et al., 2000). p63 null mutant mice fail to develop a prostate, suggesting p63 plays a critical role in prostate development (Signoretti et al., 2000). Surprisingly, embryonic UGSs from p63 null mice transplanted under the kidney capsule of adult immunodeficient male mice are able to differentiate into luminal cells and neuroendocrine cells but not basal cells, indicating that p63 is essential for the differentiation of basal cells, but p63 and thus basal cells are not required for the differentiation of luminal and neuroendocrine cells (Kurita et al., 2004). Luminal epithelial cells can therefore form through bypassing normal basal cell differentiation. The luminal cells generated from p63 null UGSs, however, show a prominent phenotype of goblet mucinous epithelial cells,
resembling the intestinal epithelium (Kurita et al., 2004); therefore, p63 and thus basal cells likely play an important role in the proper differentiation of prostate-specific luminal cells. In addition to p63, transcriptional factors such as Foxa1 were found to play an important role during prostate epithelial differentiation (Gao et al., 2005).

Mesenchymal differentiation in prostate development

There are reciprocal interactions between UGM and UGE during prostate development. UGM specifies prostatic epithelial identity and induces epithelial budding, and likewise the developing prostatic epithelium induces smooth muscle differentiation and patterning of the UGM (Cunha et al., 1996; Hayward et al., 1998). In transplantation experiments, when UGM alone is transplanted under the kidney capsule of male nude mice, only a small amount of smooth muscle differentiates in the grafts (Hayward et al., 1996c). In contrast, tissue recombinants consisting of UGM and UGE develop prostatic ducts with epithelial cells (basal and luminal) surrounded by smooth muscle bundles (Hayward et al., 1996b). Importantly, smooth muscle cells can be specified in the UGM not only by UGE, but also by epithelium from adult prostate or adult bladder, indicating common inductive signals across epithelial types and stages (Cunha et al., 1992). SHH is likely to be one of the inductive signals, as it has been postulated to play a critical role during the development of smooth muscle in bladder (Tasian et al., 2010) and gut (Mao et al., 2010).

Similar to the developmental sequence of the prostatic epithelium, smooth muscle develops in a proximal to distal order (Hayward et al., 1996b). One study of stromal development in the rat VP showed that the first mesenchymal marker to be detected is vimentin, which is initially expressed throughout the mesenchyme surrounding the UGE (Hayward et al., 1996b). Subsequently, smooth muscle markers are expressed in an orderly sequence from proximal to distal: first α-SMA, followed by vinculin, myosin, desmin, and laminin (Hayward et al., 1996b). Significantly, smooth muscle bundles are thicker in the proximal portions of the ducts than in the distal portions in the adult prostate, perhaps reflecting a longer time window for differentiation. Vimentin expression becomes largely restricted to the interdudal fibroblasts during prostate development (Hayward et al., 1996b).

In the adult mouse prostate, using molecular marker expression and cell location, we found that stromal cells can be further classified into four subtypes: subepithelial cells, smooth muscle cells, wrapping cells, and interstitial fibroblasts (Fig. 3) (Peng et al., 2013). Amongst them, subepithelial cells, wrapping cells, and interstitial fibroblasts are fibroblast subtypes. Interestingly, subepithelial cells, located between the epithelium and smooth muscle, are in close proximity to the basal cells. During prostate...
Prostate regeneration and adult stem cells

The adult mouse prostate is a quiescent organ with sparse cell proliferation being reported at the distal tips of ducts (Sugimura et al., 1986c). The maintenance of adult prostate cells and architecture requires androgen. Upon castration, about 35% of the ductal tips and branch-points are lost in distal regions of the prostatic ducts over a 2–3 week period (Sugimura et al., 1986b). The involution process is more rapid and profound in the VP than in the other lobes (Banerjee et al., 1995). Moreover, castration causes apoptotic death of both epithelial and stromal cells (Banerjee et al., 1995). In the epithelium, luminal cells are preferentially lost compared to basal cells following castration (English et al., 1987). Strikingly, 14 days after the administration of androgen to an involuted prostate, the prostatic ducts completely regenerate (Sugimura et al., 1986b).

Because cycles of involution and regeneration can be repeated over 30 times (Isaacs, 1985), it is generally agreed that a population of castration-resistant prostate stem cells exists that is capable of long-term self-renewal. To identify epithelial stem cells in the adult prostate by virtue of their slow cycling nature, Tsujimura et al. (2002) castrated male mice at 3 weeks, gave BrdU during the first round of regeneration to label almost all the epithelial cells, and then subjected the mice to up to 16 cycles of involution and regeneration. They found that slow cycling cells, detected by BrdU label-retention, are enriched in the proximal region of prostatic ducts (Tsujimura et al., 2002); moreover, epithelial cells isolated from the proximal region of ducts possess higher in vitro proliferative potential and form more elaborate branched glandular structures in collagen gels compared to epithelial cells from the distal region (Tsujimura et al., 2002). Importantly, label-retaining cells are distributed nearly equally in both luminal and basal cells in the proximal region (Tsujimura et al., 2002), providing the first in vivo evidence that epithelial stem cells may exist in both luminal and basal compartments. Based on these findings, Tsujimura et al. (2002) proposed a model in which epithelial stem cells are enriched in the proximal region of prostatic ducts, in a stem cell niche surrounded by a thick bundle of smooth muscle cells that express a high level of TGFβ (Nemeth et al., 1997). During castration, TGFβ signaling decreases proximally and increases distally (Salm et al., 2005). The increased TGFβ signaling in the distal region likely results in the apoptosis of cells in this region (Salm et al., 2005). In the proximal regions, TGFβ signaling decreases and WNT signaling increases (Placencio et al., 2008), thus favoring cell survival. In addition to WNT signaling, FGF signaling has been proposed to antagonize the effect of TGFβ (Salm et al., 2005; Franco et al., 2011), Tsujimura et al. (2002) further proposed that during regeneration, proximal epithelial stem cells give rise to young transit-amplifying cells in distal regions that repopulate the ducts. This model has had a major influence on the interpretation of studies examining the nature of epithelial stem cells and their niche; yet, it has not been rigorously tested in situ. Recently, genetic mouse models have provided a powerful approach to trace the fate of distinct cell types in situ (see below); however, currently no tools are available to preferentially label proximal versus distal cells in the prostate. When such tools are available in the future, it will be possible to test whether a proximal–distal hierarchy of cell lineage exists in the adult prostate.

Multiple stem cell markers have been identified to distinguish epithelial stem cells from the rest of the cells in the adult prostate (Fig. 3). Stem cells in the proximal region of mouse prostatic ducts can be purified by FACS (Fluorescence-Activated Cell Sorting) by virtue of their high expression of SCA1 (Burger et al., 2005; Xin et al., 2005). These SCA1high cells are enriched in proximal ducts and also express CD49f (α6 integrin) and the anti-apoptotic factor BCL2, which are characteristic markers for a variety of adult stem cells in other organs (Burger et al., 2005). Further FACS studies using different combinations of markers have identified two seemingly distinct stem cell populations (based on in vitro assays and in vivo transplant experiments): SCA1+CD49f+TROP2high (Goldstein et al., 2008) and SCA1+CD133+CD44+CD117+ (Leong et al., 2008).
Notably, these two epithelial stem cell populations mainly exist in the basal cell compartment based on immunofluorescence staining of prostate sections with CD49f (Lawson et al., 2007) or CD117 (Leong et al., 2008). These stem cell markers provide useful tools to stratify epithelial cells in terms of their potential stemness in culture or in transplant experiments. However, it is less clear how these markers relate to the machinery that empowers stemness to these epithelial stem cells, especially in vivo. A study of the Polycomb group transcriptional repressor Bmi-1 recently shed light on this matter. Cells expressing Bmi-1 are enriched in the proximal region of the prostate as well as in the SCA1+CD49f+ stem cells (Lukacs et al., 2010). Using shRNA knockdown, Lukacs et al. (2010) demonstrated that Bmi-1 plays an essential role in self-renewal of epithelial stem cells, measured by in vitro serial sphere forming assays and in vivo tissue recombination studies. Future studies are needed to establish links between signaling pathways or downstream targets of Bmi-1 that regulate stemness. Of possible relevance, signaling pathways such as Notch (Carvalho et al., 2014) and WNT (Kharashvili et al., 2011) have been implicated in the multipotency of epithelial stem cells and the lineage relationship between luminal and basal cells. For example, Notch ligands are expressed mainly by the basal cells and Notch signaling is active in both the luminal and basal cells (Valdez et al., 2012). Ectopic Notch activation in mouse prostate results in a decrease in basal cell number and an increase in luminal cell number (Valdez et al., 2012), suggesting Notch signaling plays an important role in maintaining the balance between these two cell types.

Importantly, when basal cells are isolated from the human or mouse prostate and transplanted under the mouse renal capsule together with the mouse UGM, they give rise to both basal and luminal cells (Lawson et al., 2007; Goldstein et al., 2010). However, when luminal cells are isolated and similarly transplanted, they do not give rise to basal cells (Goldstein et al., 2010). One interpretation of these experiments is that basal cells are bi-potent whereas luminal cells are uni-potent. However, this interpretation is not upheld by in situ lineage-tracing studies in mice, where both luminal and basal cells are primarily lineage restricted (see below).

More recently, mouse genetic tools have provided a powerful approach to mark cells and follow their fate in situ during androgen-mediated regeneration following castration-induced involution (Figs. 3 and 4). The homeobox transcription factor Nkx3.1 is one of the earliest specific markers for the prostate epithelium during ductal morphogenesis (Abate-Shen et al., 2008). Using Genetic Inducible Fate Mapping (GIFM) (Joyner and Zervas, 2006) and Nkx3.1CreER, R26YFP mice, Wang et al. (2009) identified a rare luminal epithelial population in the involuted prostate, termed CARNs (Castration-resistant Nkx3.1-expressing cells), that can re-populate both luminal and basal compartments during regeneration, indicating their bipotential ability. Subsequently, Choi et al. (2012) used K14-CreER and K8-CreER transgenes to fate map basal and luminal cells, respectively, in the adult prostate during two rounds of involution and regeneration. Surprisingly, they found that basal and luminal cells are independently self-sustained, indicating that cells that are lost either are replaced by unipotent stem/progenitor cells or simply by self-duplication (Choi et al., 2012). A different study using K5-CreER to fate map basal cells after more rounds of involution and regeneration found that a rare population of basal cells can contribute to both luminal and basal cells after 5 but not 3 rounds, indicating their bipotential ability when forced to regenerate to a great extent (Wang et al., 2013). It is still not clear the degree to which the cells marked in these studies overlap, and thus whether the difference in the two results is due to the different Cre driver lines used. It is important to note the number of cycles of involution and regeneration used in each experiment, as more cycles might force stem cells to regenerate to a greater extent and thus reveal bipotentiality. Nevertheless, these studies suggest that epithelial basal and luminal stem cells in the adult prostate are much more lineage-restricted when assayed in situ by genetic inducible fate mapping during involution and regeneration compared to tissue recombination studies in which UGM and isolated basal cells are mixed to induce cell expansion and differentiation in culture or after transplant.

Compared to epithelial stem cells, much less is known about stromal stem cells in the prostate. Since during castration both epithelial cells and stromal cells die (Banerjee et al., 1995; Peng et al., 2013), both cell types need to be replenished. The existence of stromal stem cells was suggested by in vitro culture of primary stromal cells from BPH tissues (Lin et al., 2007). Such stromal cells can differentiate into smooth muscle cells, osteocytes, and adipocytes under specific culture conditions (Lin et al., 2007). Our in vivo GIFM studies uncovered that all four prostate stromal subtypes are replenished by stem cells for over 12 rounds of involution and regeneration (Peng et al., 2013). To test whether multipotent or unipotent stromal stem cells exist, a Smo-CreER transgene was used to fate map smooth muscle cells over 6 rounds of involution and regeneration. The study revealed that smooth muscle cells are replenished primarily by pre-existing smooth muscle actin (Sma)-expressing cells (Peng et al., 2013). By extrapolation, four stromal subtypes are likely replenished by their own stem cells, a hypothesis that parallels the epithelial side where each epithelial subtype is replenished mainly by distinct epithelial stem cells when assayed by GIFM.

Hedgehog signaling: an overview

The most common mode of action by HH signaling is through a paracrine mode in which HH producing cells are situated close to, but are a different cell type from, those receiving HH signaling. In mammals, three homologs of the Drosophila hedgehog (HH) protein family exist: sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH). In HH producing cells, HH ligands undergo proteolytic cleavage (Perler, 1998) to generate an amino-terminal peptide that is linked to cholesterol and palmitic acid (Pepinsky et al., 1998; Taylor et al., 2001). The distance HH travels to exert its function differs between tissues but can be up to 300 μm in the limb bud of vertebrates (Briscoe and Therond, 2013). In HH receiving cells, HH signals through the core receptor Patched 1 (PTCH1), a 12-pass transmembrane protein (Marigo et al., 1996; Stone et al., 1996) and three co-receptors: CDO, BOC, and GAS1 (Okada et al., 2006; Tenzen et al., 2006; Allen et al., 2007). Mammals have a second Ptc gene, Ptc2, with an expression pattern that does not fully overlap with Ptc1 expression (Motoyama et al., 1998a, 1998c). Without HH ligand, PTCH1 represses the activity of smoothened (SMO), a seven-pass G-protein-coupled membrane protein, through an unknown mechanism. The binding of a HH ligand to PTCH1 removes the inhibition of SMO and thus turns on the signaling pathway.

SMO activation leads to a change in the balance of the repressor and activator forms of the GLI zinc-finger transcription factors. Three GLI genes are present in mammals, GLI1, GLI2, and GLI3, each with both specific and redundant functions in the nervous system (Motoyama et al., 1998b; Park et al., 2000; Bai and Joyner, 2001; Bai et al., 2002, 2004; Persson et al., 2002; Motoyama et al., 2003; Petrova et al., 2013). When the pathway is in the “off” state (SMO is inactive), GLI2/GLI3 are phosphorylated and proteolytically processed, leading to the conversion of GLI3, and to a lesser extent GLI2 into transcriptional repressors (Wang et al., 2000; Bhatia et al., 2006; Pan et al., 2006; Wang and Li, 2006). When the pathway is in
the “on” state, SMO activation inhibits GLI2/GLI3 proteolysis and promotes the formation of GLI2, and to a lesser extent GLI3 activators. GLI2/GLI3 activators turn on expression of target genes, including GlI1 (Sasaki et al., 1999; Bai et al., 2004; Smelkinson et al., 2007). GlI1 functions as a constitutive activator that exerts positive feedback on the HH signaling pathway, but it is dispensable for normal development, including the prostate (Park et al., 2000; Bai et al., 2002; Doles et al., 2006).

Vertebrate HH signaling requires the primary cilium, a slim microtubule-based organelle that projects from the surface of most vertebrate cells (Goetz and Anderson, 2010). Mouse genetics studies demonstrated that mutations affecting intraflagellar trafficking disrupt HH signal transduction and result in HH loss and gain-of-function phenotypes (Huangfu et al., 2003), providing the first evidence that cilia play an essential role for processing GLI proteins into activators and repressors. Ptc1 is enriched in primary cilia in the absence of HH and moves out of cilia in the presence of HH (Rohatgi et al., 2007). In response to HH, SMO is enriched along the primary cilium and activates the pathway by antagonizing the activity of suppressor of fused (SuFu), an important negative regulator of mammalian HH signaling (Cooper et al., 2005; Svard et al., 2006). Removal of SuFu inhibition allows GLI2/GLI3 to become activators and translocate into the nucleus, where they promote expression of HH target genes.

**Hedgehog signaling in prostate development and homeostasis**

During ductal morphogenesis, Shh is expressed in the epithelium of nascent UGS buds and in the growing tips of the elongating prostate ducts (Podlasek et al., 1999). The pattern of Shh expression is mirrored by the expression of GlI1 and Ptch1 in the adjacent UGS mesenchyme (Lamm et al., 2002; Pu et al., 2004). HH signaling was first thought to be absolutely required for inducing prostate budding based on studies of neutralizing antibody treatment of E15 UGS grafts implanted under the renal capsule and pharmacological inhibition of HH signaling in prostate organ cultures (Podlasek et al., 1999; Lamm et al., 2002). Consistent with this idea, Shh−/− embryos at E18.5 show a complete absence of prostatic ductal budding (Berman et al., 2004). However, subsequent studies showed that apparently normal glandular structures can be derived from Shh null mutant mice when the UGS is implanted under the renal capsule of adult male mice (Berman et al., 2004) or cultured in the presence of androgen (Freestone et al., 2003; Berman et al., 2004), suggesting the defect in Shh null mutants is due to insufficient levels of androgens. Ihh expression was up-regulated in both grafted and cultured Shh null mutant UGS and might therefore compensate for the loss of Shh (Doles et al., 2006). GlI2 null mutant mice exhibit reduced ductal morphogenesis and perturbed epithelial differentiation (Doles et al., 2006), which cannot be rescued by exogenous androgen supplementation, indicating that the phenotypes are not due to hypogonadism as seen in Shh null mutants (Doles et al., 2006). Thus, it is likely that HH (possibly IHH and SHH) signals through GLI2 to regulate prostate duct development.

The effects of HH signaling activation on ductal morphogenesis appear to be stage dependent. Using cultured UGS from P2 rats, Wang et al. (2003) showed that unlike with embryonic UGS, inhibition of HH signaling by cyclopamine results in enlarged ductal tips and increased ductal branching, whereas addition of SHH recombinant protein increases the number of differentiated epithelial cells (Wang et al., 2003). To resolve the different results observed with different stages of prostate tissue in culture in different studies, Yu and Bushman (2013) studied the effect of ectopic HH pathway activation on prostate ductal growth using in vitro organ cultures and in vivo transgenic mouse models. They found that co-culture of prostate epithelium with a mesenchymal cell line overexpressing SMO-M2 (an active form of SMO) results in increased ductal growth when embryonic prostate tissue is used but decreased ductal growth when postnatal tissue is used (Yu and Bushman, 2013). Moreover, they found that activation of HH signaling in the mesenchyme of mice using Fsp-Cre; R26LSL-SmoM2 mice that conditionally express SMO-M2 in the mesenchyme from mid-gestation onwards results in an increase in epithelial proliferation in embryos, but inhibition of epithelial proliferation postnatally (Yu and Bushman, 2013). The stage-dependent effects of paracrine HH signaling are likely due to differences in the response of the stroma to HH signaling at different developmental stages, for example in regulation of secretion of inhibitors of epithelial growth such as Activin A and TGFβ1 (Wang et al., 2003). Of particular interest, this plausible stage-dependent stromal response might be tightly linked to the different cell types and composition at different developmental stages. For example, undifferentiated mesenchyme might respond differently to active HH signaling compared to mature smooth muscle cells.
In the adult prostate, our recent study using knock-in reporter mice for several key components of the HH pathway has revealed that Shh is expressed specifically from the basal epithelial cells and signals to the surrounding stromal cells (Peng et al., 2013). In most regions of the adult mouse prostate, except the most proximal part, basal cells express Shh whereas luminal cells do not (Peng et al., 2013). Furthermore, a subset of the four stromal subtypes expresses Gli1, indicating cells experiencing a high-level of HH signaling, whereas most stromal cells express Gli2 indicating that they are capable of responding to HH signaling (Peng et al., 2013). Interestingly, the basal cells in addition to many stromal cells express Gli3, encoding the major repressor of the pathway (Peng et al., 2013). Considering that during involution luminal cells preferentially die compared to basal cells (English et al., 1987), it is tempting to propose that Gli3 plays a role in conferring cast ration resistance to the basal cells. The functional role of Gli3 in the basal cells requires further study. Nevertheless, it is clear that in the adult prostate, HH signals primarily from the epithelium to the stroma. Thus, the paracrine mode of action is preserved from the developing prostate to the adult prostate.

**Hedgehog signaling in human cancers**

Hedgehog signaling is aberrantly activated in a wide variety of human cancers. Its mode of action can be categorized into four classes. The first class involves cell autonomous HH signaling and results from activating mutations in HH pathway components that render tumor cells independent of HH ligands. A classic example is Gorlin syndrome, a dominant disorder characterized by heterozygous loss-of-function mutations in PTCH1 (Gorlin, 2004). Patients with Gorlin syndrome develop medulloblastoma, rhabdosarcoma and basal cell carcinoma (Gorlin, 2004). The second class of tumors involves an autocrine or juxtacrine mode of action such that tumor cells secrete and respond to HH ligands. The third class involves a paracrine mode of action in which tumor cells secrete HH ligands and signal to the surrounding stroma, which in turn secretes reciprocal paracrine signals that regulate tumor growth. In the fourth class, a reverse paracrine mode of action is active in which stromal cells secrete HH ligands and tumor cells respond. Hedgehog signaling in prostate cancer is thought to mainly function by the third mode (see below).

**Prostate cancer**

Prostate cancer is the second leading cause of cancer death in men in the United States (Siegel et al., 2013) and the second most common cause of cancer among men worldwide (Ferlay et al., 2010). Current treatments of localized prostate cancer include surgery and radiation therapy. Patients with low-risk disease can be managed with active surveillance. In cases of advanced cancer, treatments primarily include androgen deprivation therapy, which initially decreases the tumor size but castration-resistant cancer eventually evolves and becomes refractory to any current treatments (Wozney and Antonarakis, 2014). Tremendous effort has been put into understanding how prostate cancer initiates and progresses but many factors remain unclear. Relatively recently, signaling pathways that play important roles during prostate development, such as HH signaling, have drawn significant attention due to the idea that cancer cells may awaken and harness these pathways to promote their growth. Therefore, a thorough understanding of how these pathways act in prostate cancer could likely facilitate the development of more effective treatments.

Prostate adenocarcinoma can derive from both luminal and basal cells. In >95% untreated prostate cancers, tumor cells predominantly express luminal markers, whereas basal-like cells are rare (Shen and Abate-Shen, 2010). Conditional deletion of Pten in either luminal or basal cells in adult mice using K14-CreER or K8-CreER, respectively, results in prostate cancer (Choi et al., 2012). However, luminal cells are more susceptible to direct transformation, whereas basal cells appear to need to differentiate into luminal cells before they undergo oncogenic transformation (Choi et al., 2012). By contrast, tissue recombination studies have shown that human basal cells can be more susceptible to malignant transformation, at least in an ectopic graft assay. Overexpression of ERG, AKT, and AR in human prostate basal cells (isolated based on CD49high and TROP2high expression) generates prostate cancer when the cells are combined with UGM and transplanted subcutaneously into immunocompromised mice, whereas the same manipulation of luminal cells (CD49low; TROP2high) fails to generate prostate cancer (Goldstein et al., 2010). Therefore, luminal and basal cells are capable of initiation of prostate cancer but the characteristics of tumor progression likely depend on the cellular and genetic context. Of note, since in the aforementioned studies malignant transformation was induced in luminal and basal epithelial cells from different species (mouse and human), one cannot exclude the possibility that there is an intrinsic difference in the susceptibility of basal or luminal cells to malignant transformation between species.

Numerous studies have indicated the importance of reciprocal interactions between prostate stromal cells and tumor cells during prostate cancer formation (Barron and Rowley, 2012). For example, normal prostate epithelial cells give rise to intraepithelial neoplasia when combined with cancer-associated fibroblasts (CAFs) but not with normal fibroblasts (Olumii et al., 1999). In human prostate cancer, cancer-associated stroma is mainly composed of CAFs and myofibroblasts intermixed with a greatly reduced number of mature smooth muscle cells, compared to normal prostate stroma which is predominantly composed of smooth muscle (Tuxhorn et al., 2002). Myofibroblasts express vimentin and a decreased level of smooth muscle actin and are devoid of mature smooth muscle markers such as calponin and desmin (Tuxhorn et al., 2002). Significantly, the volume of cancer-associated stroma in the tumor is a predictor of disease-free survival (Ayala et al., 2003, 2011). The cell of origin for cancer-associated stroma is not clear. Many cell types have been proposed to be the source including fibroblasts, smooth muscle cells, pericytes, and mesenchymal stem cells (Barron and Rowley, 2012). However, none of the proposed sources have been rigorously tested by using in vivo fate mapping experiments.

**Hedgehog signaling in prostate cancer**

Aberrant activation of HH signaling has been implicated in human prostate carcinogenesis. However, it is not clear whether tumor cells secrete and respond to HH ligands in an autocrine fashion (class 2) or tumor cells secrete HH ligands and stromal cells respond (class 3). Studies from several groups have supported the idea that paracrine HH signaling plays an important role in prostate cancer progression. Similar to the normal adult prostate, Fan et al. (2004) Shh expression was detected by in situ hybridization in the tumor epithelium and Gli1 expression in the stroma. In addition, genetically engineered Shh overexpression in the LNCaP human cancer cell line was found to lead to increased stromal Gli1 expression and to accelerate tumor growth in a xenograft tumor model (Fan et al., 2004), supporting a paracrine mode of HH action. Furthermore, Ibuki et al. (2013) showed that the growth of LNCaP cells was significantly delayed in castrated mice treated with TAK-441, a smoothened antagonist, indicating an important role of HH signaling in the development of castration resistance. In the TAK-441-treated xenografts, expression of murine Gli1 was suppressed, while expression of human Gli1 in tumor cells was
unaffected (Ibuki et al., 2013). Since the LNCaP cancer cells were implanted under the skin, it is possible that the dermal stromal cells respond to Shh the same way as the normal prostate stromal cells and that reciprocal paracrine signaling augments tumor progression.

On the other hand, autocrine HH signaling in cancer epithelial cells has been reported (Karhadkar et al., 2004; Sanchez et al., 2004; Tzelepi et al., 2011). Aberrant activation of HH signaling, measured by GLI1 and PTC1 expression in whole tumor tissue using quantitative RT-PCR (qRT-PCR), was found to be more prevalent in metastatic prostate cancer than in localized prostate cancer (Karhadkar et al., 2004). However, since epithelial and stromal cells were not separated in this study, it is equally possible that increased stromal GLI1 expression accounts for the overall increase in GLI1 levels. Tzelepi et al. (2011) detected higher PTC1 expression in the epithelium of metastatic tissue compared to primary cancer tissue using immunohistochemistry. If the antibody is reliable, the result indicates that a shift from a paracrine to autocrine mode of action can occur during cancer progression. The seemingly different results regarding whether HH functions in a paracrine or an autocrine manner, or both, likely reflect the difficulties of detecting components of the HH pathway in human prostate cancer. Moreover, not only is there a lack of reliable antibodies to components of the HH pathway, but the heterogeneity of prostate cancer within one patient and between patients, and the difficulty of cleanly separating the epithelium from the stroma also present major challenges. In this regard, genetic mouse models provide a valuable tool to address the mode of action of HH signaling in vivo and its role during prostate cancer formation.

In the SV40 T-antigen based LADY prostate tumor model, Shh expression was found to be decreased, whereas the expression of Glitl was not altered as measured by qRT-PCR of whole tumor tissue (Gipp et al., 2007). Interestingly, Ihh expression is increased in LADY tumors compared to the normal prostate (Gipp et al., 2007). The maintenance of a normal Glitl level could actually indicate an increase in stromal Glitl expression, given that the proportion of stromal cells is usually decreased compared to epithelial cells in prostate tumors. The increased stromal Glitl expression could then be due to an increase in Ihh expression. It is unclear whether aberrant activation of the HH pathway in the stroma is sufficient to generate or accelerate progression of prostate cancer. Interestingly, overexpression of SHH ligand by electroporation of the adult mouse prostate is sufficient to cause prostate cancer in 90 days (Chang et al., 2011). In contrast, conditional expression of SMO-M2, a constitutively active form of SMO, in the postnatal prostate epithelium using a Probasin-Cre transgene does not increase epithelial proliferation or result in cancer (Mao et al., 2006). Since overexpression of SHH ligand can have effects on both the epithelium and the stroma, it is possible that activation of the HH pathway in both compartments is required to initiate prostate cancer, or that aberrant activation of the pathway in the stroma alone is sufficient to generate prostate cancer. Given that Glitl is expressed in basal cells of the epithelium (Peng et al., 2013), this epithelial cell type has at least some of the machinery necessary for HH signaling.

An important question is whether there is crosstalk between HH signaling and androgen receptor (AR) signaling in prostate cancer. As castration-resistant prostate cancer (CRPC) continues to be dependent on AR signaling which is active despite low serum levels of androgen (Chen et al., 2008), one possible mechanism of castration resistance is through activation of other signaling pathways both inside the tumor cells and in the tumor microenvironment. Therefore, inhibition of signaling pathways that assist the activation of AR signaling likely represent a compelling combined therapeutic approach for prostate cancer. There is in vitro evidence for an interaction between HH and AR signaling. When culture conditions were switched from an androgen-supplemented to an androgen-depleted medium SHH expression was increased in the LNCaP human prostate cancer cell line (Azoulay et al., 2008; Chen et al., 2009). Providing functional evidence for SHH production, conditioned medium from the androgen-deprived LNCaP cells increased Gli1 expression in cultured mouse fibroblasts (Chen et al., 2009), indicating that androgen-deprived LNCaP cells can induce paracrine HH signaling in the stromal cells. Moreover, treatment of primary human prostate stromal cells with SAG, a HH agonist, or with a lentivirus that expresses a constitutively active Gli2 leads to increased androgen production from the conversion of androgen precursors supplemented in the culture medium (Levina et al., 2012), indicating that paracrine HH signaling in stromal cells could play a role in the local production of androgen. Furthermore, combined treatment of mice with an AR inhibitor and a HH inhibitor was found to suppress CRPC growth in a xenograft mouse model of prostate cancer more effectively than treatment with either agent alone (Gowda et al., 2013). Future clinical trials of HH inhibitors with AR inhibitors will be needed to test the efficacy of this combination therapy on patients with CRPC.

Since HH signaling is active in both normal stroma and cancer-associated stroma, an important question is how stromal cells in these two different states are intrinsically different and lead to overtly different effects on the epithelium: in the normal prostate, a homeostatic balance exists between the epithelium and stroma, whereas during cancer formation the balance is disrupted such that the nature of the stroma is changed and unchecked expansion of luminal cells is favored. Considering the effects of HH activation on ductal morphogenesis are stage-dependent during prostate development, it is possible that cancer-associated stromal cells recapitulate properties of prenatal mesenchyme, where expansion of the cells is stimulated by SHH. Indeed, transcriptional studies identified common genes that are regulated by SHH in the mesenchyme of the developing prostate and in the stroma of SHH-overexpressing xenograft tumors (Shaw et al., 2009) and in primary CAFs from human prostate tumors (Wilkinson et al., 2013). However, it is equally possible that cancer-associated stromal cells resemble postnatal stroma and thus inhibiting HH signaling could potentially worsen the disease. Indeed, recent studies of pancreatic cancer provided compelling evidence that HH signaling reduces tumor progression (Lee et al., 2014; Rhim et al., 2014). To distinguish between the two possible functions of HH, it will be necessary to decipher how the network involving HH signaling is wired in the cancerous context compared to the normal prostate. In this regard, it also is important to identify the HH target genes that are differentially expressed in the normal prostate and cancer, and likely in different subtypes of prostate cancer.

**Conclusion**

Recent experiments using GEMI have uncovered that prostatic epithelial cells become lineage-restricted (from multipotent to unipotent) during postnatal development. In the adult, both epithelial and stromal stem cells are largely unipotent when assayed in vivo during a limited number of rounds of regeneration. SHH signals primarily in a paracrine mode from the epithelium to the stroma both during prostate development and in the adult. In prostate cancer, HH likely also signals mainly through a paracrine mode. Stromal cells in the cancerous tissue are phenotypically different from their normal counterparts, and thus the molecular and cellular consequences of HH signaling are likely to differ during development in comparison to adult homeostasis, and
could depend on the particular prostate tumor. Understanding the difference between normal stroma and cancer-associated stroma, and identifying the HH target genes differentially expressed between the different contexts will be key to understanding the stage and tumor-specific roles of HH signaling and for devising innovative therapies to combat prostate cancer.

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