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Differential stage-dependent regulation of prostatic epithelial morphogenesis by Hedgehog signaling

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ABSTRACT

Published studies of Hh (Hedgehog) signaling in the developing prostate have reported varying and discrepant effects on epithelial proliferation, ductal morphogenesis and growth. We report here that these differing observations accrue from stage-specific effects of Hh signaling in the developing prostate. Using *in vitro* organ cultures of the E16 UGS and P1 prostate, we show that ectopic Hh pathway activation stimulates epithelial proliferation prenatally, but inhibits epithelial proliferation postnatally. Extrapolating from previously published observations that Hh target gene expression is altered in the reactive stroma of prostate cancer, we examined and found discordant regulation of a subset of target genes by Hh signaling in the prenatal and postnatal prostate. Cell based studies and recombination assays show that these changes are not simply attributable to the age of the mesenchyme or the epithelium, but more likely reflect a complex regulation by the cellular microenvironment. To determine the *in vivo* relevance of these observations, we examined the effect of transgenic activation of Hh signaling on epithelial proliferation in the prenatal and postnatal prostate and confirmed the operation of stage-specific effects. These observations demonstrate stage-specific differences in the effect of Hh signaling on epithelial proliferation in the developing prostate and suggest that these are a product of complex interactions determined by the cellular microenvironment.

Introduction

The male sex accessory gland prostate develops from the urogenital sinus (UGS) and is located between the base of the bladder and urethra. Its development has been widely studied using rodents as models. Prostate development is initiated at E16/E18 (E16 in mouse and E18 in rat), when solid prostatic buds emerge from the UGE and invade into the surrounding mesenchyme to form the prostatic ductal buds. These buds undergo elongation, branching, cell differentiation and ductal canalization and form the prostatic ducts of the ventral, dorsolateral and anterior lobes of the adult prostate. The complex process of prostate development is androgen dependent and involves mesenchymal–epithelial interactions mediated by multiple signaling pathways.

Hedgehog (Hh) signaling controls multiple cellular events including patterning, proliferation and differentiation in a variety of embryonic developmental processes. There are three Hh ligands in mammals: Sonic hedgehog (shh), Indian Hedgehog (Ihh) and Desert Hedgehog (dhh). All three ligands bind to the transmembrane receptor, Patched (Ptc). Binding of the Hh ligand to Ptc relieves repression of another transmembrane protein, Smoothened (Krstic et al., 1995), activates the Hh signal transduction pathway and results in activation of the Gli zinc-finger family of transcription factors and transcription of Hh target genes. Gli1 and Ptc1 are two conserved target genes and increase in their expression is considered a reliable indicator of the pathway activation.

Of the two Hh ligands (Shh and Ihh), both expressed in the developing prostate, Shh is the more abundant. Localization studies showed that Shh is expressed in the prostate epithelial ducts, concentrated in the ductal tips, while its functional genes Gli1 and Ptc1 are expressed in the surrounding mesenchyme. This pattern indicates paracrine signaling from the epithelium to the mesenchyme. Gene analysis studies revealed that the expression of Shh is most abundant from E16 to P1, which is a crucial window for early prostatic duct formation, and then gradually decreases to a very low level in the adult stage. This expression pattern suggests a potential role of Hh signaling in regulation of early prostate development. The very first study using a polyclonal neutralizing Shh antibody showed that treatment of the grafted E15.5 UGS with neutralizing Shh antibody prevented formation of prostatic ducts (Podlasek et al., 1999). This observation indicated Hh signaling is

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required for prostatic ducts induction. Consistently, chemical inhibition of Hh signaling by cyclopamine in E14 explants reduced the ductal tips number and decreased cell proliferation in both epithelium and mesenchyme (Lamm et al., 2002). Those observations support a stimulatory role of Hh signaling in controlling the prostate morphogenesis, In contrast, Hh signaling was showed to inhibit both ductal branching and cell proliferation in P2 rat ventral prostate (VP) explants, suggest a negative regulation of Hh signaling on prostate morphogenesis (Wang et al., 2003). These authors found decreased epithelial proliferation and duct tips when explants were cultured in the presence of exogenous Shh, and a corresponding increase in cell proliferation and duct tips when cultured in the presence of the Hh signaling inhibitor cyclopamine. Further, when P2 VP was co-cultured with UGM cells that overexpressed activated Gli1 or Smo, the effects on ductal branching and proliferation were found to mimic the results obtained with exogenous Shh ligand. Another study using rat P0 VP explants found a suppressive effect of Hh signaling on ductal branching, but yielded inconsistent observations on epithelial cell proliferation (Freestone et al., 2003). A later study found no effect of either cyclopamine or Shh on duct tip number in cultured E16.5 mouse UGS explants (Berman et al., 2004).

We revisited the role of Hh signaling on early prostate development, focusing on its influences on epithelial proliferation and ductal branching. We utilized chemical inhibition of cultured tissues, genetic activation of the Hh pathway in stromal cells cultured together with tissues *in vitro* tissue and transgenic mice with conditional activation of the Hh pathways to compare the effects of paracrine signaling pre- and postnatal. These studies showed that Hh signaling exerts unique stage-specific effects on epithelial proliferation and prostatic ductal branching morphogenesis that correlated with stage-dependent changes in Hh target gene regulation by Hh signaling.

Materials and methods

Explant culture, primary mesenchymal cell culture and tissue recombination

E16 and P1 UGS tissues were collected from C57Bl/6 mice (Charles River) and then placed on Millicell-CM filters (Millipore) suspended on serum-free medium with 10^{-8} M DHT and other supplements as described before (Doles et al., 2006). The medium was changed every other day. For the co-culture experiment, the E16 and P1 UGS tissues were cultured together with the UGSM-2 cells infected with retrovirus that expressed GFP (green fluorescent protein) and activated SmoM2 or Gli2 at a ratio of 1×10^5 UGSM-2 cells per UGS tissue. Primary UGM cells were freshly isolated from the mouse E16/P1 UGS mesenchyme as described. Primary UGM cells were placed in cell culture dishes with Dulbecco's modified Eagles' medium/F12 containing 10% fetal bovine serum, allowing 1-week culture to reach the confluence before exposure to Shh. For the tissue recombination studies, the mesenchymal–epithelium separations were done on the E18/P1 UGS of Sprague Dawley rats (Charles River) and E16/P1 UGS of C57bl mice, as described. Basically, the UGS tissue was incubated with 1% trypsin at 4 °C for 75 min, followed by mechanical dissociation. The recombinants were constructed by wrapping the mouse E16/P1 UGM around the rat E18/P1 UGE tube, placed together on the tissue culture inserts, followed by 7 days incubation in the serum-free defined media supplemented with 10^{-8} M DHT.

AZ75 (AstraZeneca) (a cyclopamine derivative that binds to Smo as a specific inhibitor of Hh signaling) was dissolved in 95% EtOH and used in concentration at 0.5 μM; Exogenous Shh (Curis) was dissolved in PBS/0.1% BSA and diluted in the culture media to 5 nM as the final concentration.

Explants were photographed under the dissection microscope (Zeiss, Diagnostic) to quantitate the number of ductal tips (Lamm et al., 2001).

Real-time RT-PCR gene analysis

RNA was harvested from cultured tissues and UGM cells with RNeasy Mini kit (Qiagen, Valencia, CA) with optional DNase digestion to eliminate DNA contamination, followed by reverse transcription and real-time PCR as described before (Doles et al., 2006). PCR primers used are the same as listed in the previous study (Yu et al., 2009). Results were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. All experiments were performed in triplicate and reported differences were statistically significant ($P<0.05$; Student's *t*-test).

Transgenic mice and genotyping

Smo.YFP mice were obtained from the Jackson Laboratory. Fsp1.cre mice were obtained from Dr. Bhowmick (University of Vanderbilt, Nashville, TN). Rosa26 reporter mice were provided by Dr. Sun (University of Wisconsin—Madison, Madison, WI). DNA was isolated from mouse tails and genotyped by PCR analysis using the following primers:

Fsp1.cre	AGGTGTAGAGAAGGCACTTAGC (forward) CTAATCGCCATCTTCCAGCAGG (reverse), 411 bp
SmoM2.YFP	AAGTTCATCTGC ACCACCG (mutant forward) TCCTTGAAGAAGATGGTGCG (mutant reverse),173 bp CGTGATCTGCAACTCCAGTC (wild type forward) GGAGCGGGAGAAATGGATATG (wild type reverse) 410 bp

The SmoM2.YFP:Fsp1.cre mutants are showing positive bands with both cre and SmoM2.YFP mutants; the rest of the littermates are considered as controls.

BrdU incorporation and immunohistochemistry

To visualize the proliferation cells in the explants cultured tissues, 5-bromo-2′-deoxyuridine (BrdU) labeling medium (1:1000, Roche Applied Science) was added to the culture media 2 h before fixing the tissues. In the transgenic mice model, BrdU was injected into the dam (prenatal E18) or P10 mouse (10 μL undiluted per gram body weight, i.p.) 1 h before euthanasia. Explants or UGS tissues were collected and fixed in 10% formalin, followed by paraffin embedding and sectioning (5 μm/section) to perform immunofluorescence staining as described (Cook et al., 2007). The following primary antibodies were applied: mouse anti-BrdU (1:10, Roche Applied Science), rabbit anti-PanCk (1:50) and rabbit anti-P63 (1:100, Santa Cruz). Sections were counterstained with Hoechst, cover slipped and imaged by an Olympus model BX51 fluorescent microscope. To calculate the proliferation index, 10–15 digital images were randomly selected from each group. BrdU positive cells, P63 or PanCk positive cells were counted in each image and the proliferation index in the epithelium was obtained by calculating the ratio of the BrdU labeled epithelial cells to the total epithelial cells. For statistical comparisons, BrdU labeling index was analyzed by two-tailed Student's *t*-test.