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Functional compensation in Hedgehog signaling during mouse prostate development

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Abstract

Studies of hedgehog signaling in prostate development using anti-Shh antibodies, chemical inhibitors of hedgehog signaling and $Shh^{-/-}$ mutant mice have yielded conflicting data regarding the requirements of hedgehog signaling for normal ductal budding and glandular morphogenesis. We used transgenic mouse models in combination with chemical inhibitors and renal grafting to clarify the role of Hh signaling in prostate development. These studies showed that genetic loss of Shh is accompanied by an up-regulation of *Indian Hedgehog* (*Ihh*) and maintenance of Hh pathway activity. We found that while neither *Gli1* nor *Gli3* are required for normal prostate ductal budding, the urogenital sinus (UGS) of the $Gli2^{-/-}$ mutant mouse displays aberrant ductal budding in utero. When grown as a subcapsular graft, the $Gli2^{-/-}$ UGS exhibited prostatic differentiation but also displayed areas of focal epithelial hyperplasia. Functional redundancy between the three *Gli* transcription factors appears to mitigate the effect of *Gli2* LOF as evidenced by residual Hh pathway activity in the E14 $Gli2^{-/-}$ UGS that could be inhibited by cyclopamine treatment. Together, these studies reveal a surprising degree of functional redundancy operating both at the level of the ligand and at the level of transcriptional regulation that effectively mitigates phenotypes associated with Hh-signaling perturbations.

Keywords: Sonic Hedgehog; Indian Hedgehog; Prostate development; Gli1; Gli2; Gli3; Budding; Differentiation; UGS; Functional redundancy

Introduction

Sonic hedgehog (*Shh*), a homolog of the *Drosophila hedgehog* gene, encodes a secreted peptide that exerts its effects by activating gene transcription in adjacent target cells (McMahon, 2000). Binding of Shh peptide to the integral membrane receptor *Patched* (*Ptc*) on target cells initiates an intracellular signal transduction cascade activating expression of Hh target genes through the Gli family of transcription factors (Johnson and Scott, 1998; Ingham and McMahon, 2001). The importance of Hh signaling during development has been reported in many systems including the lung (Litingtung et al., 1998), gastrointestinal tract (Roberts et al., 1998), developing CNS (Echelard et al., 1993), and developing limbs (Pagan et al., 1996). In the developing prostate, *Shh* is expressed specifically in the epithelium of the urogenital sinus (Podlasek et al., 1999). Expression is increased late in gestation and localizes to the nascent ductal buds of the newborn prostate. *Shh* expression declines gradually after birth and is maintained at a low level in the adult prostate (Lamm et al., 2002).

Functional studies of Hh signaling in prostate development have used antibody blockade, chemical inhibition, and genetic loss-of-function (LOF) models (Berman et al., 2004; Freestone et al., 2003; Lamm et al., 2002; Podlasek et al., 1999). These studies have yielded conflicting data on the requirement of Shh for normal prostate development. Antibody blockade using a polyclonal antibody to Shh blocked prostate development in a sub-capsular renal graft model (Podlasek et al., 1999). Chemical inhibition of Hh-signaling with cyclopamine has produced a variety of results, including inhibition of ductal budding (Lamm et al., 2002), altered ductal bud morphology (Berman et al., 2004), increased ductal branching (Freestone et al., 2003) and altered epithelial differentiation (Wang et al., 2003).

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Studies using the $Shh^{-/-}$ transgenic mouse, however, showed that the UGS from the Shh mutant could undergo budding morphogenesis in organ culture and, when transplanted under the renal capsule of an adult male host mouse, could undergo glandular morphogenesis with normal prostate morphology (Berman et al., 2004). In these studies, however, it is important to note that while cyclopamine globally blocks Hh signal transduction, functional compensation may occur in transgenic mice lacking a single Hh ligand.

The transcriptional response to Hh signaling is mediated by three closely related Gli proteins: Gli1, Gli2 and Gli3. Gli1 and Gli2 are thought to be transcriptional activators while Gli3 acts primarily as a transcriptional repressor and may serve to balance and refine transcriptional activation by Gli1 and Gli2. In the absence of Shh, Gli3 is cleaved to a truncated form that functions as a transcriptional repressor. Hh signaling prevents cleavage of Gli3 to the repressor form thus permitting the full length, unprocessed Gli3 to participate in transcriptional regulation (Dai et al., 1999). Localization studies of *Ptc* and *Gli* gene expression in the developing prostate by whole mount in situ hybridization showed *Gli1*, *Gli2* and *Gli3* expression localized primarily to mesenchyme surrounding the epithelial buds—consistent with the concept that *Shh* is an epithelial signal which activates *Gli* expression in adjacent mesenchyme (Lamm et al., 2002; Pu et al., 2004). However, lower level expression of several *Gli* genes is also present in the epithelium of the UGS (Lamm and Bushman, 2005; Pu et al., 2004; Gao et al., 2005). Studies in several systems suggest a functional overlap in the activities of the *Gli* transcription factors—indicating potential for redundancy in Gli-mediated gene activation in addition to potential redundancy at the level of the ligand.

Our studies demonstrate that functional redundancy of Hh signaling exists both at the level of the Hh ligand and in downstream transcriptional activation by the *Gli* transcription factors during prostate development. The consequence of this is that neither genetic loss of Shh function nor disruption of a single Gli gene is adequate to completely disrupt Hh signaling. While both of these redundancies are not novel findings in the broader sense, their establishment in the context of prostate development will help in sorting out previously conflicting reports regarding the role of Hh signaling in the prostate.

Materials and methods

Tissues

Animal procedures were performed in accordance with the guidelines of the University of Wisconsin-Madison's Animal Care and Use Committee. $Gli1^{+/-}$ and $Gli2^{+/-}$ mice were generously provided by Alexandra Joyner and maintained on an outbred CD-1 background. $Gli3^{+/xt}$ mice were obtained from Jackson Laboratories and were maintained on a C57/C3H background. Timed pregnant females were generated in-house by crossing heterozygous males to heterozygous females. Adult male CD-1 nude mice were purchased from Charles River Laboratories. Mice were sedated with halothane, euthanized by cervical dislocation, and urogenital sinuses and prostates were harvested by dissection in Dulbecco's PBS (Invitrogen). For microdissection experiments, collagenase was added to PBS to facilitate quantitative counts of bud tips/branch points (Lamm et al., 2001). Tissue samples were either snap frozen in liquid nitrogen and stored at −80°C for RNA isolation or fixed and stored in 10% formalin for immunohistochemistry.

RNA isolation and RT-PCR

Frozen tissue specimens were homogenized in lysis buffer containing molecular grinding resin (Geno Technology Inc) and total RNA was isolated with RNeasy columns (Qiagen) according to the manufacturer's recommendations. Isolated RNA was subjected to DNase treatment and reverse-transcribed using standard protocols. Real-time RT-PCR was performed with gene-specific primers (Table 1) and mRNA content was normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. Products were detected by SYBR green fluorescence using an iCycler thermocycler (Bio-Rad Laboratories). For each experimental replicate, PCR reactions were run in duplicate and values were then averaged.

Whole mount in situ hybridization

In situ hybridization of 4% paraformaldehyde fixed urogenital tissues was performed according to previously described protocols with a few modifications (Wilkinson et al., 2002; Lamm et al., 2002). Briefly, the tissue was bleached for 1 h in 6% hydrogen peroxide then treated with 50 μg/ml proteinase K for 30 min. The tissue was re-fixed in 0.2% gluteraldehyde/4% paraformaldehyde then pre-hybridized at 68°C on a rotator for 1 h. Hybridization was performed at 68°C overnight. Five high stringency post hybridization washes were followed with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) overnight incubation at 4°C. The 6–8 h color reaction was developed using NBT and BCIP

Table 1
Gene-specific primers used for RT-PCR

Gene symbol	Gene name	Forward primer sequence	Reverse primer sequence
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AGC CTC GTC CCG TAG ACA AAA T	CCG TGA GTG GAG TCA TAC TGG
Shh	Sonic hedgehog	AAT GCC TTG GCC ATC TCT GT	GCT CGA CCC TCA TAG TGT AGA GAC T
Ihh	Indian hedgehog	GAG CTC ACC CCC AAC TAC AA	TGA CAG AGA TGG CCA GTG AG
Ptch1	Patched homolog 1	CTC TGG AGC AGA TTT CCA AGG	TGC CGC AGT TCT TTT GAA TG
Hhip	Hedgehog-interacting protein	CCT GTC GAG GCT ACT TTT CG	TCC ATT GTG AGT CTG GGT CA
Gli1	GLI-Kruppel family member GLI1	GGA AGT CCT ATT CAC GCC TTG A	CAA CCT TCT TGC TCA CAC ATG TAA G
Gli2	GLI-Kruppel family member GLI2	CCT TCT CCA ATG CCT CAG AC	GGG GTC TGT GTA CCT CTT GG
Pbsn	Probasin	GGA GGA GAT GAG GGA GTT CA	ACA GTT GTC CGT CTG CAT GA
Myh 11 (HCM)	Myosin, heavy polypeptide 11, smooth muscle	GCT CCA AGG ATG ATG TAG GC	TCT CTT CCA TCT GGG TCT CC
Nkx3.1	NK-3 transcription factor, locus 1	GAA AGC AGC TGT CGG AAG AC	ACA CGG AGA CCA AGG AGG TA
Nes	Nestin	GGA CAG GAC CAA GAG GAA CA	TCT CGA TCC ACC TTT TCT GG
Bmi1	B lymphema Mo-MLV insertion region 1	ATG AGT CAC CAG AGG GAT GG	AAG AGG TGG AGG GAA CAC CT
Krt1-14 (CK14)	Keratin complex 1, acidic, gene 14	CAA GGA TGC TGA GGA ATG GT	CCG GAG CTC AGA AAT CTC AC