Molecular analysis of coordinated bladder and urogenital organ formation by Hedgehog signaling

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The urogenital and reproductive organs, including the external genitalia, bladder and urethra, develop as anatomically aligned organs. Descriptive and experimental embryology suggest that the cloaca, and its derivative, the urogenital sinus, contribute to the formation of these organs. However, it is unknown how the primary tissue lineages in, and adjacent to, the cloaca give rise to the above organs, nor is bladder formation understood. While it is known that sonic hedgehog (Shh) is expressed by the cloacal epithelium, the developmental programs that regulate and coordinate the formation of the urogenital and reproductive organs have not been elucidated. Here we report that Shh mutant embryos display hypoplasia of external genitalia, internal urethra (pelvic urethra) and bladder. The importance of Shh signaling in the development of bladder and external genitalia was confirmed by analyzing a variety of mutant mouse lines with defective hedgehog signaling. By genetically labeling hedgehog-responding tissue lineages adjacent to the cloaca and urogenital sinus, we defined the contribution of these tissues to the bladder and external genitalia. We discovered that development of smooth muscle myosin-positive embryonic bladder mesenchyme requires Shh signaling, and that the bladder mesenchyme and dorsal (upper) external genitalia derive from Shh-responsive peri-cloacal mesenchyme. Thus, the mesenchymal precursors for multiple urogenital structures derive from peri-cloacal mesenchyme and the coordination of urogenital organ formation from these precursors is orchestrated by Shh signals.

KEY WORDS: Urinary bladder, External genitalia, Cloaca, Internal urethra, Pelvic urethra, Shh (sonic hedgehog), Gli, PCM (peri-cloacal mesenchyme), Smooth muscle, Exstrophy, Mouse

INTRODUCTION

During mouse development, a transient embryonic cavity called the cloaca forms at the caudal end of the hindgut and is subsequently divided into the urogenital sinus and rectum (de Santa Barbara and Roberts, 2002; Kimmel et al., 2000; Kondo et al., 1996; Penington and Hutson, 2003; Yamada et al., 2003; Yamada et al., 2006). It has been suggested that the cloaca/urogenital sinus and adjacent tissues differentiate into the urogenital and reproductive organs, including the urinary bladder (hereafter described as the bladder) in both sexes. This is supported by reports of complex congenital malformations such as: exstrophy-epispadias complex (exstrophy of cloaca or bladder and abnormal dorsal external genitalia; hereafter, dorsal external genitalia represents upper external genitalia); defective body wall, bladder and genitalia formation; and limb body wall defects that are probably caused by abnormalities in early development of the cloaca and urogenital sinus (Craven et al., 1997; Manner and Kluth, 2005; Penington and Hutson, 2003). The constellation of defects reported in affected individuals suggests that coordinated developmental programs regulate bladder and external genital morphogenesis. However, the signaling and structural contributions of the transient embryonic cloaca to formation of multiple urogenital and reproductive organs inside and outside the pelvic cavity remain obscure.

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MATERIALS AND METHODS

Transgenic and targeted mouse alleles
The Shh, Gli1 and 2, and Xr (Gli3 – Mouse Genome Informatics) mutant alleles employed here were previously described (Chiang et al., 1996; Ding et al., 1998; Hui and Joyner, 1993; Motoyama et al., 1998; Park et al., 2000), as was the Gli1-CreER<sup>2</sup> mouse line (Ahn and Joyner, 2004; Ahn and Joyner, 2005). The Del5-LacZ reporter transgenic construct included an HH-responding regulatory element (Sasaki et al., 1997) consisting of eight synthetic ‘del-5’ sequences that each contain a Gli-binding site (TTATGA CCGAGG CTAACA AGCAGG ACAAGT AGAAGC TGGCTG TC). This element was cloned adjacent to the basal gamma-crystalline promoter and inserted 5’ to the β-galactosidase coding sequence (Sasaki et al., 1997) (see Fig. 5A). This construct (total length 5.2 kb) was microinjected into fertilized C57BL/6 oocytes to generate transgenic animals using standard procedures (Sasaki and Hogan, 1996). For sampling, pregnant females were sacrificed from embryonic day (E) 10.5 to newborn, and the urogenital and reproductive tissues were examined (Haraguchi et al., 2000).

Histology

Mouse embryos were fixed overnight in 4% paraformaldehyde (PFA) or PBS, dehydrated through methanol, embedded in paraffin, and 8 μm serial sections were prepared. Hematoxylin and Eosin (HE) staining were processed by standard procedures (Haraguchi et al., 2000).

Immunohistochemistry and X-Gal staining

Immunohistochemical analysis was performed by standard procedures using the following antibodies: anti-AR (Androgen receptor) Ab (1:100; Santa Cruz, N-20) or anti-SMM (smooth muscle myosin) Ab (1:200; Biomedical Technology BF-562). β-Galactosidase activity was detected with previously described methods (Suzuki et al., 2003). X-Gal-stained embryos were postfixed with 4% PFA at 4°C overnight, embedded in paraffin, and 8 mm serial sections were counterstained with Eosin. Newborn animals were fixed in 0.2% PFA at 4°C overnight, embedded in paraffin, and 8 mm serial sections were prepared. Hematoxylin and Eosin (HE) staining were processed by standard procedures (Haraguchi et al., 2000).

In situ hybridizations for gene expression analyses

In situ hybridizations for gene expression analyses were performed as previously described (Suzuki et al., 2000). The antisense riboprobe templates for Ptc1 (Ptc1 – Mouse Genome Informatics), Gli1 and Shh were as described by previous authors (Ding et al., 1998; Haraguchi et al., 2001; Motoyama et al., 1998).

Lineage analysis of HH-responding tissues

Tissue lineage analyses were conducted by analyzing Gli1-CreER<sup>2</sup>;R26R mice (Ahn and Joyner, 2004; Ahn and Joyner, 2005). The Gli1-CreER<sup>2</sup> mice were crossed with R26R-LacZ indicator mice (Soriano, 1999) to obtain Gli1-CreER<sup>2</sup>+/R26R/R26R males, which were subsequently crossed with ICR females. Noon of the day of vaginal plug was designated as E0.5. A 20 mg/ml stock solution of tamoxifen (T-5648, Sigma) was prepared in corn oil. Two milligrams of tamoxifen per 40 g body weight was administered to the pregnant ICR mouse females using oral gavage at E8.75–9.0 to label the PCM region. Mouse embryos and newborn animals were processed for whole-mount or section X-gal staining. No overt teratologic effects were visible, although it is well developed in controls at this stage (Fig. 2).

RESULTS

Expression analysis of Shh, Ptc1 and Gli1 during urogenital organ formation

We examined histogenesis during formation of the GT, urethra, lower body wall and bladder and also assayed expression of members of the Shh signaling cascade during this period (Figs 1, 2).

At E10.5 (hereafter E represents embryonic day post coitum), the developing cloaca and caudal hindgut are apparent (Fig. 1A,D). By E11.5, the GT is observed adjacent to the cloaca as a prominent protrusion (Fig. 1B,E). Continued growth of the GT is accompanied by the development of the urethral plate (the primordia of the external urethra) and the bladder at E13.5 (Fig. 1C,F). At E10.5-13.5, prominent Shh expression was observed in the epithelium lining the cloaca, the urethral plate, the internal urethra and the luminal epithelia of the bladder (Fig. 2A-C). By assaying adjacent sections, we observed complementary expression of the HH receptor Ptc1, and HH-intracellular effector Gli1 in the PCM (Fig. 2D,G; yellow box). This finding is consistent with an epithelial-mesenchymal interaction between the cloacal epithelium and the PCM in a putative ‘cloacal field’ (Yamada et al., 2006). From E11.5 to E13.5, the Shh expression domain expanded further and included the developing urethral plate and the epithelia lining the internal urethra and bladder (Fig. 2B,C; note continuity of Shh expression in these epithelial structures). Widespread Ptc1 and Gli1 expression were detected in the mesenchyme adjacent to these epithelia (Fig. 2E,F,H,I).

Hypoplasia of urethral plate, internal urethra and bladder wall in Shh and Gli1 mutant mice

To examine how Shh signaling influences the histogenesis of the aforementioned structures, Shh null mutant embryos were examined (Fig. 3). At E13.5, the GT was hypoplastic and the UP was not visible, although it is well developed in controls at this stage (Fig.
The bladder cavity and surrounding mesenchyme were hypoplastic (Fig. 3D; blue arrow). The normal angle made by the connection between the internal urethra and the bladder (the internal urethral orifice) was apparent in wild-type embryos but was not observed in Shh null mutant mice [already prominent at early stages (Fig. 3A,B) and also clear at late stages in wild-type embryos (Fig. 3E; data not shown)]. The curved course of the internal urethra from the internal urethral orifice, under the pubic bone, to the GT is apparent in wild-type embryos (Fig. 3E, green arrows). By contrast, in Shh mutants we observed a simple bladder-like lumen attached to a rudimentary tube that extended toward the remnant of the GT (Fig. 3G). In humans, the ventral bladder is composed by two inferi- or lateral surfaces facing the pubic bones and external genitalia. Although the frontal (ventral) anatomical structures of the mouse bladder are not prominently developed, mesenchymal hypoplasia was very prominent in the frontal regions of the bladder of Shh null mutants (Fig. 3; data not shown).

HH signaling is mediated in part by the Gli family of transcription factors (composed of three members, Gli1, Gli2 and Gli3 in mammals), and studies in Gli mutants have revealed essential roles for both positive and negative transcriptional regulation mediated by these factors during normal embryogenesis (Blaess et al., 2006; Ding et al., 1998; Haraguchi et al., 2001; Hardcastle et al., 1998; Hui and Joyner, 1993; Matise and Joyner, 1999; Mo et al., 1997; Motoyama et al., 1998). During urogenital tissue formation (E10.5-13.5), Gli2 and Gli3 expression was detected in the GT and bladder mesenchyme (Haraguchi et al., 2001) (R.H. and G.Y., unpublished). To confirm a role for HH signaling in urogenital tissue development, we examined these structures in Gli1, Gli2 and Gli1;Gli2 compound mutants (Fig. 4). Gli2+/− mutants have hypoplasia of the external genitalia, internal urethra and bladder (Fig. 4B). Although Gli1−/− mutant mice were phenotypically normal (data not shown), additional loss of function of a single allele of Gli2 in a Gli1 homozygous mutant (Gli1−/−;Gli2+/−) resulted in similar urogenital malformations to those observed in Gli2−/− mutants (Fig. 4C). Previous studies of Gli compound mutants demonstrated that Gli family members have redundant functions in organogenesis (Bai et al., 2004; Hardcastle et al., 1998; Mo et al., 1997; Motoyama et al., 1998; Park et al., 2000). Our findings reveal that such redundancy also occurs during development of the urogenital tissues. In summary, mouse mutants lacking either the Shh ligand or HH transcriptional effectors display similar defects in external genitalia, internal urethra and bladder formation.

Localizing activity of the HH pathway in vivo using reporter mouse strains

Next, we employed a new reporter mouse strain to localize active HH signaling in vivo (Fig. 5). We developed an HH-responsive, LacZ marker strain called del5-LacZ (see Materials and methods) employing a Gli-responsive binding site that had been previously identified in the 5′ flanking upstream sequence of the Foxa2 gene.
Deletion analyses on the upstream sequences of Foxa2 gene revealed the sequences to detect HH signaling using in vitro systems (Sasaki et al., 1997) (H.S., unpublished). Based on such studies, an HH-responsive transgenic mouse line was generated, employing this Gli-responsive binding site (this study and J.M. and H.S., unpublished). Here, we assayed the efficacy of this reporter as a readout of intracellular HH by examining LacZ activity in the E11.5 limb and neural tube (Fig. 6), both embryonic regions of HH activities (McMahon et al., 2003) (Fig. 6A,C). Furthermore, as would be predicted, del5-LacZ activity was increased in limb buds lacking the Gli3 repressor (genotype: del5-LacZ/+;Xt/Xt, Fig. 6B) and was reduced in the neural tube and notochord of Shh null mutants (Fig. 6D).

We employed the del5-LacZ reporter mouse to assay for Gli-mediated transcriptional activation in the developing bladder, PCM and urethral plate. The mesenchymal LacZ staining observed in the del5-LacZ reporter mouse was quite specific and restricted to include the PCM adjacent to the cloaca at E10.5 (yellow box in Fig. 5B), and the mesenchyme surrounding the bladder at E12.0 and E13.5 (Fig. 5C,D). There was also prominent LacZ staining of the UP at E13.5, but not at earlier stages. The size of these del5-LacZ-positive domains was significantly reduced in Shh null mutants at both E11.5 and E13.5, (Fig. 7B,D). We attribute the residual signal detected in these regions to the other HH ligands, e.g. Ihh expression in the cloaca (data not shown).

We also noted that the intensity of del5-LacZ staining varied with the embryonic stage and tissue examined: the LacZ signal was faint in the PCM at E11.5 but quite robust in the bladder mesenchyme by E12-13.5 (Fig. 5C,D, white arrow), whereas no staining was detected in the GT mesenchyme at these stages (black arrows; see Discussion). Thus, the pattern of del5-LacZ expression is restricted within the broader Ptc1 and Gli1 expression domains in urogenital tissues at multiple stages (compare Fig. 5B with Fig. 2D,G and Fig. 5D with Fig. 2F,I; expression data not shown for E9.5). As our in vitro and in vivo data showed that the expression regulated by the del5 sequence reflects intracellular HH signaling (Sasaki et al., 1997), it is likely that restricted HH signaling revealed by the del5-LacZ staining data reflects the net of positive and negative Gli activity in this region. In fact, we detected Gli3 repressor expression during urogenital tissue development (data not shown).

**Analysis of the contribution of HH-responding urogenital tissues utilizing tamoxifen-inducible tissue labeling**

An important feature of urogenital and reproductive organ formation is the dynamic transition of the initial epithelial lined cavity (the cloaca and urogenital sinus) into bladder, GT, urethral plate and urethra. In order to follow the tissue lineages that respond to HH signals at different time points and locations during development of these structures, we used an inducible genetic labeling system. Taking advantage of the regulation of the activity of the Gli1 locus by HH signaling, the Joyner lab generated a unique HH-responding tissue lineage analysis system by targeting a tamoxifen-inducible Cre recombinase to the Gli1 locus (Gli1-CreERT2; see schematic in Fig. 8) (Ahn and Joyner, 2004). This system has been shown to be effective for analyzing the tissue lineage of HH-responding cells during limb formation and central nervous system development (Ahn and Joyner, 2004; Ahn and Joyner, 2005; Harfe et al., 2004).

Based on our data documenting the histogenesis of the cloaca and urogenital sinus and Shh expression by this tissue, we were interested in defining the contribution of early HH-responsive cells to the bladder, urethra and external genitalia. We therefore utilized tamoxifen to induce activity of Cre protein present at E8.75-9.0 (expressed from the Gli1-CreER2 allele) and then assayed LacZ activity from the Rosa26 Cre reporter (Ahn and
Joyner, 2004; Ahn and Joyner, 2005) at E10-11 and at later stages. Under these temporal conditions, we detected significant LacZ staining in the PCM [Fig. 8A,B; the LacZ signals in the yellow region correspond to the \textit{Ptc1} and \textit{Gli1} expression domains in Fig. 2D,G; this point is also suggested by the earlier expression of \textit{Ptc1} and \textit{Gli1} (data not shown)].Remarkably, assaying for LacZ activity at E13.5 revealed that ‘early-onset and long-contributing’ lineages in the PCM make a dynamic contribution to the dorsal (upper) GT and the bladder (Fig. 8C,D). This developmental LacZ transition from PCM to GT and bladder suggests a coordinated developmental formation of these organs for the first time (see below).

**Marker analysis of bladder smooth muscle and dorsal GT mesenchymal differentiation**

One of the key characteristics of bladder mesenchyme differentiation is the expression of smooth muscle markers (Baskin et al., 1996; Yamada et al., 2006). We assayed the LacZ-positive bladder mesenchyme in \textit{Gli1-CreER\textsuperscript{T2}} reporter embryos (tamoxifen induced at E8.75-9.0) for smooth muscle myosin production. Co-staining of the mesenchyme in the bladder wall at E14.5 confirms that these smooth muscle myosin-positive tissues derive from early HH-responsive lineages (genetically labeled at E9; co-staining revealed by blue LacZ signals and brown anti-smooth muscle myosin signals, Fig. 9A,B).

The external genitalia undergo hormone-dependent, dimorphic morphogenesis after E16.5, which leads to penis and clitoris formation in males and females, respectively (Cobb and Duboule, 2005; Dolle et al., 1991; Yamada et al., 2003; Yamada et al., 2006). Androgen receptor (AR) plays a fundamental role in directing the GT toward penis formation, including tubular urethra formation from the UP. AR gene is prominently expressed in male GTs later than E16.5 and also its expression is detected in the hormone-independent early phase of GT formation (R.H. and G.Y., unpublished). Thus, we examined mesenchymal AR expression in the GT mesenchyme of LacZ-stained, tamoxifen-induced HH reporter mice. At E14.5, co-staining for LacZ and AR in the dorsal GT mesenchyme reveals a developmental link between the AR-expressing dorsal GT and early HH-responding tissues derived from the PCM (Fig. 9C,D). This dorsal GT mesenchyme probably contributes to corporal bodies and penile bones (Yamada et al., 2006) (Fig. 8E). LacZ staining of the dorsal GT and bladder mesenchyme in \textit{Gli1-CreER\textsuperscript{T2}} reporter newborns that were tamoxifen-induced at E8.75-9.0 also supports the contribution of early HH-responsive PCM to these structures (Fig. 8E-G).

To further confirm these results, we assayed the effects of reduced \textit{Shh} gene dosage on the contribution of HH-responsive tissues to the GT and bladder in compound mutants (\textit{Shh}\textsuperscript{-/-}; \textit{Gli1-CreER\textsuperscript{T2}}; R26R/+, Fig. 9E-J) and noted a significant reduction in
DISCUSSION
Participation of HH signaling in bladder, urethra (external and internal) and external genitalia formation

The current analyses represent the first demonstration of the essential functions of HH signaling for bladder formation and the important structural contributions made by HH-responsive tissues to the bladder, urethra and dorsal external genitalia (often termed the BWBG region: body wall, bladder and genitalia).

The urogenital and reproductive tissue primordia ultimately form the organs necessary for uresis, ejaculation and sperm intake and urino-retention. How PCM region (partly mentioned as infra-umbilical mesenchyme; basically locating in the upper part of the cloacal field) could contribute to various urogenital tissues has remained a mystery for decades (Mildenberger et al., 1988). By using a tamoxifen-inducible genetic lineage system to label HH-responding tissues, we have been able to define the temporally distinct contributions of different HH-responding lineages to the complex urogenital organs (Fig. 9K). Labeling at E8.75-9.0 revealed that early HH-responsive tissues contribute first to the PCM and later, to the dorsal GT and bladder mesenchyme. Of the mesenchyme derived from the PCM, the dorsal GT mesenchyme displayed sexual dimorphism at late embryonic stages but bladder mesenchyme did not.

The tamoxifen-inducible labeling system described by Ahn et al. is thought to label cells within approximately one day of tamoxifen injection. In our tamoxifen/Gli1-CreERT2 labeling experiments, the LacZ signal was relatively weak in the PCM compared with the prominent staining and contribution of these cells to bladder mesenchyme and the dorsal GT. We suggest that the initial cell population within the PCM undergoes transient expansion by proliferation (data not shown). Our data obtained with the HH-responsive marker allele, del5-LacZ, also suggested very dynamic urogenital organ formation, consistent with these findings; in this system, LacZ expression is directly regulated by the HH-inducible promoter in vivo (not from the Rosa locus). In fact, the latter approach revealed prominent HH signal activation in the bladder mesenchyme, but not in the dorsal GT, at E12-13. Overall, these data illustrate the varying level of participation of HH-responsive tissues in specific aspects of urogenital organ development at different stages.

Although these data provided compelling evidence for the temporally dynamic generation of individual urogenital and reproductive tissues from HH-responsive cells, the precise source and identity of HH ligands that are required (including Shh and Ihh) and their specific targets will require further investigation, as determining the extent of redundancy between Shh and Ihh. We have found that the PCM adjacent to the cloacal epithelium is positive for SHH protein by immunostaining (data not shown). In the bladder, based on the timing of tamoxifen induction and subsequent timing of contribution of LacZ-positive cells to individual regions, most, if not all, of the required HH signaling may arise from the cloaca and urogenital sinus.

Crosstalk between HH and other signaling pathways, such as fibroblast growth factors (Fgfs), bone morphogenetic proteins (Bmps) and Wnts, and integration of these multiple inputs, is likely to be as crucial for regulating normal development of the these tissues as has been proposed in other regions of the developing embryo (Lamm et al., 2001; McMahon et al., 2003; Ovchinnikov et al., 2006; Roberts et al., 1998; Yamada et al., 2006; Zuniga et al., 1999).

Genetic cascades for urogenital organogenesis and insights into human birth defects

Orchestration of complex urogenital organ formation is one of the key events in embryogenesis. How the PCM contributes to multiple urogenital tissues has remained a mystery for decades. Our
demonstration of the contribution of early PCM to different urogenital tissues, including the bladder and external genitalia, is a unique finding that supports previous speculation in medical embryology text books (Carlson, 1994; Larsen, 1997; Moore, 1998).

In human teratology, abnormal human phenotypes reflecting simultaneous dysgenesis of the bladder and external genitalia have been described. Affected human phenotypes often display exstrophy of the bladder, epispadias, or ‘flattened or hypoplastic external genitalia’, which are intriguingly similar to phenotypes in the mutants analyzed here. These complex congenital defects prompted the idea of coordinated urogenital organ development. However, there has previously been little experimental exploration of the tissue lineages or signaling pathways involved that would provide a mechanistic basis for such a hypothesis.
Urethral birth defects are a frequent congenital defect observed in humans, and some publications suggest that their incidence may be increasing in certain geographic regions (Baskin, 2004). Moreover, significantly higher frequency of such birth defects in male infants is reported (Larsen, 1997; Moore, 1998). Defects of the dorsal external genitalia include epispadias with urethral defects and various dorsal external genitalia anomalies with relatively low frequency (Jeffer, 1987). Birth defects affecting the bladder, such as bladder extrophy with associated malformation of the urethra has been reported in 1 of about 30,000-50,000 births (Cheng et al., 2006; Jeffer, 1987). Affected children often require multiple reconstructive surgical procedures (Jeffer, 1987; Johnston, 1975), increasing the impetus for research into the developmental bases of normal and abnormal urogenital organogenesis.

Another point for consideration is the lack of understanding of the embryonic mesenchymal precursors of the bladder walls. Improving our knowledge in this regard is vital to support medical efforts in bladder reconstruction. In fact, current bladder reconstruction approaches often utilize other endodermal organs, such as the ileum and the colon as the source of autograft tissue to construct bladder-like reservoirs (Staack et al., 2005). Although the development of these endodermally derived tissues was also influenced by Shh, the ultimate structure and physiological character of mature gut and bladder are obviously significantly different. Hence, multiple complications, including electrolyte imbalance and stone formation were observed after the use of gastrointestinal mucosa or mesenchyme for bladder repair (Staack et al., 2005). Therefore, elucidation of the developmental mechanisms driving embryonic mesenchyme toward functional bladder tissues is likely to have significant impact when translated to the medical arena. Although further studies are necessary, our work has provided insight into the functions of Shh signaling during embryonic bladder formation. The current findings of the importance of HH signaling and the structural roles of HH-responsive tissues during urogenital organogenesis may not only provide clues to understanding the basis of human urogenital birth defects, but may also stimulate translational research that can have an impact on the management of these defects.

References


