Functional and phylogenetic analysis shows that *Fgf8* is a marker of genital induction in mammals but is not required for external genital development

Ashley W. Seifert[1], Terry Yamaguchi[2] and Martin J. Cohn[1,3,*

In mammalian embryos, male and female external genitalia develop from the genital tubercle. Outgrowth of the genital tubercle is maintained by the urethral epithelium, and it has been reported that *Fgf8* mediates this activity. To test directly whether Fgf8 is required for external genital development, we conditionally removed *Fgf8* from the cloacal/urethral epithelium. Surprisingly, *Fgf8* is not necessary for initiation, outgrowth or normal patterning of the external genitalia. In early genital tubercles, we found no redundant Fgf expression in the urethral epithelium, which contrasts with the situation in the apical ectodermal ridge (AER) of the limb. Analysis of Fgf8 pathway activity showed that four putative targets are either absent from early genital tubercles or are not regulated by Fgf8. We therefore examined the distribution of Fgf8 protein and report that, although it is present in the AER, Fgf8 is undetectable in the genital tubercle. Thus, *Fgf8* is transcribed, but the signaling pathway is not activated during normal genital development. A phylogenetic survey of amniotes revealed *Fgf8* expression in genital tubercles of eutherian and metatherian mammals, but not turtles or alligators, indicating that *Fgf8* expression is neither a required nor a conserved feature of amniote external genital development. The results indicate that *Fgf8* expression is an early readout of the genital initiation signal rather than the signal itself. We propose that induction of external genitalia involves an epithelial-epithelial interaction at the cloacal membrane, and suggest that the cloacal ectoderm may be the source of the genital initiation signal.

KEY WORDS: Evolution, Fgf8, Genitalia, Wnt5a, Cloaca

INTRODUCTION

The genital tubercle is the developmental precursor to the male and female external genitalia. Its outgrowth and patterning have been likened to the vertebrate limb bud, but unlike the limb, the genital tubercle is composed of cells from all three germ layers (Seifert et al., 2008). Initiation of genital outgrowth begins at approximately E10.5, when paired genital swellings appear on either side of the cloacal membrane (Perriton et al., 2002). The paired swellings are joined by an anterior swelling a day later and collectively these form the genital tubercle. Signaling between the endoderm of the embryonic cloaca (which gives rise to the urethral epithelium) and the surrounding mesoderm is necessary for outgrowth and patterning of the genital tubercle. Mice lacking both copies of the sonic hedgehog (*Shh*) gene initiate outgrowth of the paired swellings, but these fail to form a genital tubercle and the mice exhibit complete agenesis of the external genitalia (Haraguchi et al., 2001; Perriton et al., 2002). Although Shh is required for the maintenance of genital outgrowth, it is not the early initiation signal.

In the vertebrate limb bud, fibroblast growth factors (Fgfs) 8 and 10 mediate the initiation of budding, and sustained outgrowth of the limb is controlled by Fgf signaling from the apical ectodermal ridge (AER) (Mariani and Martin, 2003; Ng et al., 1999). In the genital tubercle, *Fgf8* is expressed in the distal urethral epithelium (Haraguchi et al., 2000; Perriton et al., 2002). Previous work identified Fgf8 as a genital outgrowth signal and

compared its role to that during limb development (Haraguchi et al., 2000). It was reported that removal of the distal urethral epithelium causes the arrest of genital outgrowth in organ culture, and that application of Fgf8-loaded beads can restore (or at least augment) gene expression and tubercle outgrowth (Haraguchi et al., 2000). Furthermore, treatment of cultured tubercles with Fgf8-neutralizing antibody may inhibit development (Haraguchi et al., 2000). This and subsequent studies led to the suggestion that *Fgf8* expression in the distal urethral epithelium is required for outgrowth of the genitalia (Haraguchi et al., 2000; Haraguchi et al., 2001; Haraguchi et al., 2007; Morgan et al., 2003; Ogino et al., 2001; Perriton et al., 2002; Satoh et al., 2004; Suzuki et al., 2003; Suzuki et al., 2008; Yamada et al., 2006). Although Fgf8 is widely held to be the outgrowth signal, its function in genital development has not been examined genetically, in part because *Fgf8* null embryos die prior to genital tubercle initiation (Meyers et al., 1998). Here we provide a direct test of the hypotheses that *Fgf8* is required for initiation, outgrowth and normal development of the external genitalia.

MATERIALS AND METHODS

Mice

Mouse strains used in this study have been described previously: Shh^{GFPcre} (Harfe et al., 2004); $Fgf8^{fl/fl}$ (Lewandoski et al., 2000), $Fgf4^{fl/fl}$ (Sun et al., 2000) and $Wnt5a^{-/-}$ (Yamaguchi et al., 1999). We generated mice lacking *Fgf8* in the genital tubercle by crossing $Shh^{GFPcre};Fgf8^{fl/+}$ males to $Fgf8^{fl/fl}$ females, and denote these mice as *Fgf8 cKO* (conditional knockout) mutants in the text. In one instance, a *Fgf8 cKO* embryo was recovered from a cross of a $Shh^{GFPcre};Fgf8^{fl/+};Fgf4^{fl/+}$ male to a $Fgf8^{fl/fl};Fgf4^{fl/fl}$ female (*Fgf4* is not expressed in the genital tubercle, and therefore its removal had no effect on the *Fgf8* phenotype). Genitalia of $Shh^{GFPcre};Fgf8^{fl/+}$ and $Wnt5a^{+/-}$ heterozygous animals were phenotypically normal and age-matched littermates with these genotypes were used as controls. Mice were genotyped as described previously (Harfe et al., 2004; Lewandoski et al., 2000; Yamaguchi et al., 1999).

[1]Department of Zoology and [3]Department of Anatomy and Cell Biology, UF Genetics Institute, PO Box 103610, University of Florida, Gainesville, FL 32611, USA. [2]Cancer and Developmental Biology Laboratory, Center for Cancer Research, NCI-Frederick, NIH, Frederick, MD 21702, USA.

*Author for correspondence (e-mail: mjcohn@ufl.edu)

In situ hybridization and analysis of cell death

In situ hybridization was performed as described previously (Perriton et al., 2002). In order to detect *Fgf8* expression in *Sus scrofa* we used a pig-specific probe cloned and kindly provided by Brooke Armfield (NEOUCOM, Rootstown, OH, USA) and Zhengui Zheng (University of Florida, Gainesville, FL, USA); for detection in *Monodelphis domesticus* we used an opossum-specific probe kindly provided by Anna Keyte and Kathleen Smith (Duke University, Durham, NC, USA); for detection in both *Trachemys scripta* and *Alligator mississippiensis* we used an *Fgf8* probe cloned from *T. scripta* and kindly provided by Scott Gilbert (Swarthmore College, Swarthmore, PA, USA). Mouse probes used in this study were *Fgf8* (G. Martin, UCSF, San Francisco, CA, USA), *Shh*, *Wnt5a* (A. McMahon, Harvard University, Cambridge, MA, USA), *Bmp4*, *Etv4*, *Etv5* (B. Hogan, Duke University, Durham, NC, USA), *Dusp6* (J. C. Izpisua Belmonte, Salk Institute, La Jolla, CA, USA), *Spry4* (B. Harfe, University of Florida, Gainesville, FL, USA) and *Hoxd13* (D. Duboule, University of Geneva, Geneva, Switzerland). Cell death was assayed using Lysotracker Red (Invitrogen) according to the manufacturer's protocol.

Immunohistochemistry

Embryos were fixed in 2% paraformaldehyde overnight, washed in PBS, cryoprotected in 30% sucrose and embedded in OCT. Sections were cut at 10 ⬜m. We performed immunohistochemistry as described previously (Thewissen et al., 2006). Briefly, we performed antigen retrieval by autoclaving in sodium citrate, followed by cooling on ice, washing with PBT, quenching of endogenous peroxidase with 2% hydrogen peroxide, blocking in 3% rabbit serum/PBT, and overnight incubation at 4°C with anti-fibroblast growth factor 8 (1:1000, Santa Cruz, sc-6958). Sections were washed in PBT, incubated with Vectastain HRP anti-rabbit ABC kit and detected with activated DAB. In addition, negative controls were performed without a primary antibody.

RESULTS

Fgf8 expression is associated with genital tubercle initiation in *Wnt5a* null mice

In order to address the role of *Fgf8* during genital tubercle outgrowth, we first examined *Wnt5a*$^{-/-}$ mice, in which genital outgrowth is initiated but not maintained (Yamaguchi et al., 1999; Suzuki et al., 2003). Surprisingly, in newborn (P0) male and female *Wnt5a*$^{-/-}$ mice, we found that external genitalia can develop, although with variable penetrance (Fig. 1). Out of 15 *Wnt5a*$^{-/-}$ mice examined at birth, six had external genitalia with varying degrees of outgrowth (Fig. 1). We examined mice maintained on the original C57BL/6 background (Yamaguchi et al., 1999) and on C57BL/6 × CD1 mixed backgrounds. *Wnt5a* mutants on both backgrounds exhibited a similar range of phenotypes, and the phenotypic spectrum did not vary with the genetic sex of the animal (Fig. 1; data not shown). In *Wnt5a* null mice that developed external genitalia, the limbs were positioned normally (Fig. 1C). In the majority of cases in which external genitalia were absent, the hindlimbs were displaced medially (Fig. 1G), and in a few cases were fused into a single structure complete with digits (Fig. 1I). Although medial displacement of the limbs was generally associated with reduction of the genitalia, some *Wnt5a*$^{-/-}$ embryos exhibited complete agenesis of the external genitalia, despite having normally positioned hindlimbs (Fig. 1E).

Given the variable degree of genital outgrowth in *Wnt5a*$^{-/-}$ embryos, we asked whether the extent of outgrowth correlated with *Fgf8* expression in the cloacal epithelium. Examination of *Fgf8* expression and tubercle morphology in *Wnt5a*$^{-/-}$ embryos at E11.5 (when *Fgf8* is expressed in normal embryos) revealed a range of expression patterns consistent with the morphological variation observed in mutants at postnatal day 0 (P0) (Fig. 1D,F,H,J). *Fgf8* expression was detectable in *Wnt5a*$^{-/-}$ embryos that had a tubercle
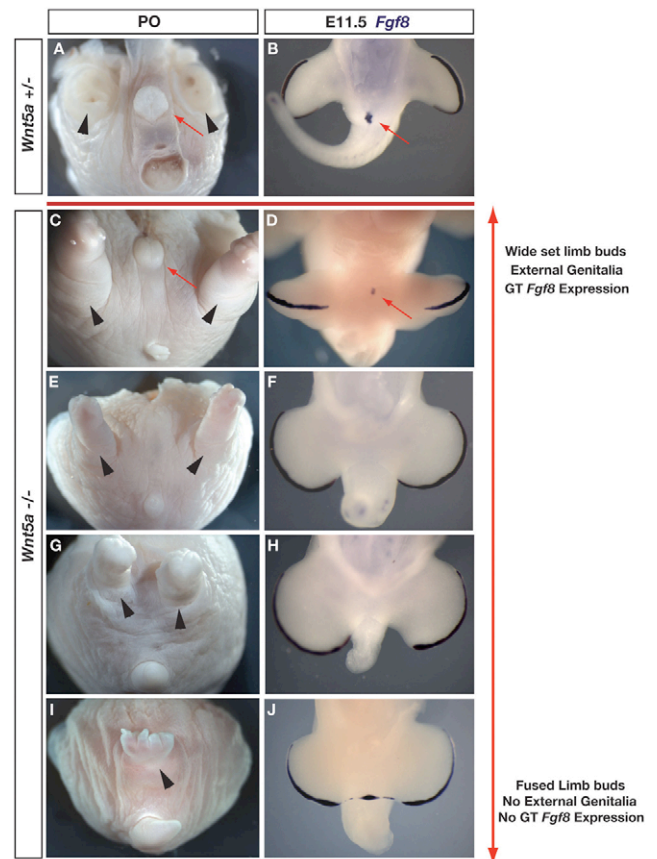

Fig. 1. Variably penetrant development of external genitalia in *Wnt5a*$^{-/-}$ mice correlates with *Fgf8* expression. *Wnt5a*$^{+/-}$ (**A**,**B**) and *Wnt5a*$^{-/-}$ (**C-J**) mutants at P0 (A,C,E,G,I) and E11.5 (B,D,F,H,J). Ventral views; tail is at the bottom of each panel. Red arrows mark genitalia and black arrowheads mark positions of hindlimbs (tail and limbs dissected in A to show genitalia). (A,B) *Wnt5a*$^{+/-}$ mice showing normal position of external genitalia, hindlimbs, anus and tail (A), and normal *Fgf8* expression in the distal genital tubercle (B). (C,E,G,I) *Wnt5a*$^{-/-}$ mice showing the range of phenotypes affecting the hindlimbs and external genitalia, which includes normally positioned hindlimbs and external genitalia (C), normally positioned hindlimbs and no external genitalia (E), medially displaced hindlimbs and no external genitalia (G) and a single fused hindlimb with digits and no external genitalia (I). No anal opening was found in all *Wnt5a*$^{-/-}$ mice examined. (D,F,H,J) Examination of *Fgf8* expression in *Wnt5a*$^{-/-}$ embryos with varying degrees of hindlimb bud displacement shows *Fgf8* expression in the distal genital tubercle (D) and no *Fgf8* expression in mutants lacking genital tubercles (F,H,J). (J) *Fgf8* expression in center of embryo marks continuous AER-like structure across the midline (also see Fig. S1 in the supplementary material).

positioned centrally between the hindlimb buds (Fig. 1D) (Yamaguchi et al., 1999). In other embryos, the hindlimb buds were displaced medially and the tubercle was either absent or severely reduced (Fig. 1F,H,J). In these embryos, *Fgf8* expression was undetectable in the genital region, although expression was robust in the AER (Fig. 1F,H,J). In the most extreme cases, medial displacement of the hindlimb buds was so severe that they appeared to meet in the midline, and in these cases we saw no evidence of genital outgrowth (Fig. 1J). Embryos with such medially displaced hindlimb buds showed a single contiguous AER that expressed *Fgf8* and had a stratified or pseudostratified columnar epithelial character

DEVELOPMENT

(Fig. 1J; see Fig. S1A-C in the supplementary material). In these mutants, the *Shh* domains were also contiguous between the two limb buds. Our finding that *Fgf8* is expressed only in those *Wnt5a* mutants that initiate genital outgrowth raised the possibility that loss of *Fgf8* expression might underlie the absence of external genitalia in the most severely affected mutants.

Fgf8 is induced at the endodermal-ectodermal boundary of the cloacal membrane

In order to conditionally remove *Fgf8* from the genital tubercle, it was first necessary to map the origin of *Fgf8*-expressing cells. Previous reports have localized *Fgf8* expression to the cloacal epithelium prior to genital tubercle outgrowth (Gofflot et al., 1997) and to the distal urethral epithelium following emergence of the tubercle (Haraguchi et al., 2000; Haraguchi et al., 2001; Morgan et al., 2003; Ogino et al., 2001; Perriton et al., 2002; Satoh et al., 2004). However, in the absence of cell lineage data, it has remained unclear to what extent *Fgf8* is expressed in endoderm, ectoderm, or both cell types. We recently reported that the cloacal membrane consists of two lineage-restricted cellular compartments derived from endoderm and ectoderm (Seifert et al., 2008). To determine the origin of the epithelial cells that express *Fgf8*, we used *LacZ* expression in *Shh^{GFPcre}*;*R26R* mice to mark the endodermal compartment, and in situ hybridization to detect *Fgf8* expression (Fig. 2A-C). During initiation and early outgrowth of the genital tubercle, *Fgf8* is restricted to the distal-most endoderm immediately beneath the surface ectoderm. The endodermal expression domain extended up to its boundary with the ectoderm, but the ectodermal cells themselves were negative for *Fgf8* (Fig. 2A,C).

We then mapped the dynamics of *Fgf8* expression beginning at E10, and found that *Fgf8* is not detectable in the cloacal endoderm before the onset of budding, although it was expressed in the endoderm and chordoneural hinge of the tailbud (Fig. 2D, and inset). From E10.5, *Fgf8* is expressed in a broad ventral portion of the cloacal epithelium immediately subjacent to the ectoderm and, as the genital tubercle grows out, the domain becomes restricted to the distal urethral epithelium, disappearing after E14.5 (Fig. 2A,C,E,F) (Perriton et al., 2002). Thus, although *Fgf8* is expressed within some primitive streak cells that give rise to the definitive endoderm (Crossley and Martin, 1995), it does not appear to be expressed continuously. Rather, its expression is switched back on in cloacal endodermal cells at the endoderm-ectoderm boundary of the cloacal membrane during initiation of genital outgrowth, in the cells fated to form the distal urethra (Seifert et al., 2008).

This comparison of *Fgf8* expression and cell lineage shows that *Fgf8* is activated in the cloacal endoderm that makes contact with the ectoderm, and it is therefore possible that *Fgf8* expression is a response to an inductive signal from the adjacent ectoderm. As the embryonic cloaca becomes subdivided into the urogenital sinus (ultimately giving rise to the urethra and bladder) and the anorectal sinus (ultimately giving rise to the rectum and anus), the posterior-ventral endoderm remains in contact with the surface ectoderm at the cloacal membrane (Seifert et al., 2008). If endodermal-ectodermal signaling at the cloacal membrane is required for the activation of *Fgf8*, then one would expect embryos in which the cloacal endoderm fails to contact the ectoderm also to lack *Fgf8* expression. We tested this hypothesis in *Wnt5a^{-/-}* embryos in which anterior-posterior (AP) axis elongation is disrupted, which affects the posterior position of the embryonic cloaca. In order to identify the posterior limit of the gut we used the expression of *Shh* because it labels the entire gut endoderm prior to and during development of the genital tubercle (Fig. 3A-C′). *Shh* also marks the zone of polarizing activity (ZPA) in the limb buds, which provides a morphological landmark for the posterior limit of the hindlimb bud (Fig. 3A-C′). Comparisons of the position of the embryonic cloaca and ZPA at E10.5 in wild-type and heterozygous embryos (which are phenotypically normal) show that the cloacal endoderm is positioned posterior to the ZPA and that *Fgf8* is expressed where the endoderm is in contact with the surface ectoderm (Fig. 3A,A′,D; see Fig. S2 in the supplementary material). In comparison, in *Wnt5a^{-/-}* embryos that initiate genital outgrowth, the cloacal endoderm does not extend as far posteriorly but comes to lie anterior to the ZPA (Fig. 3B,B′; compare position of green and red lines relative to control embryos in Fig. 3A,A′). In these mutants the cloacal endoderm remains in contact with the ectoderm and *Fgf8* is expressed, albeit in a smaller domain (Fig. 3B′,E; compare distance between black arrows in Fig. S2 in the supplementary material). In those *Wnt5a^{-/-}* embryos that fail to initiate genital budding, the gut terminates anterior to the hindlimbs (Fig. 3C), the hindgut endoderm does not reach the cloacal ectoderm (Fig. 3C′; see Fig. S2 in the supplementary material) and *Fgf8* is not expressed (Fig. 3F). Thus, *Fgf8* is expressed in cases in which endodermal-ectodermal contact occurs, whereas in more extreme cases the gut terminates anterior to the cloacal ectoderm and *Fgf8* is not expressed. These findings suggest that during initiation of genital tubercle outgrowth, *Fgf8* expression is switched on only in the endodermal cells that contact cloacal ectoderm, perhaps in response to an ectodermally derived genital initiation signal.
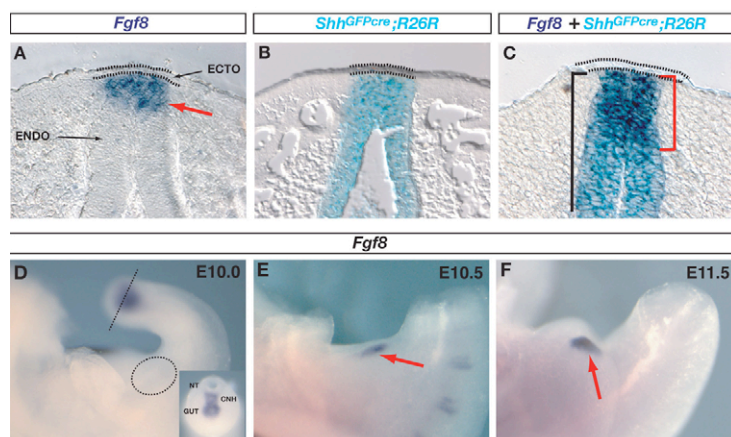

Fig. 2. *Fgf8* expression in normal genital tubercles is restricted to *Shh*-expressing cloacal endoderm. (**A**) *Fgf8* expression (red arrow) in a section of an E11.5 normal embryo is localized exclusively to the cloacal endoderm (ENDO) and is excluded from the ectoderm (ECTO; dotted lines). (**B**) X-Gal-stained *Shh^{GFPcre}*;*R26R* cells are restricted to the cloacal endoderm. (**C**) In situ hybridization and X-Gal staining of an E11.5 genital tubercle shows that *Fgf8* expression (red bracket) is restricted to *Shh^{GFPcre}*-expressing cloacal endodermal cells. Black bracket marks X-Gal-stained endoderm. (**D-F**) Dynamics of *Fgf8* expression during initiation of genital outgrowth. (D) *Fgf8* is not expressed in the cloacal endoderm at E10 (dotted circle), before initiation of genital outgrowth. Expression is detected in the tailbud (inset; position of section indicated by dotted line), where it localizes to the chordoneural hinge (CNH) and tailgut. NT, neural tube. (E,F) *Fgf8* is expressed during initiation (E) and early outgrowth (F) of the genital swellings (red arrows).
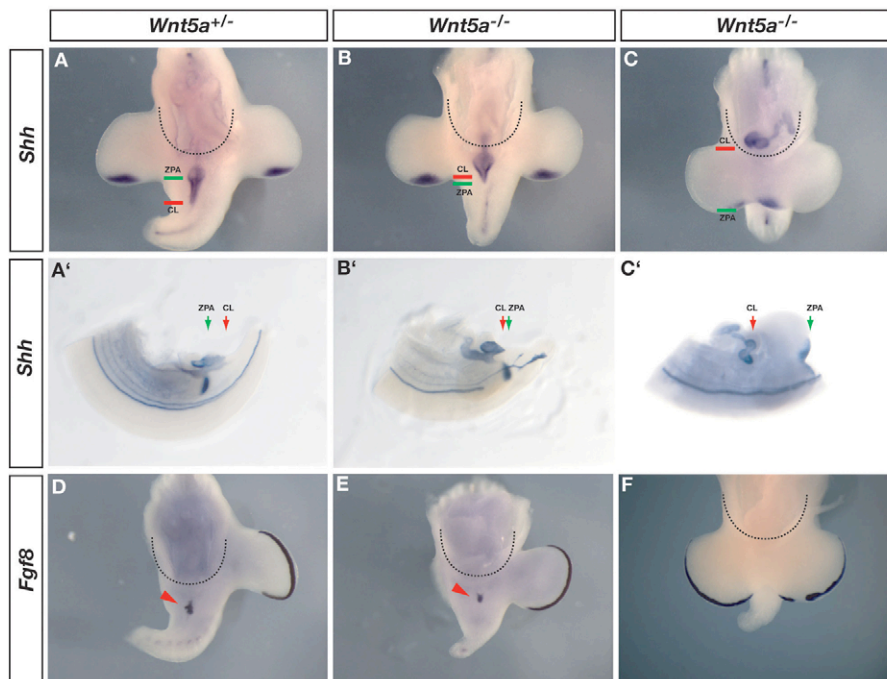
Fig. 3. *Fgf8* **is activated following endodermal-ectodermal contact at the cloacal membrane.** (**A-C'**) In situ hybridization for *Shh* shows the posterior limit of the gastrointestinal tract (cloaca) in *Wnt5a*^{+/–} and *Wnt5a* [–/–] mice. Dotted semicircles mark posterior boundary of the peritoneal cavity. (A'-C') Cleared specimens reveal posterior limit of the embryonic cloaca (CL; red arrows) in relation to the zone of polarizing activity (ZPA; green arrows) of the hindlimb bud. The gut normally extends posterior to the ZPA (A'), but is anterior to the ZPA in some *Wnt5a*^{–/–} mutants (B') and is displaced anterior to the entire hindlimb buds in others (C'; compare position of green and red arrows). The endodermal-ectodermal boundary of the cloacal membrane has not formed in C,C'; also see Fig. S2 in the supplementary material. (**D-F**) Endodermal *Fgf8* expression is detected in *Wnt5a* heterozygous embryos (D; red arrowhead) and in those *Wnt5a* null embryos where the cloacal endoderm is in contact with surface ectoderm at the cloacal membrane (E; red arrowhead). (**F**) *Fgf8* expression is not detected in *Wnt5a*^{–/–} embryos where the endoderm has failed to contact the cloacal ectoderm.

Fgf8 is not necessary for outgrowth of the genital tubercle

The observations that *Fgf8* is expressed in *Wnt5a*^{–/–} embryos that develop external genitalia, and that mutants lacking genital tubercles also lack *Fgf8* expression, led us to ask whether *Fgf8* is required for initiation and outgrowth of the external genitalia. In order to test this hypothesis directly, we conditionally removed *Fgf8* from the cloacal endoderm prior to the initiation of the genital tubercle. Based on our finding that *Fgf8* is expressed exclusively in *Shh*-expressing cells of the urethra, we used *Shh*[GFPcre] to inactivate floxed alleles of *Fgf8* in the cloacal epithelium, and we refer to these mice as *Fgf8 cKO* (conditional knockout; see Materials and methods for details). Mice carrying *Shh*[GFPcre] and two floxed alleles of *Fgf8* underwent initiation of the paired genital swellings and developed a morphologically normal genital tubercle (Fig. 4A). To confirm that this strategy results in the complete deletion of *Fgf8* from the urethral epithelium, we used a riboprobe designed to detect exons 2 and 3, which are internal to the loxP sites in the conditional *Fgf8* allele. Using this deletion probe, *Fgf8* expression is undetectable in the genital tubercles of *Fgf8 cKO* mice before, during and after initiation of outgrowth (Fig. 4A). *Fgf8* expression in the AER, which lacks Cre, served as a positive internal control, and the deletion probe revealed strong expression in the AER. In littermates lacking the *Shh*[GFPcre] allele, the deletion probe showed the normal pattern of *Fgf8* expression in the genital tubercle and in the AER (Fig. 4A). The finding that *Fgf8 cKO* mice form a genital tubercle in the absence of *Fgf8* demonstrates that *Fgf8* is not required for initiation or outgrowth of the genital tubercle.

Does Fgf8 regulate *Bmp4, Wnt5a, Hoxd13* and *Shh* expression in the genital tubercle?

Fgf8 has been placed at top of the genetic cascade that regulates genital tubercle outgrowth, and has been proposed to coordinate gene expression in the mesenchyme flanking the urethral epithelium (Haraguchi et al., 2000; Haraguchi et al., 2001;

Morgan et al., 2003; Perriton et al., 2002; Satoh et al., 2004; Suzuki et al., 2003; Suzuki et al., 2008; Yamada et al., 2006). Manipulations of genital tubercles in organ culture suggested that the expression of *Bmp4, Msx1 Fgf10* and *Hoxd13* is regulated by Fgf8 from the distal urethral epithelium; removal of distal tissue led to varying degrees of downregulation, and implantation of Fgf8 beads augmented their expression (Haraguchi et al., 2000). Interestingly, genital tubercles cultured with an Fgf8-neutralizing antibody maintained normal expression of *Msx1* and *Fgf10*, and *Bmp4* showed only a minimal response (Haraguchi et al., 2000). Thus, it is unclear to what extent gene expression in the genital tubercle is regulated by Fgf8. To test this directly, we collected *Fgf8 cKO* embryos between E11.0 and E12.5 to examine whether deletion of *Fgf8* affects the expression of *Bmp4, Wnt5a, Hoxd13* or *Shh*. In *Fgf8 cKO* embryos, the expression domain of each of these genes in the genitalia was maintained (Fig. 4B), indicating that Fgf8 is not required for the expression of *Bmp4, Wnt5a, Hoxd13* or *Shh* in the genital tubercle. These findings are consistent with the recent report that removal of β-catenin in the cloacal endoderm results in the loss of *Fgf8* expression, but *Hoxa13, Hoxd13, Msx2* and *Wnt5a* expression remains unaffected in the genital tubercle (Lin et al., 2008).

Fgf8 is not required for cell survival in the genital tubercle

Fgf8 functions as a cell survival factor in the limb bud (Sun et al., 2002), and it has been reported that Fgf8 inhibits apoptosis during outgrowth of the genitalia (Suzuki et al., 2003). Cell death is normally detected most prominently in the distal portion of the urethral epithelium and in the adjacent distal mesenchyme (Fig. 4C) (see also Morgan et al., 2003; Perriton et al., 2002; Suzuki et al., 2003). In contrast to the situation in the limb, we did not detect an increase in cell death at the distal tip of the genital tubercle following removal of *Fgf8* (*n*=6; Fig. 4C). Cell death was still observed at the distal tip, although the apoptotic domain appeared smaller than that
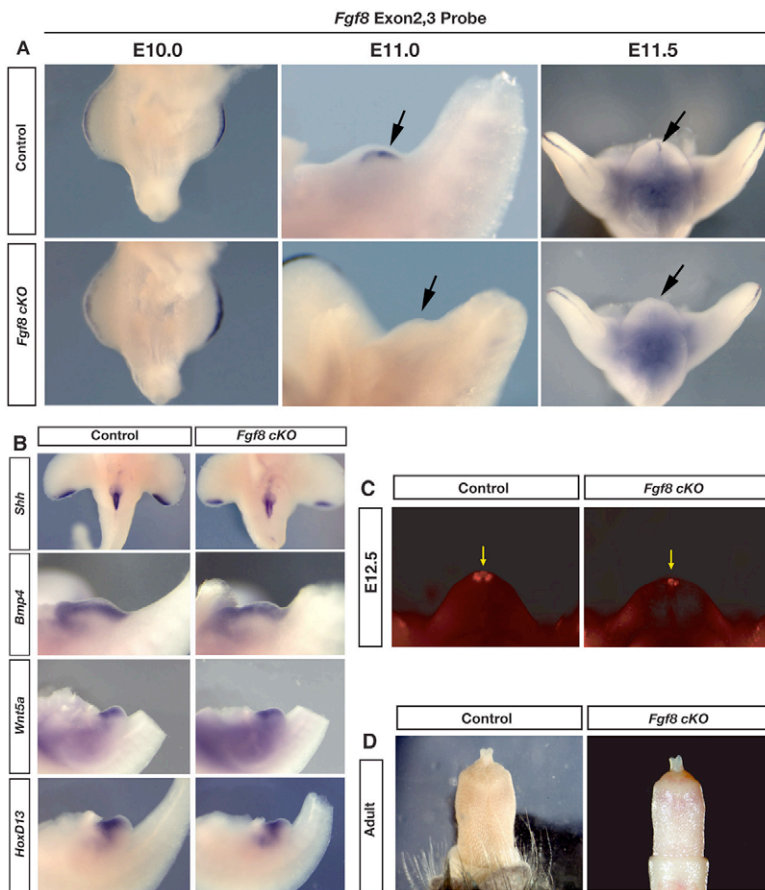
Fig. 4. External genitalia develop normally in the absence of *Fgf8*. (**A**) An *Fgf8* deletion probe specific to the floxed exons 2 and 3 shows absence of expression in both *Fgf8 cKO* and control embryos at E10.0. Expression is absent from the genital tubercle of *Fgf8 cKO* embryos during initiation (E11.0) and early outgrowth (E11.5), but is present in control embryos (arrow). Note expression in the AER of *Fgf8 cKO* embryos. Dark coloration of deep tissue at E11.5 is nonspecific background staining. (**B**) In situ hybridizations of *Fgf8 cKO* and control genitalia showing normal expression of *Shh*, *Bmp4*, *Wnt5a* and *Hoxd13*. (**C**) Analysis of cell death using Lysotracker Red in *Fgf8 cKO* and control littermates at E12.5 shows that apoptosis is not increased in the absence of Fgf8 (arrow). (**D**) Penis morphology is normal in *Fgf8 cKO* and control adults.

observed in wild-type littermates (Fig. 4C). Previous work in organ culture showed that antagonism of *Shh* results in the downregulation of *Fgf8* and an increase in cell death (Haraguchi et al., 2000). Our finding that the genetic removal of *Fgf8* in vivo results in neither increased cell death nor altered morphology of the tubercle indicates that Fgf8 is not required as a cell survival factor during genital development, and suggests that diminished *Fgf8* expression in *Shh*$^{-/-}$ genitalia is unlikely to account for the increased apoptosis in those mutants. Why deletion of *Fgf8* would result in a slight decrease in apoptosis is more difficult to explain, however, it is interesting that mice lacking noggin, a Bmp antagonist, also show reduced *Fgf8* expression and decreased apoptosis in the distal genital tubercle (Suzuki et al., 2003).

Conditional removal of *Fgf8* results in normal development of the penis

Fgf8 expression normally persists in the distal urethral epithelium through E14.5. Therefore, we wanted to determine whether Fgf8 plays a later role in genital development and patterning. To investigate this possibility we generated *Fgf8 cKO* mice and raised them to adulthood to assess the morphology of the penis. We found that outgrowth, axial patterning and urethral tube closure were unaffected by the removal of *Fgf8* (Fig. 4D). In addition, we analyzed the bacula (os penis) of *Fgf8 cKO* males and found that they were indistinguishable from wild-type males (data not shown). The genitalia of *Fgf8 cKO* males are functional, as they are able to urinate, copulate and produce a semen plug, although they appear to be sterile. These results show that *Fgf8* is not required for outgrowth, patterning or differentiation of the external genitalia.

Is Fgf8 signaling active during normal genital development?

To determine whether other Fgfs might compensate for Fgf8 during genital initiation, we examined all 22 known Fgfs in the genitalia of wild-type embryos, however, none was expressed in the cloacal endoderm prior to E12.5 (data not shown; a complete description of all Fgf and Fgfr expression patterns in the genitalia will be described elsewhere). As an additional test for Fgf pathway activation, we monitored the expression of four targets of Fgf signaling during normal genital development. *Dusp6*, *Spry4*, *Etv4* and *Etv5* have been shown to be transcribed in response to Fgf signaling (Kawakami et al., 2003; Mao et al., 2009; Mariani et al., 2008; Minowada et al., 1999; Roehl and Nusslein-Volhard, 2001; Sharrocks, 2001; Taniguchi et al., 2007; Zhang et al., 2009). *Dusp6* is induced through the phosphoinositide 3-kinase/Akt pathway in response to Fgfs expressed in the AER (*Fgf4*, *-8*, *-9* and *-17*) and is particularly sensitive to *Fgf8* (Kawakami et al., 2003; Mariani et al., 2008). *Dusp6* expression was undetectable during early outgrowth of the genital tubercle (prior to E12.5), although robust expression was observed near other areas of *Fgf8* expression, including the distal limb mesenchyme and dermamyotome (Fig. 5). We first observed *Dusp6* expression in the urethral epithelium at E12.5, two days after initiation of genital outgrowth, in a domain that partially overlapped with the *Fgf8* domain (Fig. 5).

Sprouty 4 negatively regulates MAPK/ERK activation in response to Fgf signaling and has been used as a readout of *Fgf8* expression in many cell populations during mouse embryogenesis (Minowada et al., 1999; Sasaki et al., 2003). We monitored the expression of *Spry4* and, like *Dusp6*, it was undetectable in the genital tubercle at E10.5 (Fig.
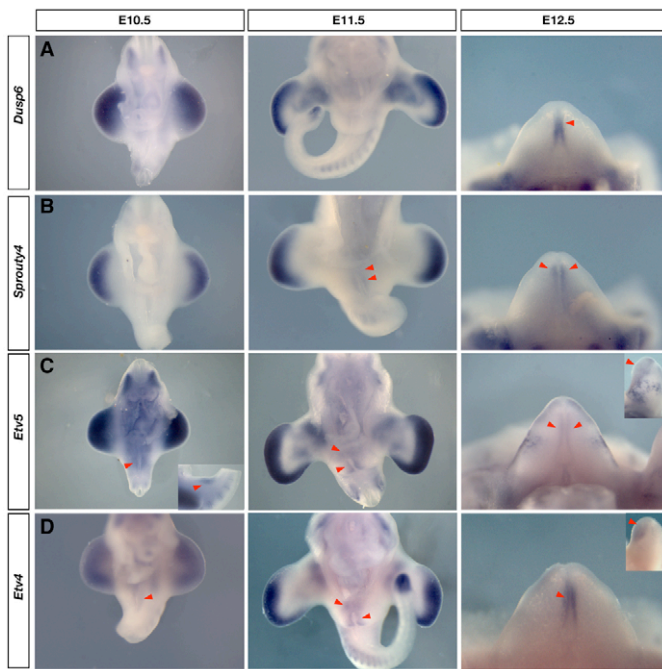
Fig. 5. Analysis of targets of Fgf signaling in the genital tubercle. (**A-D**) Expression of proposed targets of Fgf signaling: *Dusp6*, *Spry4*, *Etv5* and *Etv4* from E10.5 to E12.5. Red arrowheads mark expression in the external genitalia. (A,B) Neither *Dusp6* nor *Spry4* was detected during initiation of genital budding at E10.5, although both genes were expressed in the limb buds, somites and tailbud between E10.5 and E11.5. (C,D) By contrast, both *Etv5* and *Etv4* were expressed in broad domains around the cloacal membrane at E10.5 and E11.5. At E11.5, *Dusp6* expression was not detected in the genital tubercle (A), whereas expression of *Spry4* was detected distally (B). At E12.5, *Dusp6* and *Etv4* were expressed in the urethral plate endoderm (A,D), whereas *Spry4* and *Etv5* were expressed lateral to this domain (B,C). In addition, *Etv5* and *Etv4* expression covered the entire dorsal swelling (insets in C,D). Detection of these targets at E12.5 coincided with the expression of other Fgfs.

5). Expression of *Spry4* was first observed around the dorsal swelling of the tubercle at E11.5, with robust expression observed lateral to the urethral plate at E12.5 (Fig. 5; see Fig. S3 in the supplementary material). The absence of *Spry4* and *Dusp6* in early genital tubercles suggests an absence of *Fgf8* activity during initiation and outgrowth of the external genitalia.

ETS transcription factors are expressed in response to ERK activation, and the expression of both *Etv4* and *Etv5* has been shown to mirror *Fgf8* expression in zebrafish embryos and to be dependent on Fgf signaling in vertebrate limb buds (Mao et al., 2009; Roehl and Nusslein-Volhard, 2001; Zhang et al., 2009). We detected expression of both *Etv4* and *Etv5* at very low levels in a broad pattern surrounding the cloacal membrane at E10.5 (Fig. 5). By E11.5, both genes were expressed broadly in the genital mesenchyme lateral to the endoderm and in the dorsal swelling (Fig. 5; see Fig. S3 in the supplementary material). The expression of both ETS factors remained strong in the dorsal swelling at E12.5, with *Etv5* expression now partially overlapping the *Fgf8* domain in the urethral endoderm and *Etv4* expression strongest in the mesenchyme lateral to the urethral plate (Fig. 5). To determine whether the activation of *Etv4* and *Spry4* was regulated by *Fgf8*, we assayed for the expression of both genes in *Fgf8 cKO* embryos. Both *Etv4* and

Spry4 were expressed in the appropriate patterns in *Fgf8 cKO* embryos at E11.5 (see Fig. S4 in the supplementary material). Taken together, the findings that neither *Dusp6* nor *Spry4* are activated prior to E11.5, and that *Etv4* and *Spry4* are unresponsive to the deletion of *Fgf8*, suggest that Fgf8 does not participate in initiation or outgrowth of the genital tubercle.

Given that *Fgf8* is expressed robustly in the distal urethra but fails to activate genes reported to be Fgf targets, we next asked whether Fgf8 protein was present. We used an antibody against the N-terminal domain of Fgf8, which has been shown to detect Fgf8 protein in the limbs and fins of several vertebrate species (Freitas et al., 2006; Thewissen et al., 2006). Although Fgf8 protein was present in and immediately beneath the limb ectoderm, it could not be detected in the genital tubercle at either E11.0 or E11.5 (Fig. 6A-D). These results suggest that Fgf8 is not translated in the distal urethra, or that the protein is present at such low levels that it cannot be detected by immunohistochemistry.

Phylogenetic distribution of *Fgf8* in amniote genital tubercles

The results of our experiments indicate that *Fgf8* is transcribed in response to, but is not required for, initiation of the genital tubercle in mouse embryos. External genitalia are found in every class of amniotes, and comparative developmental data indicate that amniote genitalia share a common embryonic origin and utilize a common suite of molecular developmental mechanisms (A.W.S. and M.J.C., unpublished observations). To determine whether *Fgf8* expression is a conserved feature of amniote external genital development, we examined its expression in the genital tubercles of a diverse array of amniote embryos, including a rodent, an artiodactyl, a marsupial, an archosaur and a chelonian. *Fgf8* expression was observed in the genital tubercles of the opossum *Monodelphis domestica* (a marsupial), the pig *Sus scrofa* (an artiodactyl) and the mouse (a rodent), indicating that *Fgf8* expression is a conserved feature of mammalian external genital development (Fig. 7). *Fgf8* expression in the pig genital tubercle was confined to a single domain in the urethral plate (Fig. 7). Opossums develop a bifid glans penis in which the distal urethral tube bifurcates into a pair of urethral grooves, and we observed two domains of *Fgf8* at the distal end of the opossum genital tubercle (Fig. 7). In contrast to the three mammalian embryos, *Fgf8* expression was not detectable in the genital tubercles of either the red-eared slider *Trachemys scripta* (a chelonian) or the alligator *Alligator mississippiensis* (an archosaur), although in both taxa we detected *Fgf8* expression in the AER (Fig. 7). The finding that both turtles and alligators develop external genitalia in the absence of *Fgf8* expression, along with our functional analysis of *Fgf8* in the mouse, suggests that *Fgf8* is not required for the development of external genitalia across amniotes.

DISCUSSION

Conditional deletion of *Fgf8* in the mouse genital tubercle demonstrates that Fgf8 is not required for any aspect of external genital development. Although *Fgf8* is not necessary for outgrowth of the genitalia, its expression serves as a useful marker for genital induction, as illustrated in the variably penetrant phenotype of *Wnt5a$^{-/-}$* mice. The analysis of *Wnt5a$^{-/-}$* embryos suggests that *Fgf8* expression is a readout of endodermal-ectodermal signaling at the cloacal membrane. As such, examination of *Fgf8* in mouse mutants that exhibit cloacal membrane defects might be useful for elucidating the role of the cloacal ectoderm during induction of genital outgrowth. The conclusion that *Fgf8* is simply a response to the initiation signal is consistent with the observation that in *Shh$^{-/-}$*
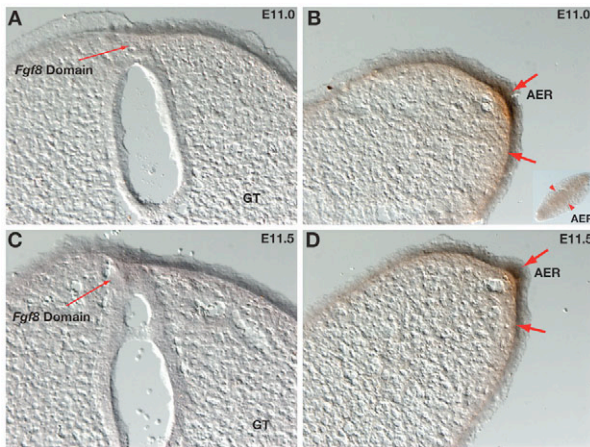
Fig. 6. Fgf8 protein is undetectable in the genital tubercle.
(**A-D**) Immunohistochemical analysis of Fgf8 protein in the genital tubercle (A,C) and hindlimb buds (B,D) at E11.0 and E11.5. Fgf8 protein was detected in and near the AER of the hindlimb buds (thick arrows) but was undetectable in the genital tubercles (GT) of the same embryos. Thin arrows mark regions of *Fgf8* mRNA expression but absence of detectable Fgf8 protein.

embryos *Fgf8* expression is activated in the cloacal endoderm and external genital budding is initiated, although outgrowth is not maintained (Perriton et al., 2002). Thus, both Fgf8 and Shh can be eliminated as candidates for the genital initiation signal.

Previous work reported that surgical removal of the distal epithelium from genital tubercles in organ culture led to decreased growth, and that this can be reversed by the addition of Fgf8-loaded beads (Haraguchi et al., 2000). Because the distal urethral endoderm is contiguous with the distal ectoderm, surgical excision of the distal tip likely resulted in the removal of both ectoderm and endoderm, each of which expresses numerous signaling molecules and transcription factors that might be involved in outgrowth. Although the published interpretation of those manipulations is that *Fgf8* produced from the distal tubercle is required for proliferation and outgrowth of the genital mensenchyme, our results indicate that those truncations cannot be explained by loss of Fgf8 alone.

During limb development several Fgfs are expressed in the AER, and this redundancy results in compensation following loss of any one Fgf (Boulet et al., 2004; Lewandoski et al., 2000; Mariani et al., 2008; Moon et al., 2000; Sun et al., 2000; Sun et al., 2002; Sun et al., 1999). We investigated whether such redundancy among Fgfs could account for the ability of *Fgf8 cKO* mice to form genital tubercles, but our in situ hybridization analysis of all known Fgfs during initiation and early outgrowth of the genitalia indicated that no other Fgf family member is expressed in a pattern similar to *Fgf8* prior to E12.5 (although other Fgfs were detected in the mesenchyme and in the ectoderm). This highlights an interesting difference between the distal urethral epithelium of the genital tubercle and the AER of the limb bud. Whereas the AER is a site of extensive redundancy of Fgfs that synergistically control proximodistal outgrowth of the limb, the distal urethral epithelium shows no such redundancy during early stages of genital outgrowth. Therefore, the normal development of a genital tubercle in *Fgf8* conditional mutants cannot be explained by compensatory Fgf signaling from the urethral endoderm.

After outgrowth is initiated, the expression of *Spry4*, *Etv4* and *Etv5* in the dorsal swelling coincides with the expression of several Fgfs in that region, suggesting that these target genes might be activated by other members of the Fgf family or other receptor tyrosine kinase pathways (Fig. 5, insets; data not shown). This is further supported by the finding that the expression of both *Spry4* and *Etv4* persists in the absence of Fgf8. When Fgf4/8 or Fgfr1/2 are inactivated during limb development, *Etv4* and *Etv5* expression is diminished but not completely abolished (Zhang et al., 2009). The expression of both genes can be completely abolished in zebrafish and chick limb buds following treatment with SU5402 (Mao et al., 2009; Roehl and Nusslein-Volhard, 2001). It is noteworthy that SU5402 does not specifically inhibit Fgfr signaling; previous studies have shown that it can inhibit both Pdgf and Vegf signaling (Mohammadi et al., 1997). In addition, several secreted signaling molecules are expressed in domains that overlap with *Fgf8*, including *Bmp2*, *Bmp7* and *Tgfb2*, all of which are capable of ERK activation (Derynck and Zhang, 2003; Grijelmo et al., 2007; Jin et al., 2006). Thus, it is possible that ERK activation of *Etv4* and *Etv5* in the genital tubercle might occur in response to other factors present in or around the urethral epithelium.
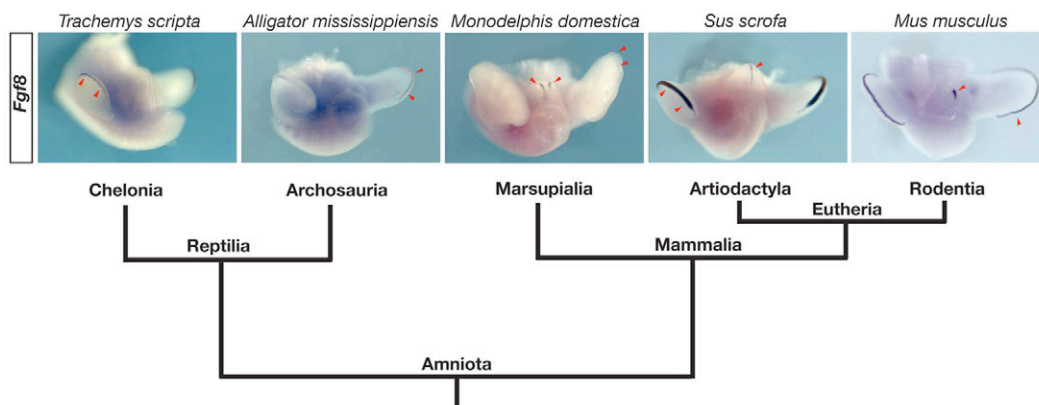

Fig. 7. Phylogenetic distribution of *Fgf8* expression in amniote genitalia. *Fgf8* was expressed in the genital tubercles of all mammalian embryos examined, including *Monodelphis domestica* (opossum), *Sus scrofa* (pig), and *Mus musculus* (mouse). By contrast, *Fgf8* was undetectable in the genital tubercles of *Trachemys scripta* (turtle) and *Alligator mississippiensis* (alligator), although expression was detected in the AER of the limb buds. Phylogenetic relationships are based on Hedges and Poling and Beck et al. (Hedges and Poling, 1999; Beck et al., 2006). Red arrowheads indicate *Fgf8* expression domains.

The finding that Fgf8 protein is undetectable during initiation and early outgrowth of the genital tubercle suggests possible post-transcriptional regulation of *Fgf8*. Interestingly, analysis of the mouse *Fgf8* sequence using MicroInspector software (Rusinov et al., 2005) revealed 155 miRNA binding sites, with 27 detected in exons 2 and 3, which are present in all of the potential *Fgf8* splice variants (A.W.S. and M.J.C., unpublished observations). Further analysis of *Fgf8* regulation, both at the transcriptional and translational levels, will be interesting in this context.

Why *Fgf8* would be transcribed in the urethral epithelium of mammals but not reptiles is unclear. A major distinction between the genitalia of mammals and other amniotes is that only mammals undergo urethral tubulogenesis; in non-mammalian amniotes the urethral epithelium persists as an open sulcus. The phylogenetic distribution of an *Fgf8* expression domain in the urethral epithelium suggests that the evolutionary origin of its expression domain coincided with the evolution of a urethral tube. The mechanism responsible for the evolutionary transition from a sulcus to a tube is unknown, but it is possible that *Fgf8* expression is a reflection of this new developmental process. Whatever the evolutionary basis of this novel domain of expression, the results presented here suggest that *Fgf8* expression is a consequence, rather than a cause, of external genital development. The nature of the external genital initiation factor remains to be discovered, and our findings raise the possibility that the cloacal ectoderm might be the source of this signal.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/15/2643/DC1

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