Phallus Development in the Turtle

Trachemys scripta

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Abstract
Development of a phallus occurs in almost all amniotes; however, considerable variation in phallus morphology among different amniote lineages has contributed to the debate about their structural homology. Mammals are the only amniotes that form a closed urethral tube within the penis. In contrast, the phallus of reptiles and birds has an open urethral groove, or sulcus spermaticus, that facilitates directional flow of sperm along the penis. One condition of structural homology is that the organs should share a common developmental origin; de novo development from different embryonic progenitors would indicate that the structure re-evolved in a new position. Although a common developmental origin does not itself demonstrate homology, different origins could indicate a lack of homology. To further understand how development of external genitalia evolved in amniotes, we examined this in the turtle Trachemys scripta. We found that phallus development in the turtle closely resembles that of mice at the tissue, cellular, and molecular levels, consistent with the hypothesis that their phalluses are homologous structures. We find that acquisition of specialized characters, such as a closed urethral tube, involved lineage-specific specialization of the common plan for amniote phallus development.

The transition of tetrapods from an aquatic to a terrestrial environment resulted from the evolution of a suite of traits that enabled land dwelling. A key innovation that allowed reproductive success in a terrestrial environment was the evolution of the amniotic egg which does not require an aquatic environment for fertilization or development but instead requires internal fertilization [Wake, 1979; Kardong, 2002]. For internal fertilization to occur efficiently, an intromittent organ is usually needed for the male to deliver sperm into the female. Almost all amniotes have an intromittent organ, the phallus or penis, although it has been secondarily reduced or lost in some taxa, such as most birds [King, 1981; Romer and Parsons, 1986; Briskie and Montgomerie, 1997; Brennan et al., 2008; Herrera et al., 2013].

Considerable variation exists in phallus structure and function, from the shape of the phallus to the method of erection [Wake, 1979; King, 1981; Kelly, 2002]. Indeed,
external genitalia evolve with spectacular rapidity and are often key characters used to make taxonomic distinctions [Hosken and Stockley, 2004]. For example, testudines (turtles, tortoises, and terrapins) have a single medial phallus on the anterior ventral side of their cloaca (the common outlet of the gut, urogenital, and reproductive sinuses), whereas squamates have paired phalluses, the hemipenes, which are situated on the lateral sides of the cloaca [King, 1981; Raynaud and Pieau, 1985; Romer and Parsons, 1986]. Most birds have reduced or lost their phalluses, although basal birds and some waterfowl have well-developed external genitalia [King, 1981; Briskie and Montgomerie, 1997; Brennan et al., 2008; Herrera et al., 2013]. Even the structure of the urethra, which is important for expelling urine and depositing semen into the female, differs among the amniotes. Mammals are the only group with a closed urethral tube along the length of the phallus; all other amniotes have an open urethral groove, or sulcus spermaticus [Wake, 1979; King, 1981; Raynaud and Pieau, 1985]. In amniotes with a sulcus, the cavernous bodies fill with blood (in reptiles) or lymph (in birds) during erection, causing the urethral groove to essentially become a closed tube and allowing the transport of sperm into the female [Wake, 1979; King, 1981]. These differences in phallus structure and function raise questions about the developmental mechanisms responsible for the evolutionary diversification of external genital organs and contribute to the debate about whether phalluses evolved independently in each group or whether they are homologous structures derived from the phallus of their common ancestor [Kelly, 2002, 2004].

In humans, one of the most common birth defects is hypospadias, a condition in which the penile urethra is open at an inappropriate position along the ventral side of the penis [Paulozzi et al., 1997; Baskin et al., 2001]. Interestingly, in severe hypospadias the urethra can be open along the length of the penis, reminiscent of the normal state in non-mammalian amniotes. Thus, the developmental defects that lead to hypospadias in humans could be interpreted as reversions to the primitive condition found in non-mammalian amniotes. Therefore, understanding how a urethral groove develops in non-mammalian species will be important for determining how this common defect arises in humans and will also contribute to our understanding of phallus evolution.

To gain insight into the evolution of urethral tube closure, we examined external genital development in the turtle *Trachemys scripta* (the red-eared slider). Development of the genitourinary system has been described for the turtles *Testudo graeca* and *Emys orbicularis* [Raynaud and Pieau, 1985], but a detailed analysis of turtle external genital development at the tissue, cellular, and molecular levels has not been done. Here, we report that the turtle phallus develops with striking similarity to that of mammals, from embryonic origins to gene expression patterns. However, we also identify patterns of apoptosis in the turtle genital tubercle that differ from patterns described in the mouse [Perriton et al., 2002; Morgan, 2003; Suzuki et al., 2003], specifically in the region that will form a urethral sulcus in the turtle versus a urethral tube in the mouse. These differences in cell death correlate with differential expression patterns of *Bmp4* and its downstream target *Msx2*, a pathway known to promote apoptosis. Our comparative analysis of external genital development provides new insights into how a closed urethral tube may have evolved in mammals and into the morphogenetic processes that underlie urethral tube closure defects in humans.

**Materials and Methods**

**Turtle Egg Incubation**

Freshly laid *T. scripta* eggs were purchased from commercial sources, incubated at a male-producing temperature (26 °C), and harvested at stages 12 through 17 of development (between 2 weeks and 4 weeks at the incubation temperature). Stages were determined according to the Yntema and Greenbaum staging series [Yntema, 1968; Greenbaum, 2002].

**LysoTracker and Histology**

For LysoTracker staining, live embryos were incubated in LysoTracker Green (Molecular Probes, L-7526) in PBS at 37 °C for 1 h. Embryos were then washed in PBS and fixed in 4% paraformaldehyde (PFA) prior to imaging.

For histology, embryos were fixed overnight in 4% PFA and dehydrated through a graded ethanol series (25, 50, 75, 100%) followed by Xylene washes. The embryos were incubated in 2 changes of paraaffin wax at 55 °C, with the final incubation under vacuum. The embryos were then embedded in paraaffin wax and sectioned at 10 μm. Sections were stained with hematoxylin and eosin prior to imaging.

**In situ Hybridization**

Embryos were fixed overnight in 4% PFA, dehydrated through a graded methanol series, and stored in 100% methanol. In situ hybridization was carried out according to previously published protocols [Nieto et al., 1996] with the following modifications: proteinase K concentration was increased to 70 mg/ml [Laufner, 1997], KTBT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl) contained 1% Triton X-100, alkaline phosphatase buffer (NTMT) contained 0.1% Tween-20, and the NBT-BCIP staining solution contained 0.01% dimethylformamide. *Msx2* and *Bmp4* probe plasmids were kindly provided by J. Moustakas [Moustakas, 2008]. *T. scripta* cDNA was used to PCR clone *Shh* (Genbank AY327848), *Ptc1* (Genbank KF646775), *Fgfr2* (Genbank KF646777), and *Suzuki et al., 2003*.
Hoxd13 (Genbank KF646776), which were cloned into the pGEM-T Easy vector (Promega, A1360). Antisense probes for in situ hybridization were made from the cloned PCR products.

**Scanning Electron Microscopy**

Turtle embryos were fixed at a minimum of overnight in 1% glutaraldehyde in PBS at 4°C. Embryos were then washed, treated with 2% osmium tetroxide for 1 h, and dehydrated to 100% ethanol. The samples were then critical point dried, sputter coated with metal, and imaged on a Hitachi S-4000 scanning electron microscope at the University of Florida Interdisciplinary Center for Biotechnology Research Electron Microscopy Core.

**Results**

**Morphological Development of Male Turtle External Genitalia**

We used scanning electron microscopy and histology to examine the morphological development of male *T. scripta* external genitalia. At stage 12, paired swellings emerge on either side of the cloacal membrane, where the hindgut endoderm meets the ventral ectoderm (fig. 1A, A’, G). The cloacal membrane forms at the apex of an anteroposterior ridge along the ventral midline of the embryo by the hindlimb buds (fig. 1A, A’, G). By stage 13, the paired genital swellings and anterior cloacal ridge have grown and fused to form a single genital tubercle (fig. 1B, B’, H). At this stage, lateral swellings begin to emerge at the base of the tubercle and will form the adult cloacal collar that envelopes the phallus. Sections show that the embryonic cloaca endoderm remains in contact with the ventral ectoderm of the genital tubercle (figs. 1H, 2B). By stage 14, further outgrowth of the cloacal collar swellings delineates the margin of the future cloaca outlet (fig. 1C, C’, I). At this stage, growth and anterior-posterior curvature of the embryo causes the tail to begin to fold towards the external genitalia (fig. 1C). By stage 15, a furrow appears between the base of the tail and tubercle (fig. 1D’). The ventral margin of the endoderm remains in contact with the ventral surface ectoderm of the genital tubercle (figs. 1J, 2D). At stage 16, the ventral side of the phallus is now covered by the ventral side of the tail, and the collar continues to grow around the phallus (fig. 1E, E’). After stage 16, the cloacal membrane ruptures, revealing the underlying endoderm of the sulcus (fig. 1, compare E to F and K to L). Removal of the tail shows that the large opening of the distal sulcus is contiguous with the.
narrow urethral groove that extends proximal-distally along the ventral side of the phallus (fig. 1F’’).

Cloacal Septation in the Turtle

The embryonic cloaca is the common outlet for the urogenital and anorectal canals; however, in therian mammals, this outlet becomes partitioned by the urorectal septum, which results in the formation of separate openings for the anorectal and urogenital sinuses [Romer and Parsons, 1986; Lombardi, 1998]. By contrast to therian mammals, in monotremes, reptiles, most birds, and amphibians, a cloaca persists in adults [Romer and Parsons, 1986; Lombardi, 1998; Kardong, 2002]. In mammals with severe cases of hypospadias, there is also a persistent cloaca or a failure to septate the anorectal and genitourinary outlets [Yucel et al., 2007; Seifert et al., 2009a; Wu et al., 2009]. Given that turtles retain a cloaca as adults and do not develop a urethral tube in the phallus, we investigated the degree of septation that develops in the turtle embryonic cloaca. Serial sagittal sections of turtle embryos were collected from stages 12 through 17 so that a urorectal septum would be visible along the anterior-posterior axis. At stage 12, the urorectal septum can be seen at the anterior limit of the cloaca (fig. 2A). On the ventral posterior end of the cloaca, the cloacal endoderm abuts the ventral surface ectoderm to form the cloacal membrane (fig. 2A). By stage 16, the urorectal septum is in close proximity to the base of the developing genital tubercle (fig. 2E), dividing the embryonic cloaca into urethra and rectum, indicating that the turtle embryonic cloaca undergoes an unexpected degree of septation. It is not clear whether the urorectal septum actively grows to reach the base of the tubercle or if this reflects passive displacement of the septum as a secondary effect of curvature of the embryo. The finding that the urorectal septum almost completely divides the embryonic hindgut into anorectal and genitourinary sinuses, which approaches the degree of septation that occurs in therian mammal embryos, suggests that the depth of the cloacal outlet in adult turtles is achieved by growth of the cloacal collar beyond the terminus of the embryonic cloaca.

Apoptosis during Genital Tubercle Development of Male Turtle

In turtles, the phallic urethra opens completely to form the sulcus spermaticus, whereas in mice, the ventral seam of the urethra remains intact throughout development, resulting in a closed urethral tube [Perriton et al., 2002; Suzuki et al., 2002]. Based on these observations, we hypothesized that apoptosis is involved in rupturing the cloacal membrane to expose the sulcus in turtles. To test this, we stained stage 12–17 turtle embryos with LysoTracker Green, which detects lysosomal activity associated with programmed cell death [Zucker et al., 2000]. At stages 12 and 13, LysoTracker stained cells along the anteroposterior ridge of the cloacal membrane (fig. 3A, B). At stage 14, we detected LysoTracker activity along the ventral midline of the tubercle, in the region where the endoderm...
meets the ectoderm, and this domain continued to be stained at stage 15 (fig. 3C, D). At stage 16, programmed cell death occurred along the margins where the urethra opened to form the sulcus (fig. 3E). LysoTracker staining had diminished by stage 17 although some staining was found in the anterior collar (fig. 3F). Thus, high levels of programmed cell death localize to the position of sulcus formation in the turtle genital tubercle.

**Gene Expression Patterns in Genital Tubercle of Male Turtle**

Next, we examined the expression patterns of several genes known to be important for the development of external genitalia in mammals. *Shh* is expressed in the mouse urethral endoderm and is required for cloacal separation and maintenance of a closed urethral tube [Harguchi et al., 2001; Perriton et al., 2002; Lin et al., 2009; Seifert et al., 2009b]. In turtle embryos, we found that *Shh* is expressed throughout the cloacal epithelium at stage 12, and expression persists in the developing urethral sulcus through stage 16 (fig. 4 A–C). *Ptc1*, a transcriptional target of Shh signaling, was expressed in the mesenchyme adjacent to the cloacal/urethral epithelium, and staining persisted after the sulcus opened (fig. 4 A–F).

*Hoxd13* is required for outgrowth of the genital tubercle in the mouse and is associated with external genital defects, including hypospadias, in humans [Tuzel et al., 2007]. In mice, *Hoxd13* is expressed throughout the genital tubercle, in both the mesenchyme and the urethral epithelium [Perriton et al., 2002; Cobb and Duboule, 2005]. When we performed in situ hybridization for *Hoxd13* in *T. scripta* embryos, we detected a similar pattern of expression to that reported for mice. At stage 12, *Hoxd13* marked the cloaca and the genital field from which the paired genital swellings emerge (fig. 4G). As these swellings merge to form a single outgrowth, the genital tubercle continued to express *Hoxd13* (fig. 4H, I).

*Fgfr2* encodes 2 alternatively spliced Fgf receptors: *Fgfr2IIIb*, which is expressed in the urethral epithelium and ectoderm of mice and is required for urethral tubulogenesis, and *Fgfr2IIIc*, which is expressed in the genital tubercle mesenchyme [Satoh et al., 2004; Petiot et al., 2005]. Our analysis of *Fgfr2* expression (both isoforms) in the turtle revealed weak staining in the cloacal region between the paired genital swellings (fig. 4J). At stage 14, *Fgfr2* expression was strongest in the distal genital tubercle (fig. 4K), and at stage 16, *Fgfr2* expression remained strong distally, and more diffuse staining was detected proximally (fig. 4L).

*Bmps* are important regulators of apoptosis in many developmental contexts. Given that apoptosis is elevated in the turtle cloacal membrane and that *Bmp4* has been associated with cell death in the genital tubercle of other amniotes [Suzuki et al., 2003; Herrera et al., 2013], we investigated whether differential expression of *Bmp4* is associated with high levels of cell death in the ventral midline of the turtle tubercle. *Bmp4* was expressed in the mesenchyme adjacent to the urethra, as well as in the distal mesenchyme of the tubercle through stage 15 (fig. 4M–O) which resembles the pattern described for the mouse. Interestingly, we also detected a stripe of *Bmp4* in the center of the urethral sulcus epithelium (fig. 4N), a domain not seen in the mouse [Perriton et al., 2002; Suzuki et al., 2003]. This region of *Bmp4* expression along the urethral sulcus epithelium corresponds to the region where programmed cell death occurs at these stages (fig. 3). At stage 16, *Bmp4* was localized to the mesenchyme adjacent to the distal urethra, with the highest levels around the developing sulcus (figs. 4O, 3E). This expression pattern suggests that *Bmp4* may be involved in regulating apoptosis in turtles. We next examined *Msx2*, a direct target of Bmp signaling, and detected expression in the distal tubercle mesenchyme at all stages examined (fig. 4P–R). In addition, *Msx2* expression was detected at a site where the urethral epithelium is in contact with the ventral ectoderm at stages 12 and 14 (fig. 4P, Q), which corresponds to the region of *Bmp4* expression and apoptosis. Although *Msx2* expression in the distal genital tubercle is similar to that reported in the mouse, expression along the urethral sulcus occurs in turtles but not in mice [Perriton et al., 2002; Seifert et al., 2010].
Discussion

One of the most common congenital defects in humans is hypospadias, a failure of urethral-tube closure. Mutations in developmental control genes, such as HOXA13, which causes hand-foot-genital syndrome [Mortlock and Innis, 1997; Wang and Baskin, 2008; Kalfa et al., 2009], and androgen receptor, have been implicated in some cases of hypospadias, and there is increasing evidence that exposure to environmental endocrine disruptors may be involved. In most cases, however, the cause of hypospadias is unknown, and identification of genes that regulate development of a closed urethral tube remains important. Because the turtle normally develops an open urethra, which is phenotypically similar to the most severe forms of hypospadias in humans, the turtle can serve as a new model to identify processes that differ during development of an open versus a closed urethra. Comparison of turtle and mammalian external genital development has the potential to uncover the mechanisms that led to the evolution of a closed urethral tube, and these may be the same mechanisms that are disrupted in human hypospadias. Moreover, because defects in cloacal septation have been associated with a failure to close the urethral tube in humans, analysis of cloaca development and septation in the turtle has similar translational potential.

In this study, we provided a detailed characterization of penis development in T. scripta at the tissue, cellular, and molecular levels. Our results showed that early development of the turtle genital tubercle is similar to that of the mouse. At later stages, we identified a domain of cell death in the turtle phallus that is associated with formation of the sulcus, and this differs from the patterns of cell death described for the mouse. We also found that cell death in this region is preceded by activation of the Bmp4 pathway, which has been shown to promote cell death in the genital tubercle in the mouse [Suzuki et al., 2003]. Finally, our analysis of cloacal septation showed that the early phase of this process is similar in turtle and mouse.
embryos, but in mice, septation leads to formation of the perineum and continues into the male genital tubercle where it masculinizes the penile urethra [Seifert et al., 2008].

**Cloaca Development in the Turtle: Embryonic versus Adult**

The cloaca is the common outlet for the urinary, rectal, and genital ducts and is found in all vertebrates embryonically. In therian mammals, the cloaca undergoes septation during development, leading to separate anorectal and urogenital sinuses in the adult [Romer and Parsons, 1986; Lombardi, 1998]. Because adult non-therian amniotes have a cloaca, the adult cloaca has been described as a persistence of the embryonic cloaca [Romer and Parsons, 1986]. Our finding that the embryonic cloaca of turtles undergoes septation (more than expected based on the depth of the mature cloaca) suggests that outgrowth of the cloacal collar, which envelops the phallus, urinary and rectal outlets, beyond the limit of the embryonic cloaca contributes to the formation of the adult cloaca. Thus, the adult cloaca is not simply the result of persistence of the embryonic cloaca and degradation of the cloacal membrane [King, 1981; Raynaud and Pieau, 1985; Lombardi, 1998].

We were surprised by the degree of similarity between turtle and mammalian cloacal development at early stages. We found that during development of *T. scripta*, there is a urorectal septum that reaches the base of the phallus by stage 17 (fig. 2F). This septation looks remarkably similar to what has been found in mice at e13.5, the stage when the urorectal septum has just reached the base of the developing phallus [Sasaki et al., 2004; Seifert et al., 2008]. In contrast to turtles, however, the mammalian septum continues to grow, further separating the anorectal and urogenital openings and, in males, extending into the phallus to masculinize the urethra [Nievelstein et al., 1998; Qi et al., 2000a, b; Hynes and Fraher, 2004; Sasaki et al., 2004; Seifert et al., 2008]. Although we did not examine embryos beyond stage 17 in *T. scripta*, we hypothesize that one difference between the urorectal septum in turtles and in therian mammals is that the former does not continue to grow beyond this initial separation of the anorectal and urogenital sinuses, whereas in the latter, sustained outgrowth gives rise to the perineum and internalizes the male urethra [Seifert et al., 2008]. It will be interesting to compare growth of the urorectal septum among other taxa to determine how the extent of septation varies across amniotes and how this relates to closure of the urethra.

Turtle cloacal collar development is reminiscent of a later event in rodent external genital development. Although mice do not have a cloacal collar, the prepuce similarly develops from swellings at the base of the genital tubercle and envelops the phallus (but excludes the anorectal opening). The extent to which developmental or evolutionary relationships exist between the cloacal collar of non-mammalian amniotes and the prepuce of mammals is an open question and fertile area for future investigation. Together, our results suggest that cloaca development in turtles and mammals may be more similar than previously appreciated.

**Evolution of the Phallus and Urethral Tube Closure**

Despite the presence of a phallus in most amniotes, there have been questions of the homology of the phallus between different amniote lineages [Kelly, 2002, 2004]. Based on the diversity of phallus morphologies (including absence of a phallus) in different taxa, it has been suggested that the phallus evolved independently in each of the major amniote lineages [Kelly, 2002, 2004]. However, despite this extensive morphological diversity, it remains possible that the phallus is an amniote synapomorphy that has undergone rapid diversification. Our results suggest that the turtle phallus is derived from the same embryonic tissues as the mouse phallus. In both taxa, a genital tubercle develops from paired swellings that emerge from the pericloacal mesenchyme, contains a urethral epithelium that is derived from cloacal endoderm, and is covered by ventral ectoderm that ruptures in turtles but remains intact in normal mice. In addition to their conserved embryonic origins and pattern of morphogenesis, turtle and mouse genital tubercles show strikingly similar patterns of gene expression. These similarities in embryonic origin, cellular composition, and molecular markers in turtles and mice, together with similar findings in birds and alligators [Herrera et al., 2013, Gredler et al., this issue], suggests that the genital tubercle is a plesiomorphic character.

We favor the view that an open sulcus (as found in turtles, birds, and crocodilians) is the primitive condition and that the transition to a closed tube occurred within mammals because the alternative – that a closed tube is primitive and was lost independently in all of the other amniotes – is not parsimonious. During mouse external genitalia development, the urethra is closed within the genital tubercle and opens at only 2 positions, at the base of the tubercle to form the proximal urethral opening (which eventually closes in males but remains open to form the vaginal opening in females) and at the distal tip.
of the urethra, where the definitive urethral meatus forms [Perriton et al., 2002; Suzuki et al., 2002; Seifert et al., 2008]. The turtle urethra is also closed initially, but, in contrast to mice, it opens along the entire ventral side of the phallus. The gene expression patterns that occur in T. scripta generally were similar to the patterns that have been reported for mammals, and the few differences may be associated with formation of a urethral groove rather than a tube. For example, Shh is expressed in the urethral endoderm in both turtles and mice, and Bmp4 is expressed in the mesenchyme adjacent to the urethra in both groups [Haraguchi et al., 2001; Perriton et al., 2002], but turtles also show Bmp4 expression in the developing sulcus. Moreover, Msx2, a Bmp target, is expressed initially along the entire cloacal membrane and later in the developing sulcus of turtles. Bmp4 and Msx2 expression along the ventral seam marks the position where apoptosis occurs during sulcus formation, suggesting a potential role for apoptosis in the formation of the ventral urethra. Moreover, apoptosis in turtles and mammals will be important in order to test whether these signaling events are responsible for an open urethral phenotype and, by extension, evolution of a closed urethral tube.

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