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The development of the bladder trigone, the center of the anti-reflux mechanism

Renata Viana¹, Ekatherina Batourina¹, Hongying Huang², Gregory R. Dressler³, Akio Kobayashi⁴, Richard R. Behringer⁴, Ellen Shapiro², Terry Hensle¹, Sarah Lambert¹ and Cathy Mendelsohn^{1,*}

The urinary tract is an outflow system that conducts urine from the kidneys to the bladder via the ureters that propel urine to the bladder via peristalsis. Once in the bladder, the ureteral valve, a mechanism that is not well understood, prevents backflow of urine to the kidney that can cause severe damage and induce end-stage renal disease. The upper and lower urinary tract compartments form independently, connecting at mid-gestation when the ureters move from their primary insertion site in the Wolffian ducts to the trigone, a muscular structure comprising the bladder floor just above the urethra. Precise connections between the ureters and the trigone are crucial for proper function of the ureteral valve mechanism; however, the developmental events underlying these connections and trigone formation are not well understood. According to established models, the trigone develops independently of the bladder, from the ureters, Wolffian ducts or a combination of both; however, these models have not been tested experimentally. Using the Cre-lox recombination system in lineage studies in mice, we find, unexpectedly, that the trigone is formed mostly from bladder smooth muscle with a more minor contribution from the ureter, and that trigone formation depends at least in part on intercalation of ureteral and bladder muscle. These studies suggest that urinary tract development occurs differently than previously thought, providing new insights into the mechanisms underlying normal and abnormal development.

KEY WORDS: Bladder, Reflux, Trigone, Ureter, Urinary tract formation, Mouse, Human

INTRODUCTION

A crucial feature in embryonic development is the assembly of independently formed organs into complex systems that conduct substances such as food, air and waste into and out of the embryo. The organs that comprise the upper (kidney and ureter) and lower (bladder and urethra) urinary tract form independently, connecting at mid-gestation to form an outflow tract that conducts urine from the kidneys to the bladder for storage and excretion. The kidneys, ureters and Wolffian ducts, paired epithelial tubes that form most of the male genital tract, are largely derived from intermediate mesoderm, a strip of tissue lying between the lateral plate and the paraxial mesoderm. Wolffian ducts open into the cloaca, which differentiates into the urogenital sinus, the primordium of the bladder and urethra. The ureteric bud, which will give rise to the renal collecting duct system and extra-renal ureter, forms as a caudal sprout from the Wolffian duct that invades the metanephric blastema and undergoes successive rounds of branching morphogenesis in response to signals from the metanephric mesenchyme. The portion of the ureteric bud lying outside the kidney differentiates into the ureters, which are muscular tubes that mediate myogenic peristalsis, propelling urine from the renal pelvis to the bladder.

The upper and lower urinary tract compartments join when the ureters undergo transposition, moving from their primary insertion site in the Wolffian ducts to the urogenital sinus epithelium, where

they make final connections in a triangular structure, known as the trigone, situated between the bladder and urethra (Fig. 1). Our previous studies suggest that formation of these final connections involves apoptosis, which enables the ureters to disconnect from the Wolffian ducts, and fusion, in which the ureter orifice inserts into the urogenital sinus epithelium at the level of the trigone (Batourina et al., 2005). Precise connections between ureters and the trigone are crucial for function of the valve mechanism that prevents back flow of urine from the bladder to the ureters, a major cause of reflux and obstruction, which can damage the kidney and cause severe health problems including end-stage renal disease.

Despite its central importance in urinary tract function, the origin and role of the trigone in the anti-reflux mechanism remains controversial. Analysis of human and animal specimens has led to the suggestion that the trigone is structurally distinct from the bladder and urethra, differentiating from the common nephric duct and ureter (Hutch, 1972; Tanagho, 1981; Weiss, 1988; Wesson, 1925). Other studies suggest that the bladder muscle (detrusor) might also be part of the trigone structure (Meyer, 1946). Hence, a number of questions remain: what is the derivation of the trigone, how is the anti-reflux mechanism established, and how do positional abnormalities of the ureteric bud translate into reflux and obstruction? To begin to address these questions, we used mouse models to study the structure of the trigone and to determine which lineages contribute to its formation. We find, unexpectedly, that the trigone derives largely from bladder muscle and that ureteral fibers are an important contributor to trigone structure. A number of studies also suggest that the ureteral pathway through the bladder is formed by a sheath of ureteral muscle (Waldeyer, 1892) (reviewed by Hutch, 1972). We find, paradoxically, that the ureteral pathway is present in the bladder wall and forms independently of the ureter. These studies elucidate important mechanisms controlling urinary tract assembly that are also important for formation of the ureteral valve that is crucial for preventing reflux and preserving renal function.

¹Columbia University, Department of Urology, 650 West 168th Street, New York, NY 10032, USA. ²Department of Urology, New York University School of Medicine New York, NY, USA. ³Department of Pathology, University of Michigan, MSRB1, BSRB 2049, 109 Zina Pitcher Dr, Ann Arbor, MI 481093, USA. ⁴Department of Molecular Genetics, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA.

* Author for correspondence (e-mail: hendelsohn@gmail.com)

MATERIALS AND METHODS

Immunostaining

For cryosections (10 μm), tissue was fixed in 4% paraformaldehyde (PFA) for 1-3 hours at 4°C and embedded in OCT compound. For vibratome sections (100-150 μm), tissue was fixed overnight in 4% PFA, washed in PBS and then embedded in 3% agarose. Sections were then permeabilized with 0.3% hydrogen peroxide in cold methanol for 20 minutes, washed in PBS/0.1% Triton X-100 for 30 minutes then processed for immunostaining. For double staining with uroplakin and smooth muscle alpha actin, samples were incubated in blocking solution (2% horse serum in washing buffer) then primary uroplakin antibody, a marker of urothelial terminal differentiation (Wu et al., 1994). UP3 antibody (clone #744) was a gift of Dr T. T. Sun (New York University, NY) was applied overnight at 4°C. After washing, the secondary antibody (donkey anti-rabbit IgG) was applied for 2 hours at room temperature. After washing and reblocking, the tissue was incubated in (ASMA)FITC- or Cy3-conjugated antibodies (Sigma) overnight at 4°C then washed and mounted.

Human tissues

With approval from the New York University Institutional Board of Research Associates, lower urinary tracts were removed from four human fetuses ranging in gestational age from 19 to 22 weeks. Informed consent was obtained by the consulting obstetrician. The gestational ages were estimated from date of last menstrual period as well as from sonographic measurements of crown rump and foot length. Specimens were formalin-fixed, paraffin-embedded and serially sectioned at 4 μm .

Immunohistochemistry for smooth muscle actin

Representative tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes. Antigen retrieval was performed by incubating paraffin sections with antigen unmasking solution (Vector Labs #H-3300) and microwave treatment (900 W) for 20 minutes, followed by blocking with 10% normal goat serum. Mouse monoclonal antibody (M0851, Dako, Carpinteria, CA) was used to detect the human smooth muscle actin. After overnight incubation at 4°C with anti-smooth muscle actin, a biotinylated goat anti-mouse secondary antibody was applied. Slides were then treated with avidin-biotinylated peroxidase complex and developed in a solution containing 3,3'-diaminobenzidine (DAB). All sections were counterstained with Hematoxylin, dehydrated, mounted and observed by light microscopy.

X-Gal histochemistry

To reveal *lacZ* expression, vibratome or cryostat sections were fixed in cold 2% PFA in PBS for 5 minutes at 4°C, washed in PBS, and stained in X-Gal solution for 2-5 hours at 37°C (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS and 1.2 mg/ml X-Gal in dimethyl sulfate). After staining, samples were washed 2-3 times with PBS, post-fixed with 4% PFA and stored at 4°C in 80% glycerol.

Animals and genotyping

For timed matings, males and females were placed in a cage together at 16.00-17.00 h, and the morning when the vaginal plug was visualized was taken to be E0.5. *Hoxb7-Gfp* mice (Srinivas et al., 1999) were a kind gift from Dr Frank Costantini (Columbia University, New York, NY). Genotyping was with PCR using primers: 5'-AGCGCGATCACATGGTC-CTG-3' and 5'-ACGATCCTGAGACTTCCACACT-3'. *Pax2* mutant mice were genotyped using the following three primers: *Pax2F*, 5'-CCCAC-CGTCCCTTCCCTTTTCTCCTCA-3'; *Pax2R*, 5'-GAAAGGCCAGTGTG-GCCTCTAGGGTG-3'; and *PGK*, 5'-AGACTGCCTTGGGAAAAGCGC-3'. *Sm22-Cre* mice (Holtwick et al., 2002) were obtained from the Jackson Laboratory and genotyped by PCR using: 5'-CAGACACCGAAGCT-ACTCTCCTTCC-3' and 5'-CGCATAACCAGTGAAACAGCATTGC-3'. *Rosa26 lacZ* mice (Soriano, 1999) were also obtained from the Jackson Laboratory and genotyped using: 5'-AAAGTCGCTGAGTTGTAT-3', 5'-GCCAAGAGTTTGTCTCAACC-3' and 5'-GGAGCGGGAGAAA-TGGATATG-3'. *Rarb2-Cre* mice were genotyped as described (Kobayashi et al., 2005).

RESULTS

In newborn mouse urogenital tracts, the bladder is encircled by a thick layer of muscle called the detrusor and the ureters enter the trigone at the base of the bladder between the bladder and urethra (Fig. 1A,C,D). The trigone can be visualized in dissected urogenital tracts as a smooth triangular shaped region bounded by the ureters laterally, terminating at the bladder neck where the urethra begins (Fig. 1D,E). The surface of the urethra and ureters, like the bladder, is covered by the urothelium, a specialized transitional epithelium that prevents leakage and damage (Fig. 1D, the urothelium is red). The intramural ureters pass through the bladder muscle and submucosa and open into the trigone at its lateral edges (Fig. 1E). Higher magnification reveals the eyelet-shaped ureter orifice opening into the urothelium (Fig. 1F). Unlike the bladder, which is covered by folds, the trigone is generally smooth, which has led to the suggestion that its origin might be distinct from the bladder.

Development of the trigone

The trigone has been defined in a number of ways; here, we will consider the trigone to be the muscular triangle bounded laterally by the ureter orifices extending posteriorly to the urethra (Fig. 1C). The unique features of the trigone including its appearance and physiological properties have led to the idea that the trigone originates from non-urogenital sinus tissue, in particular from the common nephric duct that is the caudal-most segment of Wolffian duct. However, our previous studies suggest that this is not the case because the common nephric duct undergoes apoptosis during ureter transposition, hence the trigone is likely to form in a different manner than previously thought. Other studies suggest that the trigone is formed in large part from ureteral fibers that fan out laterally forming an inter-ureteric ridge and posteriorly forming Bell's muscle (Fig. 1C). To begin to address this question we first established which muscles are present in the trigone by analyzing its formation in mouse urogenital tracts at different developmental and post-natal stages. At E15, analysis for expression of smooth muscle alpha actin revealed extensive smooth muscle differentiation (green) in the bladder, urethra and in the extra-vesicular ureters (the portion of ureter outside the bladder), but there was little if any detectable smooth muscle lining the intramural ureter (the portion of the ureter within the bladder) in the trigonal region (Fig. 2A).

Analysis of urogenital tracts at P0 revealed a thick smooth muscle coat surrounding the extra-vesicular ureter and a few longitudinal fibers surrounding the intramural ureter extending through the detrusor and submucosa (Fig. 2B,E,F). Analysis at adult stages revealed additional smooth muscle lining the intramural ureter. The trigone appeared at this stage to be a hybrid between the bladder and urethra. Its surface was smooth and free of folds like the urethra was covered by a thick muscularis submucosa, similar to that in the bladder (Fig. 2C,D,G,H). The ureteral wall outside the bladder is thick, containing at least three layers of circular and longitudinal muscle (Fig. 2E). However, as reported by other groups (Yucel and Baskin, 2004), only a small subset of longitudinal ureteral fibers extend into the intramural region, where they appear to intercalate with the bladder muscle and terminate in the submucosa, below the urothelium (Fig. 2F,G). These findings suggest that two major muscle types are present in the trigone: the bladder muscle (detrusor) and the muscle associated with the intramural ureter. Extensive analysis of whole-mount urogenital tracts, cryosections and vibratome sections did not reveal additional muscle groups reported to be part of the trigone, including an intra-ureteric bar which is said

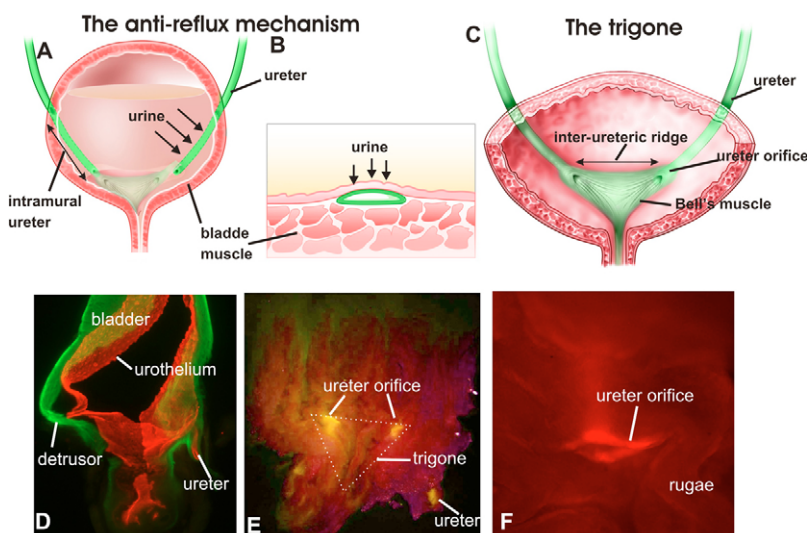


Fig. 1. The trigone is the site of the anti-reflux mechanism. (A) Schematic of the trigone at the bladder base and its connections with the ureters showing the intramural ureter segment that is normally compressed to prevent back-flow of urine to the ureters and kidneys. (B) Schematic showing compression of the intramural ureter. (C) A detailed representation of the trigone, which is thought to be composed of ureteral fibers that enter the bladder via Waldeyer's sheath, fan out across the base to form the inter-ureteric ridge and extend down toward the apex to form Bell's muscle. (D) A vibratome section from an adult mouse stained for uroplakin (red) to reveal the urothelium, and for smooth muscle alpha actin (green) to reveal smooth muscle. (E) Opened bladder showing the trigone in an adult *Hoxb7-Gfp* mouse. The ureter orifices (yellow) are located at the base of the trigone. (F) High magnification of the ureter orifice, showing its eyelet shape at the point it opens into the urothelium (red, uroplakin). Magnification: $\times 100$ in D,E; $\times 200$ in F.

to extend laterally between the two ureter orifices, and Bell's muscle which is said to extend caudally from the ureter orifices to the trigone apex (Tanagho et al., 1968).

The trigone is evolutionarily conserved

The failure to identify structures in the mouse thought to be associated with the trigone suggests that either the trigone is formed differently than previously thought, or that there are substantial differences in the structure of the mouse and human trigone. To address this question, we compared the trigone in human and mouse. Sections through the trigone of a 22-week human fetus stained for smooth muscle alpha actin revealed the ureter passing through the bladder muscle and into the submucosa (Fig. 3A). The morphology of the bladder muscle, which is organized in bundles, was seen to be distinct from the thin longitudinal smooth muscle fibers that surround the ureter (Fig. 3A,C). Analysis of the mouse trigone at similar stages revealed few, if any, differences. The ureter is ensheathed in a thin

layer of longitudinal smooth muscle one or two cell layers thick, surrounded by and distinct from the bladder muscle (Fig. 3B). Cross-sections through the ureter as it passes through the bladder revealed extensive similarity across species. The intramural ureter in the human trigone is surrounded by a thin layer of longitudinal fibers that are most likely ureteral smooth muscle, similar to that in the section through the mouse trigone at a comparable level (Fig. 3C,D). The observation that the mouse trigone displays similar morphology and muscle arrangement to that in human suggests that the trigone develops in a similar manner in both species, and is likely to be formed primarily from the ureter and bladder muscle.

Lineage analysis reveals the origin of trigonal muscle

Ureteral muscle is thought to make a major contribution to the trigone (Roshani et al., 1996; Tanagho et al., 1968; Woodburne, 1964). However, given the complexity of the trigonal region it is not

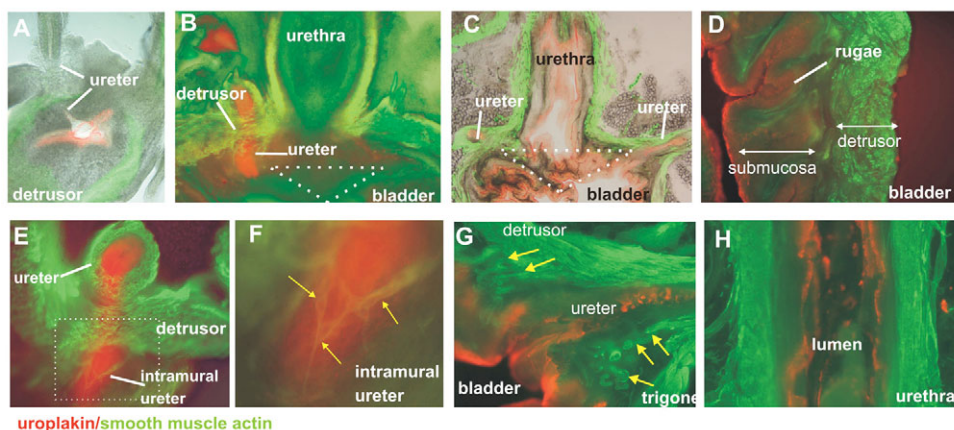


Fig. 2. Development of the trigone. (A) Brightfield/darkfield composite showing a frontal section through an E15 embryo stained for uroplakin (red) to reveal the urothelium, and smooth muscle alpha actin (green) to reveal smooth muscle. Note the absence of muscle surrounding the intramural ureter compared with the extra-mural ureter, which already has a thick smooth coat. (B) The trigone in a newborn mouse showing the intramural ureter crossing the bladder muscle and submucosa. Note the longitudinal muscle fibers surrounding the intramural ureter. (C) The trigone in an adult mouse. (D) The bladder of a newborn mouse showing the deep folds of the lining, and the muscularis mucosa and smooth muscle layers below. (E) Higher magnification of the ureteral tunnel shown in B. (F) High-magnification image of the intramural ureter showing the longitudinal muscle fibers (green). (G) Higher magnification of the region in C showing the position in the trigone where the ureter joins. Note the longitudinal fibers that intercalate with the bladder muscle (yellow arrows). (H) The urethra in a newborn mouse showing the thick muscle coat (green) and smooth urothelial surface (red). Magnification: $\times 50$ in A-C; $\times 100$ in D,E,G,H; $\times 200$ in F.

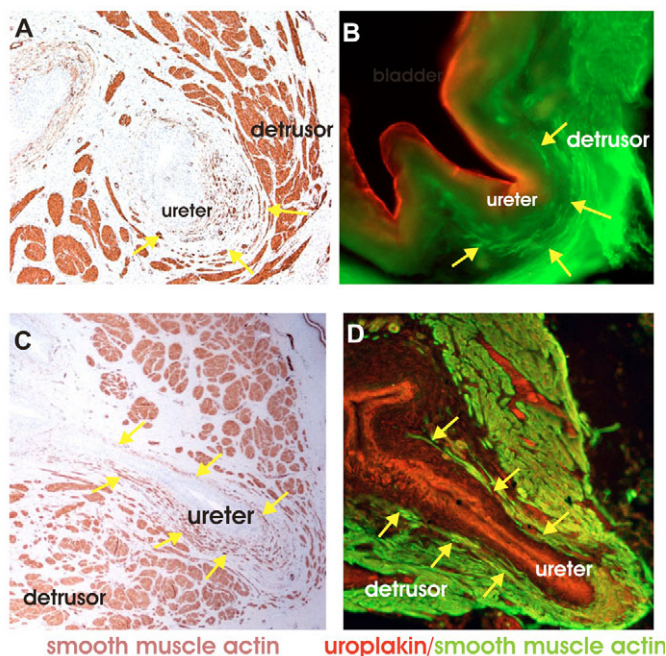


Fig. 3. Comparison of the trigone in humans and mice. (A) A section through the human trigone at the level of the intramural ureter stained for smooth muscle alpha actin (brown). Black arrows point to the intramural muscle fibers. (B) A section through a newborn mouse showing the trigone stained for smooth muscle alpha actin (green) and the urothelium stained for uroplakin (red). The yellow arrows point to the longitudinal ureteral muscle fibers that encircle the intramural ureter. (C) Section through a human trigone showing the intramural path of the ureter and its surrounding thin layer of fibers (black arrows). (D) Section through the mouse trigone at birth showing the path of the intramural ureter, stained for uroplakin (red) to reveal the urothelium and smooth muscle alpha actin (green). The yellow arrows point to the longitudinal muscle fibers associated with the intramural ureter. Magnification: $\times 20$.

possible to determine whether this is the case by visual inspection. To address this question, we performed lineage studies permanently labeling smooth muscle progenitors in the ureter using the Cre-lox recombination system. We then followed the fate of ureteral mesenchymal cells at late stages of development to determine whether their descendants populate the trigone. We crossed *Rarb2-Cre* mice (Kobayashi et al., 2005), which express the Cre recombinase in mesonephric mesenchyme surrounding the nephric duct, in mesenchymal cell types within the kidney and in ureteral mesenchyme (Kobayashi et al., 2005), with *Rosa26 lacZ* reporter (*R26RlacZ*) mice (Soriano, 1999). *lacZ* expression is permanently activated in cells expressing both the *Rosa26* reporter and the *Rarb2-Cre* transgene and in their descendants, enabling us to determine the contribution of ureteral muscle to the trigone.

Analysis of *Rarb2-Cre;R26RlacZ* embryos at E14 revealed *lacZ* expression in mesenchymal cells around the ureters, but not in smooth muscle progenitors in the bladder and trigone (Fig. 4A,B). At birth, *lacZ* expression persisted in smooth muscle cells in the extra-vesicular ureter coat in both circular and longitudinal fibers, which were most likely descendants of the labeled mesenchymal cells observed at E14, but not in the bladder or urethra (Fig. 4C). In the trigonal region, careful analysis revealed *lacZ* activity in the longitudinal fibers surrounding the ureter that extended into the bladder muscle and submucosa (Fig. 4D,E). Despite the large

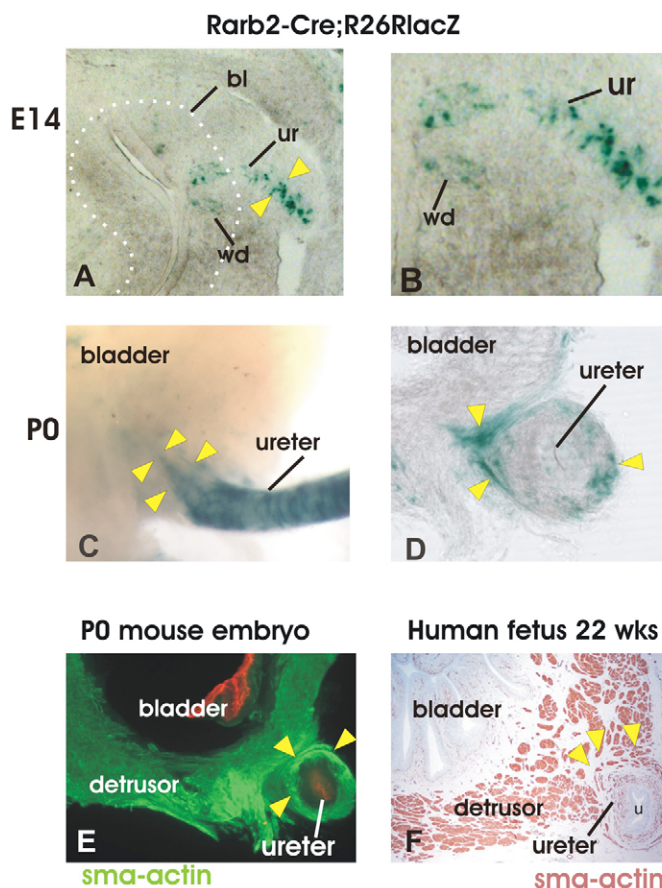


Fig. 4. Ureteral fibers contribute to the trigone. (A) Sagittal section through a *Rarb2-Cre;R26RlacZ* embryo at E14 showing *lacZ*-expressing mesenchymal cells surrounding the ureter (yellow arrowheads in all panels). Note the absence of *lacZ*-expressing cells in the bladder, trigone and urethra. (B) Higher magnification of a region of A. (C) Whole-mount of a newborn *Rarb2-Cre;R26RlacZ* urogenital tract showing *lacZ*-expressing smooth muscle cells lining the extra-mural and intramural ureter. (D) A section through the trigone showing *lacZ*-expressing cells surrounding the intramural ureter. (E) Smooth muscle uroplakin staining of a section serial to D, showing that the *lacZ* activity in D corresponds to smooth muscle. (F) Section through a human fetus at the same level as E, showing the ureteral muscle embedded in bladder muscle in the trigone. wd; Wolffian duct. Magnification: $\times 100$ in A; $\times 200$ in B-F.

amount of muscle in this region, we did not observe ureteral fibers extending further into the trigone, which have been postulated to generate the inter-ureteric bar, nor into the posterior trigone extending toward the urethra, which have been postulated to form Mercier's bar (Fig. 4C,D). Comparison of the distribution of muscle in the mouse and human trigone at this stage revealed few, if any, differences (Fig. 4E,F), suggesting that the failure to identify a more extensive contribution from ureteral fibers is not due to interspecies differences. These findings suggest that the trigone is formed predominantly from bladder muscle, with a contribution from ureteral fibers that is much more limited than previously thought.

The trigone is formed predominantly from bladder muscle

Histological studies suggest that two muscle groups reside in the trigonal region: the detrusor muscle of the bladder and longitudinal ureteral fibers. To assess the contribution of bladder muscle to the

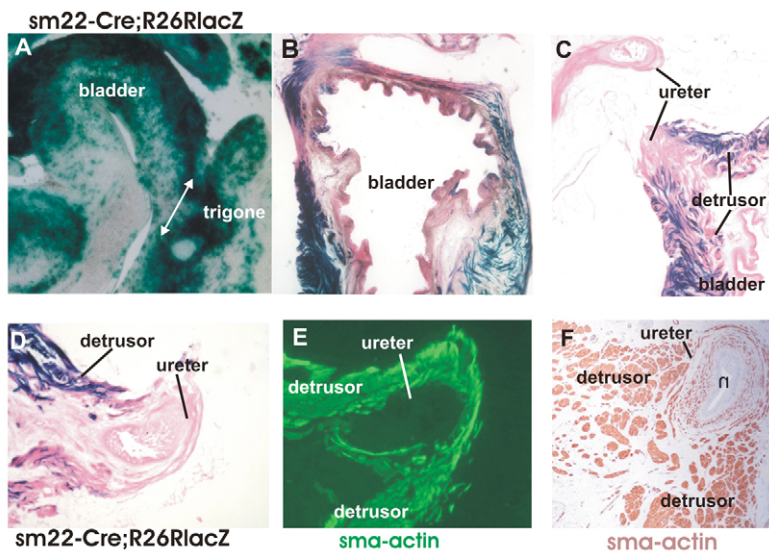


Fig. 5. The trigone is formed predominantly from bladder muscle. (A) A sagittal section through a *Sm22-Cre-R26RlacZ* embryo at E14. *lacZ*-expressing mesenchymal cells are visible in the bladder, urethra and trigone (white arrow), but not in the ureter or Wolffian duct. (B) Section through the bladder and urethra of an adult *Sm22-Cre-R26RlacZ* mouse showing descendants of the urogenital sinus mesenchyme that have differentiated in the bladder and urethra muscle. (C) Section through an adult *Sm22-Cre-R26RlacZ* mouse showing the ureter, which has few if any *lacZ*-expressing cells, and its path through the bladder muscle that is extensively labeled by the *Sm22-Cre* transgene. (D) A section through the intramural portion of the ureter in an *Sm22-Cre-R26RlacZ* adult. (E) A section from the same sample as in D, stained for smooth muscle alpha actin to reveal muscle of the intramural ureter, unlabeled by the *Sm22-Cre* transgene. (F) Section through a comparable level of a human embryo showing the path of the intramural ureter through the bladder muscle of the trigone. Magnification: $\times 100$ in A-C; $\times 200$ in D-F.

trigone, we permanently labeled bladder and urethral mesenchymal muscle progenitors by crossing *R26RlacZ* reporter mice with a *Sm22-Cre* mouse line in which the Cre recombinase is expressed in urogenital sinus mesenchyme but not in ureteral mesenchyme (Kuhbandner et al., 2000) (Fig. 5). Beginning at E12, *Sm22-Cre;R26RlacZ* embryos displayed extensive *lacZ* activity in mesenchymal cells in the bladder, the trigone and the urethra, but not in the ureters or Wolffian ducts (Fig. 5A and data not shown). By birth, expression was throughout the muscle in the bladder, trigone and urethra, but there were few if any *lacZ*-labeled smooth muscle cells in the ureter, including the intramural ureter in the trigonal region (Fig. 5B,C). The distribution of *lacZ* activity in the trigonal region of *Sm22-Cre;R26RlacZ* mice was compared with that of smooth muscle alpha actin in wild-type embryos. This revealed that there is indeed muscle present in this lateral portion of the trigone at the ureteral junction, and that these unlabeled cells are likely to correspond to ureteral muscle (Fig. 5D,E). Comparison with sections from human trigone revealed remarkable similarity in the smooth muscle configuration: ureteral muscle was clearly present, embedded in the bladder wall, corresponding to the unlabeled portion of the trigone in the *Sm22Cre;R26RlacZ* mouse (Fig. 5D-F). Hence, ureteral fibers make a contribution to the trigone, which is formed mainly from bladder muscle.

Ureters enter the trigone through a tunnel and ureteral fibers intercalate with bladder muscle

One piece of evidence supporting the idea that ureteral muscle is important for formation of the trigone is the observation that ureter agenesis results in an abnormally shaped ipsilateral hemitrigone.

Ureteral muscle is thought to contribute extensively to the trigone itself and, according to the literature, the ureteral passageway to the trigone is encased in a sheath that is formed from ureteral musculature (Waldeyer, 1892) (reviewed by Hutch, 1972). Analysis of muscle differentiation in sagittal sections of wild-type E18 embryos revealed that the ureter passes through a tunnel in the bladder wall in parallel with blood vessels. Ureteral muscle fibers terminate in the trigone and intersect with ureteral and bladder muscle exclusively at its lateral edges. These findings suggest that the trigonal structure might be formed from this pathway of the ureter through the bladder and intercalation of the ureteral and urogenital sinus-derived fibers. (Fig. 6A). To further address this question, we analyzed trigone formation in the absence of the ureter in *Pax2* mutants, which display apparently normal urogenital sinus differentiation but lack ureters and kidneys owing to agenesis of the caudal Wolffian duct. The trigone in the *Pax2* mutant shown (Fig. 6B) contains bladder muscle that appeared to completely encircle the bladder neck. Interestingly, both in *Pax2* mutants and in wild-type littermates, a gap was present in the bladder wall, which probably corresponds to the ureteral tunnel. In wild-type mice, the tunnel contained the intramural ureter and blood vessels that pass through the muscle and submucosa into the urothelium. In *Pax2* mutants, the tunnel was also present, but contained only blood vessels owing to the absence of the ureter. The presence of the ureteral tunnel in the absence of ureters indicates that it is almost certainly derived from the bladder/trigone. The observation that intercalation of ureteral and bladder muscle occurs only at the lateral sides of the trigone is consistent with the requirement for the ureter to maintain the raised

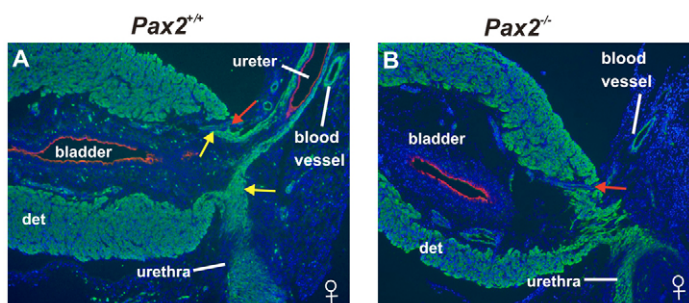


Fig. 6. The structure of the trigone is likely to depend on intercalation of ureteral and bladder muscle. (A) A sagittal section through an E17 *Pax2*^{+/+} embryo showing the point at which the ureteral longitudinal fibers join the bladder detrusor (yellow arrows). (B) A sagittal section through a *Pax2*^{-/-} littermate of that shown in A, showing the structure of the trigone region in the absence of the ureter. Note the abundant bladder and urethral muscle, and the tunnel through the bladder (red arrow) present in both wild type (A) and mutant (B). det, detrusor. Magnification: $\times 100$.

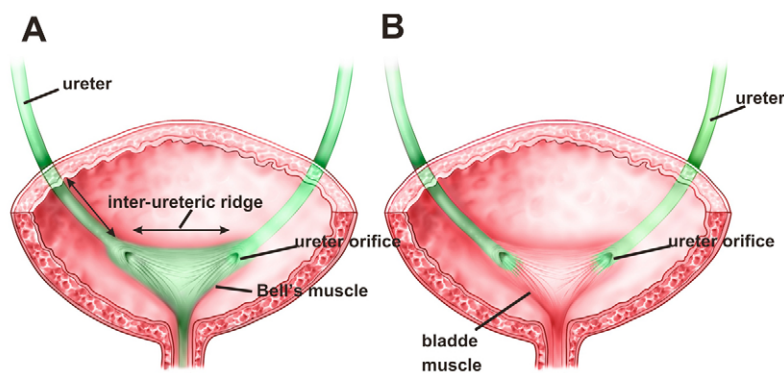


Fig. 7. Models of trigone formation. (A) Old model of trigone formation, showing the trigone to be continuous with the ureters (green), formed in large part from ureteral fibers that fan out across the surface generating the inter-ureteric ridge and Bell's muscle. Note that the trigone has been considered to form independently of the bladder. (B) Current model of trigone formation, showing a small contribution from ureteral fibers (green) and the bulk of the structure derived from bladder muscle and the space around the ureter that functions as a tunnel.

triangular structure normally associated with the trigone, explaining why the absence of the ipsilateral ureter results in deformation of the trigone.

DISCUSSION

Rethinking urogenital tract formation

According to the literature, the structure of the trigone is complex, derived predominantly from ureteral muscle that stretches across the base to form the ureteral ridge, and also toward the trigone base to form Bell's muscle (Fig. 7). The ureters are said to penetrate the bladder via a tunnel (Waldeyer's sheath or space) derived from the ureter (Brooks, 2002; Tanagho et al., 1968; Wesson, 1925). The common nephric duct, which is the most caudal Wolffian duct segment, is thought to contribute to the trigone as it differentiates and expands during ureter transposition, repositioning the ureter orifices in the bladder neck. However, it is unclear which portion of the trigone this tissue would form, as the common nephric duct is an epithelial tube, an extension of the Wolffian duct, whereas the trigonal muscle is likely to be derived from mesenchyme, as are other muscular tissues in the embryo. Our previous findings and the current lineage study suggest an alternate model of urinary tract formation. We have established that the common nephric duct does not contribute to any part of the bladder, trigone or urethra, but instead undergoes apoptosis during ureter transposition (Batourina et al., 2005). Here, using Cre-lox recombination, we followed the fate of ureteral and bladder muscle progenitors and find that the trigone is formed predominantly from bladder muscle, with a contribution from ureteral longitudinal fibers at the lateral edges that is much more limited than previously thought (Fig. 7B). The intercalation of ureteral and bladder muscle is likely to be crucial for trigone formation and for maintaining the ureteral anti-reflux mechanism. These studies also suggest that muscles such as Mercier's bar and Bell's muscle, which have been considered to be formed from the ureter, are in fact derived from the bladder (Fig. 7), as suggested by others (Woodburne, 1964). The observation that the trigone is formed from the same primordial tissue as the rest of the bladder (the urogenital sinus) is consistent with studies demonstrating that the urothelial covering of the trigone is indistinguishable from that of the bladder, but is distinct from that of the ureter (Liang et al., 2005).

Distinct patterning along the urinary outflow tract

Recent studies indicate that most, if not all, of the mesenchymal muscle progenitors lining the ureter and urogenital sinus derive from the tail bud or cloacal mesoderm (Brenner-Anantharam et al., 2007; Haraguchi et al., 2007). However, the morphology of these tissues

is diverse. Ureters are ensheathed by 3-4 layers of muscle that mediate myogenic peristalsis. The bladder is surrounded by a thick layer of smooth muscle, a muscularis mucosa and a surface composed of deep folds that enable contraction and expansion. The trigone is smooth and has a distinctive shape probably generated by interaction between bladder and ureteral muscle fibers at its lateral edges. Its cellular morphology is likely to depend not on its embryological origin, as has been suggested, but on spatially expressed signaling molecules, including Hox genes, *Bmp4*, *Tbx18* and *Shh*, that are crucial for patterning other urinary tract tissues (Airik et al., 2006; Brenner-Anantharam et al., 2007; Haraguchi et al., 2007; Raatikainen-Ahokas et al., 2000; Scott et al., 2005; Yu et al., 2002). Future studies will determine the role of these pathways in normal trigone development and whether mutations in these genes also lead to trigone abnormalities.

Application of this new model to human disease

The pathway taken by the ureter through the bladder muscle and submucosa is thought to be important for function of the anti-reflux mechanism, which normally prevents back-flow of urine to the ureters and kidney by compressing the intramural ureter against the smooth muscle bladder wall. The ability to effectively compress this terminal ureteral segment is thought to depend on several factors, including sufficient length of the intramural segment, its pathway through the bladder and insertion of the ureter orifice at a stereotypical position in the trigone (King et al., 1974; Stephens et al., 1996) and innervation that regulates opening of the ureteral orifice (reviewed by Radmayr, 2005).

A shortening of the intramural segment, or ureter orifices joining the trigone abnormally, can be caused by sprouting of the ureteric bud from the Wolffian duct from a location more cranial or caudal than normal (Mackie and Stephens, 1975; Pope et al., 1999; Stephens, 1983) as seen in several mouse models (Basson et al., 2005; Batourina et al., 2005; Grieshammer et al., 2004; Kume et al., 2000; Lu et al., 2007; Miyazaki et al., 2000; Yu et al., 2004), or by abnormalities in ureter transposition, at the time when the ureter normally separates from the Wolffian duct (Batourina et al., 2005). Intrinsic ureteral abnormalities, such as a failure in muscle differentiation, can also result in reflux owing to faulty urine transport or peristalsis (Airik et al., 2006; Chang et al., 2004; Yu et al., 2002).

The trigone is the site at which surgery is performed to correct reflux, whereby the refluxing ureter is detached from its original insertion site and reinserted in the trigone in such a way that the length of the intramural segment is increased and has improved muscular backing. The observations from our studies that trigone formation and, by default, ureteral valve function, depend on

intercalation of ureteral fibers with bladder muscle, suggest that in addition to increasing the length of the intramural ureter, reimplantation of ureters might also inadvertently help establish better connections with underlying bladder muscle and the trigone. This will further our understanding of the anti-reflux mechanism that is paramount for renal function.

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