Smooth Muscle Differentiation and Patterning in the Urinary Bladder

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Abstract

Smooth muscle differentiation and patterning is a fundamental process in urinary bladder development that involves a complex array of local environmental factors, epithelial-mesenchymal interaction, and signaling pathways. An epithelial signal is necessary to induce smooth muscle differentiation in the adjacent bladder mesenchyme. The bladder epithelium (urothelium) also influences the spatial organization of the bladder wall. Sonic hedgehog (Shh), which is expressed by the urothelium, promotes mesenchymal proliferation and induces differentiation of smooth muscle from embryonic bladder mesenchyme. Shh, whose signal is mediated through various transcription factors including Gli2 and BMP4, is likely also important in the patterning of bladder smooth muscle. However, it is not known to what extent early mediators of mesenchymal migration, other Shh-associated transcription factors, and crosstalk between the Shh signaling cascade and other pathways, are involved in the patterning of bladder smooth muscle. Here we review the role of epithelial-mesenchymal interaction and Shh signaling in smooth muscle differentiation and patterning in the bladder. We also discuss emerging signaling molecules, transcription factors, and mesenchyme properties that might be fruitful areas of future research in the process of smooth muscle formation in the bladder.

Keywords

bladder; smooth muscle; differentiation; patterning; sonic hedgehog

Introduction

The function of the adult human bladder is to store and empty urine at socially appropriate times. Despite this seemingly simple function, micturition is a complex anatomic and physiologic function that involves interaction between the urothelium, bladder smooth muscle, afferent and efferent nerves, spinal cord, and higher-level cerebral processing. The urinary bladder is a dynamic organ that is both compliant in order to allow storage of urine and contractile to allow for coordinated emptying. The detrusor, which is the muscle that contracts to expel urine, is comprised of randomly-oriented smooth muscle fibers and, under

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normal conditions, is under autonomic control. In conjunction with a normally functioning nervous system, the physiologic properties of the bladder are derived from the anatomic properties of the bladder smooth muscle. During filling, the normal adult bladder maintains a low intravesical pressure (< 10cm H_2O) and has a capacity of 350-500cc. At a socially appropriate time, urethral pressure decreases and intravesical pressure increases as the bladder smooth muscle undergoes coordinated contraction, allowing for effective emptying. Concomitant with expelling urine through the urethra, detrusor contraction also closes the muscular tunnel through which the ureters traverse as they join the bladder. This prevents retrograde flow (vesico-ureteral reflux), which protects the kidneys from the pressure generated by detrusor contraction.

Over the last forty years, significant advances have been made in the understanding of how smooth muscle develops in the embryonic bladder. Observational studies of developing human embryos have shown that smooth muscle differentiates from embryonic mesenchyme by the twelfth week of gestation.[1] While such descriptive studies are useful in establishing the timeline of bladder organogenesis, they do not provide insight into the molecular mechanisms that underlie smooth muscle differentiation. Use of transgenic and knockout mice, tissue recombination experiments, and enhanced molecular biologic and immunohistochemical techniques have greatly increased our understanding of bladder smooth muscle development. The focus of this review is the complex array of local environmental factors, epithelial-mesenchymal interaction, and signaling pathways that are critical for smooth muscle differentiation and patterning in the urinary bladder.

Bladder Embryology

The bladder arises from the urogenital sinus (UGS) after the urorectal septum partitions the cloaca into the urogenital sinus ventrally and the rectum dorsally. The bladder then forms from the superior part of the UGS, which is of endodermal origin. (Figure 1) The adjacent mesenchyme, derived from splanchnopleural mesoderm, coalesces to surround the endodermal-derived epithelial lining of the lumen of the UGS and establishes the reciprocal epithelial-mesenchymal relationship that is critical for bladder development. There is emerging evidence that the epithelium of the bladder trigone, which classically is thought to be of mesodermal origin, is also derived from endoderm, as is the rest of bladder.[2-4] Similarly, recent studies have shown that the smooth muscle of the trigone arises primarily from the same primordial mesenchyme as the rest of the detrusor, which counters the previous hypothesis that the smooth muscle of the trigone was derived primarily from incorporation of ureteral smooth muscle.[5]

The full thickness of bladder mesenchyme, from the subepithelium to the sub-adventitial layers, has the potential to develop into smooth muscle. During normal development, bladder smooth muscle forms first in the outer zone (adventitial side) of peripheral mesenchyme, then proceeds towards the inner zone (luminal side). However, the region of the mesenchyme immediately adjacent to the urothelium, the subepithelium, remains mostly devoid of smooth muscle. The location of the urothelium on the luminal surface of the bladder is critical to this patterning of the bladder wall.[6]

Smooth Muscle

Embryology, Anatomy, and Physiology

In humans, bladder smooth muscle has been reported to form between 7 – 12 weeks of gestation.[1, 7] The paucity of human tissue has obviously necessitated using animal models to investigate the molecular mechanisms of bladder differentiation. Although many models have been used, the mouse is most often used to study smooth muscle differentiation in the

bladder. In the mouse, which has an approximate 20-day gestation, differentiation of smooth muscle in the mesenchyme begins at E13.5.[7] (Figure 2) The difference in timing of organogenesis between mice and humans is readily apparent; in mice, the bladder forms at the beginning of the third trimester while, in humans, the bladder forms in the first trimester. Such differences limit the generalizability of animal experiments to human development; however, they also have greatly increased our knowledge of bladder smooth muscle differentiation by allowing investigators to isolate and manipulate the mouse bladder prior to the onset of smooth muscle differentiation.

Although smooth muscle anatomy and physiology is beyond the scope of this review, a brief overview is warranted. Bladder smooth muscle fibers have a fusiform shape, are 300-400 μm in length, are of the single unit type, and have a greater ratio of actin to myosin and a larger length-tension curve than striated muscle.[8, 9] In single-unit smooth muscle, an autonomic nerve innervates a single cell within a bundle of muscle cells. The action potential is then is then propagated to neighboring cells through gap junctions, which causes a coordinated contraction. These properties allow bladder smooth muscle to stretch (necessary for bladder filling) but still maintain contractility (necessary for bladder emptying). As in striated muscle, the major structural components of smooth muscle are actin and myosin. However, in smooth muscle, contraction is regulated by calmodulin, which, after binding calcium, activates myosin light-chain kinase (MLCK). MLCK then phosphorylates the light chain component of the myosin head, which allows muscle fiber contraction. Caldesmon, a component of thin filaments in smooth muscle cells, binds calmodulin and inhibits actin-activated ATP hydrolysis by smooth muscle myosin.[10, 11] Calponin, after binding calcium, also tonically inhibits the ATPase activity of myosin.

Knowledge of these proteins is critical to the interpretation of many smooth muscle developmental biology experiments because the stage of smooth muscle differentiation can be characterized by its protein expression profile. Generally, smooth muscle alpha actin (SMAA) is considered a marker of early smooth muscle cell differentiation, calponin and SM22α, intermediate markers, and smooth muscle myosin heavy chain (SM-MHC) a marker of advanced differentiated smooth muscle cells.[12-15] SMAA was utilized as the marker of smooth muscle differentiation in the bladder for all of the experiments described in this review except in cases where use of other specific markers are noted.

Differentiation

Epithelium

An epithelial signal is necessary for induction of smooth muscle differentiation from bladder mesenchyme (BLM).[16, 17] Tissue recombination experiments have revealed the importance of epithelial-mesenchymal interaction during bladder development. Intact bladders harvested at embryonic day (E) 12, which is prior to the onset of smooth muscle formation, survive and undergo smooth muscle differentiation when grown as grafts beneath the kidney capsule of nude mice for two weeks whereas isolated E12 BLM does not develop and does not survive. However, E12 BLM onto which urothelium has been grafted survives and undergoes normal smooth muscle differentiation.[16] This epithelial signal necessary for mesenchymal survival and smooth muscle differentiation is neither restricted to embryonic urothelium, as fetal, newborn, and adult bladder epithelium (BLE) are all able to induce smooth muscle differentiation from BLM nor is it specific to BLE; ureteral, gastric, and corneal epithelium have been shown to induce smooth muscle differentiation from BLM, albeit to varying degrees.[17] The same has been shown in uterine smooth muscle (myometrium) development.[18] The resulting smooth muscle pattern also depends on epithelial-mesenchymal interaction. In the bladder, the pattern of smooth muscle appears to be governed by the mesenchyme (i.e. bladder mesenchyme plus gastric epithelium produces

bladder-like smooth muscle pattern).[17] This mesenchymal-specific patterning of smooth muscle is somewhat different than the gut in which intestinal epithelium alone can induce differentiation and direct intestinal-like patterning of adjacent stroma.[19] However, for the bladder, it appears that most types of endodermal-derived epithelium are able to induce BLM to undergo smooth muscle differentiation and the pattern of smooth muscle organization is largely intrinsic to the mesenchyme.

Although it appears that BLM dictates the spatial organization of smooth muscle layers, Cao *et al.* demonstrated that the location of the bladder epithelium significantly influences its ultimate pattern.[6] With the urothelium in its orthotopic (luminal) location, smooth muscle develops throughout the bladder wall except for a thin subepithelial layer. However, when urothelium was placed on both the luminal and adventitial aspects of the bladder, smooth muscle differentiation was inhibited immediately adjacent to both urothelia. (Figure 3) Epithelial-mesenchymal interaction has also been found to be important in patterning mesenchyme in other hollow-organ systems such as the intestine.[20]

The robust epithelial-mesenchymal interaction that results in smooth muscle differentiation in embryonic BLM diminishes with age. Grafting E14 BLM recombined with embryonic BLE within the wall of adult bladders significantly decreased SMAA expression compared to the same recombinants grafted underneath the kidney capsule.[21] This indicates that the signals that induce smooth muscle differentiation in the embryonic bladder are inhibited by the local environment of the adult bladder. This finding may have important implications for development of cell or gene-based therapies for repair of the adult bladder, which likely does not have the same plasticity seen in the developing bladder.

Sonic Hedgehog

Signaling

Sonic hedgehog (Shh) is a growth factor expressed by many types of epithelia that promotes mesenchymal proliferation [7, 22-25] and is necessary for the differentiation of smooth muscle from embryonic mesenchyme in diverse organ systems including the bladder, lung, and vasculature.[6, 7, 25-30] Shh is expressed by the bladder urothelium and is necessary for bladder development and smooth muscle differentiation.[7, 30, 31] Shh functions by binding to its membrane-bound receptor, Patched (Ptc1). In the unbound state, Ptc1 suppresses the activity of Smoothened (Smo); once Ptc1 binds Shh, the inhibitory activity on Smo is suppressed. The binding of Shh initiates an intracellular signal transduction cascade that activates three highly regulated *Gli* transcription factors: *Gli1*, *Gli2* and *Gli3*. Both *Gli1* and *Gli2* are transcriptional activators that activate Shh target genes such as the Wnt family and the bone morphogenetic proteins (BMPs) of the TGFβ superfamily.

In the presence of Shh signaling, *Gli2* is stabilized, and translocates to the nucleus to regulate numerous target genes involved in a number of different Shh-mediated cell processes including cell survival, cellular proliferation, and differentiation. (Figure 4) After nuclear translocation, *Gli2* upregulates transcription of *foxf1* and *foxl1*, which then go on to upregulate *BMP4* transcription. In turn, BMP4 provides negative feedback on *foxf1* transcription.[32, 33] Many experiments in the bladder have shown that BMP4 is found early in the developing bladder with highest levels in the subepithelial layer and significantly lower levels in the peripheral mesenchyme.

Downstream signaling from BMP4 activation is mediated by receptor-associated Smad proteins (R-Smad) 1, 5, and 8, which are distinct from the Smad proteins that mediate TGF-β signaling (R-Smad 2 and 3). Association of the R-Smad with Smad4, which is shared by both BMP4 and TGF-β signaling pathways, allows translocation of the Smad complex into

the nucleus. Association of the R-Smad:Smad4 complex with different nuclear cofactors determines the ultimate transcriptional effect.[34] Further research is needed to elucidate the downstream mediators of BMP4/R-Smad/Smad4 in bladder development.

Mesenchymal proliferation

It is known that Shh acts as a mitogen during development. Shh expands and maintains cell subpopulations within the somite [35] and has been shown to promote mesenchymal proliferation in diverse tissues, including developing tooth, kidney, and ureter.[36, 37] Ptc1 mediates the effect of Shh on cellular proliferation. In its unbound state, Ptc1 sequesters M-phase promoting factor (MPF). MPF is a complex of *cdc2* and *cyclin B1*, and has an important role in regulating the cell cycle. Ptc1, after binding Shh, releases MPF, which then translocates to the nucleus and drives the cell through the M-phase of mitosis.[38] It plausible that this mechanism is preserved in the bladder. Future research on the role of Shh, Ptc1, and MPF in regulating bladder mesenchyme proliferation might prove fruitful.

In a series of *ex vivo* experiments, progressively higher Shh concentrations up to 48nM increased the number of cells and organ size of E12 bladders after 72 hours in culture. These experiments demonstrated that increasing Shh concentrations increase bladder size and cell number and that there appears to be a threshold of the proliferative effect Shh has on bladder mesenchyme. It is known that urothelium expresses Shh and the adjacent mesenchyme, which responds to the Shh signal, co-express Ptc1 and BMP4 (Figure 5). Results from other studies raise BMP4 as a candidate gene that might mediate the effect of Shh on mesenchymal proliferation in the bladder. In the developing kidney, BMP4 inhibits mesenchymal proliferation; however, the overall mesenchymal response to the Shh signal is an increase in proliferation. This suggests that Shh promotes mesenchymal proliferation through BMP4-independent pathways and Shh can overcome the inhibitive effect of BMP4 on mesenchyme proliferation. [37] Although, to our knowledge, no studies have been performed in the fetal bladder, the similar expression pattern of Shh, Ptc1, and BMP4 in the embryonic kidney and bladder raise the possibility that a similar inhibitory feedback mechanism of BMP4 on the Shh-mediated promotion of mesenchymal proliferation might exist.[7] See Table 1 for organ systems in which Shh-BMP4 is known to be important for epithelial-mesenchymal interaction.

Also in kidney organogenesis, deletion of *smad4* results in disorganized nephrogenic mesenchyme, which illustrates the central role of Smad4 in mediating the cellular response to BMP4 and TGF-β signaling. In the same study, *smad4* deletion did not produce a phenotypic change in the ureteric bud.[39] Given that BMP4 is necessary for ureteric bud morphogenesis, this suggests that an alternative pathway that does not involve the Smad complex can mediate the BMP4 signal, at least in the ureter.[40] The alternative pathway hypothesis is supported by the finding that diverse mesodermal patterning processes, which are regulated by TGF-β and BMP4 are unaffected by Smad4 deletion.[41] It is not known what effect *smad4* deletion has in the bladder.

Smooth muscle differentiation and patterning

In addition to promoting mesenchymal proliferation, Shh regulates smooth muscle differentiation in the bladder. Smooth muscle differentiation occurs in the peripheral mesenchyme of intact bladders harvested at E12 when grown in organ culture without Shh, whereas isolated E12 BLM cultured without Shh does not undergo smooth muscle differentiation. However, E12 BLM cultured with exogenous Shh express markers of advanced smooth muscle differentiation, including SM-MHC, at 72 hours.[42] These results demonstrate that either Shh or an intact urothelium is necessary to support bladder growth

and induce smooth muscle differentiation. (Figure 6) Given the localization of Shh to the urothelium, it is highly likely that the urothelium is the source of Shh.[7]

In vivo, Shh expression by the urothelium changes over time during bladder organogenesis. mRNA levels of Shh, *Ptc1*, *Gli1*, and *BMP4* decrease linearly after peaking at E12.5. The converse is true for smooth muscle proteins. mRNA transcript levels for SMAA, smooth muscle gamma actin (SMGA), SM-MHC, and Calponin-1 are not detectable at E12.5 but increase in inverse proportion to the Shh pathway gene transcripts. (Figure 7) The temporal relationship between Shh signaling cascade proteins and smooth muscle marker expressions supports the hypothesis that Shh of epithelial origin induces and orchestrates smooth muscle differentiation in the bladder. As is the case with mesenchymal proliferation, it is likely that BMP4 is one of the prime mediators of the Shh signal in regulating smooth muscle differentiation. *In vivo* evidence is available in the ureter, where conditional *bmp4* knockouts have significantly decreased and disorganized smooth muscle.[43]

In the same set of *ex vivo* experiments that demonstrated that increasing Shh concentration increased bladder explant size and cell count, increasing Shh concentrations also increased SMAA expression. Similar to the effect seen on cellular proliferation, SMAA expression did not significantly change with an increase in Shh concentration from 48nM to 480nM, **which may be due to receptor saturation**.[42] One hypothesis is that all the observed effects of varying Shh concentrations on mesenchymal proliferation and smooth muscle differentiation are due to its graded proliferative effect on bladder mesenchyme. Smooth muscle differentiation occurs after mesenchymal expansion during bladder development. Consequently, the increase in α-actin expression by E12 bladder explants cultured in high concentrations of Shh may be a direct reflection of the population of mesenchymal cells available to undergo differentiation into smooth muscle. However, it is uncertain to what extent smooth muscle differentiation is dependent upon mesenchymal proliferation. Yu postulated that smooth muscle differentiation begins only when mesenchymal precursors reach a specific density.[37] However, there is also evidence that smooth muscle differentiation and patterning are distinct from mesenchymal proliferation and are mediated through different pathways.[44, 45] For example, while bladders of *Gli2* null mice have a normal number of proliferating mesenchymal cells, these cells fail to pattern appropriately. [44]

Other signaling pathways are involved in bladder smooth muscle differentiation. Inhibition of Shh signaling with cyclopamine, which inhibits hedgehog signaling by binding to Smo, decreases or eliminates expression of *Gli1* and *Bmp4*, as well as early markers of smooth muscle differentiation, such as SMAA. However, inhibition of Shh did not decrease expression of SM-MHC.[7] Therefore, while Shh and/or urothelium is necessary for the induction of smooth muscle differentiation, it is clear that other pathways are involved in the development of mature smooth muscle cells. Currently, it is not known what transcription factors, local environmental cues, or signaling cascades mediate these later phases of smooth muscle differentiation.

BLM is exquisitely sensitive to Shh and may have differential responses to Shh concentration. One hypothesis is that high levels of Shh inhibit smooth muscle differentiation. This can be inferred from urothelium recombination experiments in which smooth muscle differentiation was inhibited immediately adjacent to the urothelium where Shh concentrations are presumed to be highest, while smooth muscle differentiation was induced distant from the urothelium where Shh concentrations are presumed lowest.[6] Furthermore, Ptc1 is highly expressed in the mesenchyme immediately adjacent to the urothelium but has markedly decreased expression in the periphery of bladder where smooth muscle differentiation first occurs.[7] An alternative hypothesis is that the fate of cells at

different positions within the bladder wall are determined by transcription factors active earlier in embryogenesis and Shh is simply the universal "on" switch that induces differentiation into previously determined cell types.

Shh has been shown in other organ systems to pattern tissue during embryogenesis; in the nervous system, cells from different areas in the developing brain differentiate into distinct cell types upon exposure to Shh-producing cells.[28, 29] In the gut, smooth muscle differentiation is inhibited in cells in closest proximity to the Shh source.[46] These findings are consistent with Shh acting as a paracrine factor that determines cell fate, possibly as a function of concentration gradient.[28, 29] Shiroyanagi proposed that Shh is necessary for the induction of smooth muscle differentiation but, after induction, smooth muscle differentiation can proceed throughout the mesenchyme only when Shh levels and its downstream mediators fall below the point at which smooth muscle differentiation is inhibited.

The temporal and spatial expression of Shh and its downstream mediators also indirectly support the hypothesis that Shh concentration affects patterning of bladder smooth muscle. At E12.5, *Ptc1*, *Gli1*, and *Bmp4* mRNA is much more highly expressed in the sub-urothelial layer (which is devoid of smooth muscle) than the outer zone of the peripheral mesenchyme (where smooth muscle differentiation begins and is most robust). *Gli1* expression is highly expressed in the subepithelial mesenchyme from E12.5-E13.5 but then decreases after E13.5. The converse is found in the peripheral mesenchyme: *Gli1* expression increases from E12.5 to E15 but never reaches the level of expression seen in the subepithelial layer at E12.5. The expression profile of *BMP4* in the peripheral mesenchyme follows the trend of *Gli1* but is delayed by one day.[47] (Figure 8) These findings support the hypothesis that high levels of BMP4 inhibit smooth muscle formation in the subepithelial layer but permit and/or promote smooth muscle differentiation, as assessed by SMAA and SM-MHC, in the peripheral mesenchyme. Similar to the inhibitory effect it has on mesenchymal proliferation, high levels of BMP4 may inhibit smooth muscle differentiation. If this is confirmed, BMP4 would be a prime candidate for the mediator of Shh on smooth muscle differentiation and patterning in the bladder.[7]

Cheng et *al.* has provided further insight into the Shh-Ptc1-Gli2-BMP4 pathway's role in patterning of bladder smooth muscle by demonstrating that *Gli2* mediates the inductive effect of Shh on mesenchymal proliferation, as well as the radial patterning of smooth muscle seen in the bladder, possibly through regulation of BMP4.[44] Cell proliferation, especially in the subepithelial mesenchyme of the bladder, was significantly reduced in *Gli2*−/− mice. In WT mice, BMP4 co-localized with Gli2 and was highly expressed in the subepithelial mesenchyme of the bladder. In *Gli2* knockouts, BMP4 levels were decreased and smooth muscle formed in the subepithelial mesenchyme where smooth muscle formation is inhibited in WT mice. Some caution must be exercised: while *Gli1* and *Gli2* generally mediate the Shh signal and *Gli3* represses the Shh signal, the interaction between the different Gli signaling molecules and other components of the Shh cascade make interpretation of single knockout mutants difficult.[48, 49] However, the role of Gli2, BMP4, and the Smad pathway, which is activated by Shh, is most likely central to the process of smooth muscle differentiation and patterning in the bladder.

Despite these advances, it is not known what early mediators of mesenchymal migration, other Shh-associated transcription factors, and crosstalk between the Shh signaling cascade and other pathways, are involved in the patterning of bladder smooth muscle. Looking at different organ systems such as the nervous system in which the role of the Shh pathway is better understood may provide areas of investigation that could advance our understanding

of how smooth muscle differentiation and patterning is regulated in the bladder (for review of Shh in brain development, see Ruiz I Altaba).[50]

TGF-β

TGF-β is known to induce smooth muscle differentiation in prostatic stromal cells

[51] TGF-β is also expressed in the developing bladder, with the highest expression levels found at E13.5 in the peripheral mesenchyme.[47] The temporal and spatial similarities of TGF-β expression in the bladder with known mediators of smooth muscle differentiation make TGF-β an attractive candidate for mediating smooth muscle differentiation. TGF-β signaling is primarily transduced by R-Smad 2 and 3; however, members of the TGF-β family have been shown to promote intestinal development and terminal smooth muscle differentiation in 10T1/2 cells through the hedgehog pathway.[13, 52-55]. Lien *et al* demonstrated that TGF-β stimulation of this cell line increased SMAA and SM22α through the PI3K/Akt pathway.[54] Furthermore, it has recently been shown that TGF-β induces expression of *Gli1* and *Gli2* through the Smad pathway, which could serve as possible points of cross-talk with the Shh pathway.[56]

SRF

Serum response factor (SRF) is a DNA-binding phosphoprotein that is a key controller of muscle-specific gene expression. Although it is expressed in a number of organs, SRF is important in muscle cell-specific gene expression and smooth muscle differentiation.[57-59] SRF-mediated transcription of smooth muscle genes depends on the interaction of SRF with appropriate cofactors, the specific combination of which determines the transcriptional target.[60] Myocardin, which is expressed abundantly in smooth muscle cells, is one of these cofactors and has been shown to be important in SRF-dependent gene transcription during smooth muscle differentiation.[61, 62]

SRF is present in E12.5 bladders and localizes to the peripheral mesenchyme.[63] It appears to be required for the initial differentiation of smooth muscle cells and has been proposed to have a role in the expression of SMAA during early smooth muscle differentiation.[63] The SMAA and SM22-α promoters both have binding sites for SRF.[64] SRF localizes to the peripheral bladder mesenchyme prior to SMAA expression.[63] Additionally, SRF is an attractive candidate gene for a downstream mediator of Shh on terminal smooth muscle differentiation as it is expressed most highly in the peripheral mesenchyme of E15 bladders, where SMAA expression has already begun and SM-MHC expression is peaking.[14, 47]. Shiroyanagi *et al.* have proposed that SRF is upregulated in the peripheral mesenchyme where Shh, Ptc1, and BMP4 levels are low, which seems to be necessary for smooth muscle differentiation.[7] (Table 2)

Dlgh1

Discs-large homolog 1 (Dlgh1), which is highly expressed in urothelium but not in smooth muscle, is important for patterning smooth muscle in the ureter. *Dlgh1*–/– mice have a normal urothelium but lack a subepithelial layer and have misaligned ureteral smooth muscle.[65] It is possible that *Dlgh1* regulates epithelial-mesenchymal signaling in the developing ureter and/or is involved in forming cytoskeletal architecture. Although, to our knowledge, the role of *Dlgh1* in the bladder has not been examined, the similarities between mesenchymal proliferation and smooth muscle differentiation in the bladder, kidney, and ureter make *Dlgh1* an attractive candidate gene in bladder development.

DNA-binding proteins

Investigations into mesenchymal development and differentiation in the genitourinary system and other organs have revealed that DNA-binding proteins such as T-box transcription factors are important in early mesenchymal migration and patterning.[66-68] In the ureters of *Tbx18*–/− mice, *Ptc1* is down-regulated and mesenchymal *BMP4* expression is lost. However, unlike *Dlgh* mutants, the epithelium of *Tbx18* knockouts fails to differentiate into a functional urothelium. This results in decreased cell migration from the metanephric mesenchyme to the ureter; the mesenchymal cells that do migrate fail to differentiate into smooth muscle. Although *Tbx18* is not expressed elsewhere in the urogenital system, it is likely that similar gene(s) are active in initial mesenchymal-epithelial interaction in the bladder; however, they have yet to be identified.

Other DNA-binding proteins appear to act later in smooth muscle differentiation. The zinc finger DNA binding protein Teashirst3 (*Tshz3*) is required for smooth muscle differentiation from mesenchyme in the cranial ureter but is not necessary for the earlier event of mesenchymal proliferation.[45] Unlike *Tbx18*, *Tshz3* is expressed in the bladder. At E15.5, *Tshz3* is expressed in the peripheral mesenchyme, which has already differentiated into smooth muscle. At E18.5, *Tshz3* is more highly expressed in the subepithelial layer than in smooth muscle cells.[45] *Tshz3* mediates the Shh signal downstream from *Ptc1* and *BMP4*, and is expressed in both undifferentiated peripheral mesenchyme and differentiating smooth muscle cells. *Tbx18* mutants fail to express myocardin and have been shown to activate non-BMP4 mediated pathways involved in smooth muscle differentiation. This raises the possibility that *Tshz3* is involved in SRF-mediated smooth muscle cell differentiation.

Summary and Future Directions

The research conducted over the past 30 years has greatly advanced our understanding of the developmental biology of smooth muscle in the urinary bladder. In the bladder, as is true in most epithelialized organs, the interaction between the epithelium and mesenchyme is critical to the differentiation and organization of smooth muscle. The urothelium, which secretes Shh, acts in a paracrine fashion on the adjacent mesenchyme. It is likely that Shh is the factor that links mesenchymal proliferation and smooth muscle differentiation during normal bladder development.

First, Shh induces cellular proliferation, possibly by regulating the mitotic cycle through the interaction between Ptc1 and cell cycle regulatory proteins as well as through the Gli pathway. It is possible that BMP4 may indirectly inhibit mesenchymal proliferation through as yet unidentified mechanisms. Then, once a critical mass of mesenchymal cells has been achieved, Shh acts along the well-described pathway through the *Gli* proteins and BMP4 to regulate smooth muscle differentiation and patterning. Further work should be done to elucidate what is assuredly a complex interaction between genes that orchestrate early mesenchymal migration and patterning, Shh and its known downstream mediators, and as yet unidentified proteins, during the fluid process of bladder development.

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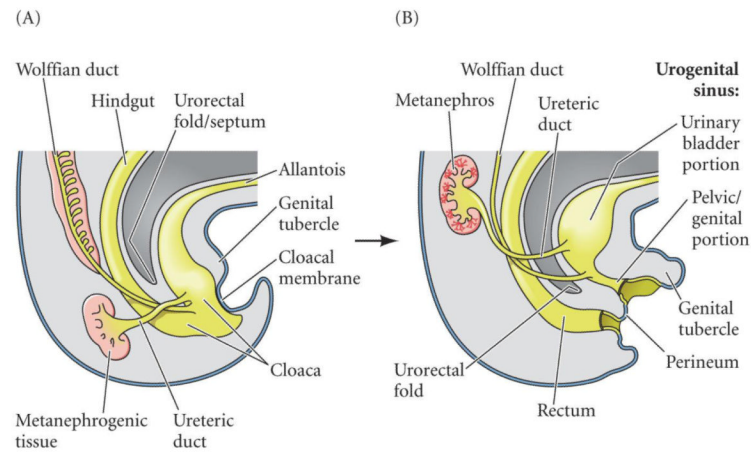
Figure 1. Bladder embryogenesis
A) In humans, the division of the cloaca into the urogenital sinus (UGS) and the rectum occurs during the 6th week of gestation. The mesodermal-derived urorectal fold consists of two structures: the upper fold (Tourneux) divides the cloaca in a cranial-caudal direction and fuses in the midline with the lower fold (Rathke), which partitions the UGS and hindgut in a lateral-medial direction. B) The bladder then forms from the cranial aspect of the UGS. The allantois becomes the urachus and, in the adult, the median umbilical ligament. Reproduced with permission from Developmental Biology, Eighth Edition, 2006, Sinauer Associates Inc.
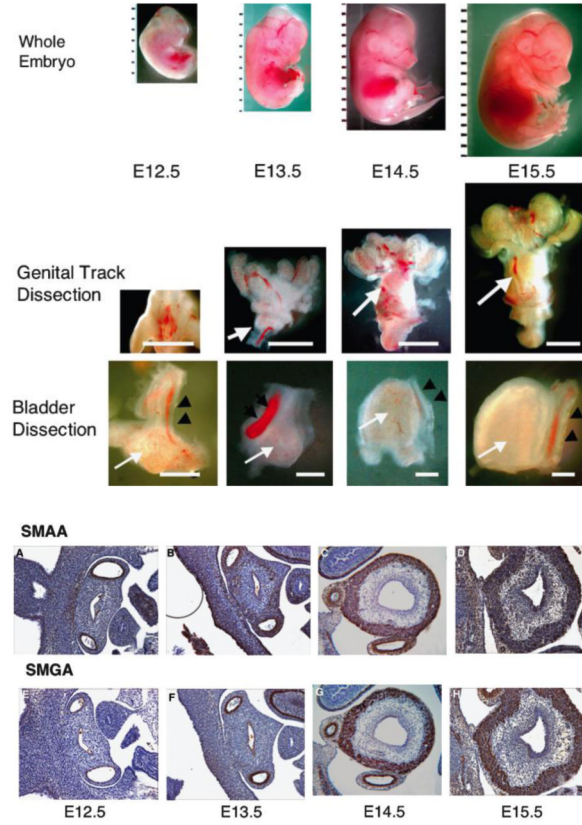
Figure 2. Time course of bladder organogenesis and smooth muscle differentiation in the mouse
Gross dissections of mouse embryos: whole embryo (upper panels), genital tracts (middle panels), and bladder with umbilical arteries (lower panels). The white arrows indicate the urinary bladder. The black arrowheads indicate the umbilical arteries. Top panel: Ruler = 1 mm. Bars in the middle panels are 1 mm. Bars in the lower panels are 100 mm. Smooth muscle (SM) actin staining of the embryonic bladder. (A–D) Smooth muscle α-actin (SMAA), (E–H) smooth muscle γ-actin (SMGA). E12.5 (A and E), E13.5 (B and F), E14.5 (C and G), and E15.5 (D and H). Note the positive staining of the umbilical arteries. Final magnifcation 100X A-H. Reproduced with permission from Shiroyanagi et al., Differentiation 2007. [7]
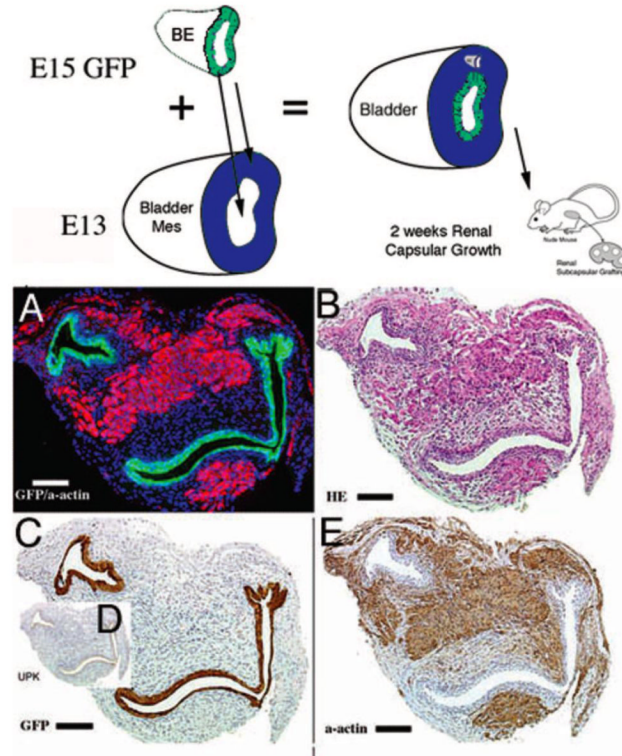
Figure 3. Epithelium patterns bladder smooth muscle
Schematic and results of urothelial recombination with bladder mesenchyme in the orthotopic location. Histologic serial sections: (*A*) Color triple florescent stain, GFP is green, alpha-smooth muscle actin is pink and Hoescht dye is blue representing the zone of smooth muscle inhibition or submucosa; (*B*) H&E dbff hematoxylin and eosin; immunohistochemistry: (*C*) GFP = green fluorescent protein; (*D*) UPK= uroplakin and (*E*) α-actin = smooth muscle alpha-actin. (magnification bar = 100 μm). Reproduced with permission Cao et al., Pediatric Research 2008. [6]
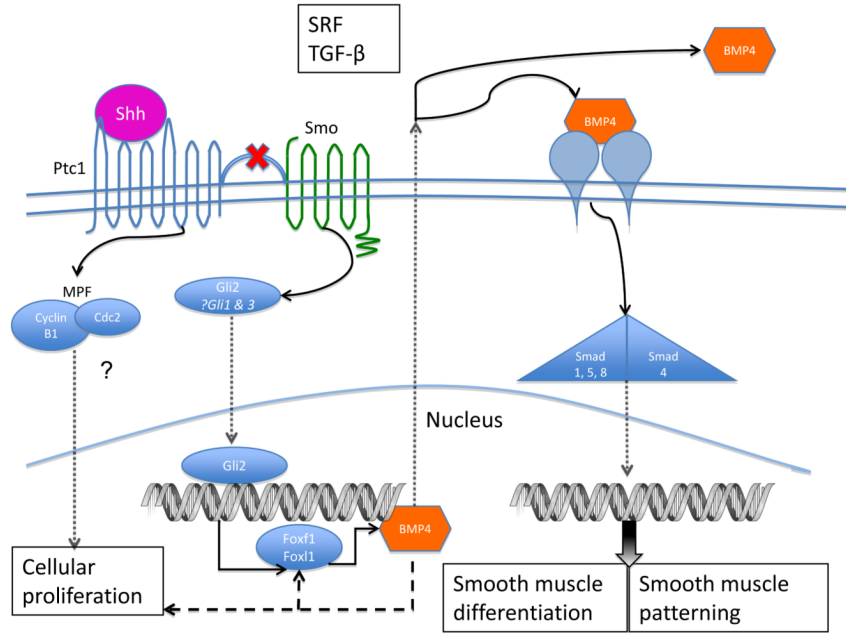
Figure 4. Proposed Shh signaling pathway in bladder mesenchymal cells
Upon binding Shh, Ptc1 releases the inhibition of Smo. The effect of Shh on smooth muscle differentiation and patterning is mediated by *Gli2*, which, upon translocation into the nucleus, regulates downstream genes including BMP4. BMP4 may inhibit cellular proliferation and is a prime candidate for mediating the effect of Shh on smooth muscle patterning during bladder development. Other signaling molecules, such as TGF-β and SRF, likely have a role in bladder smooth muscle differentiation and may interact with the Shh pathway. The mechanism through which Shh affects cellular proliferation in the bladder is uncertain but MPF has been shown to be a Shh-responsive complex that regulates mitosis in other systems.
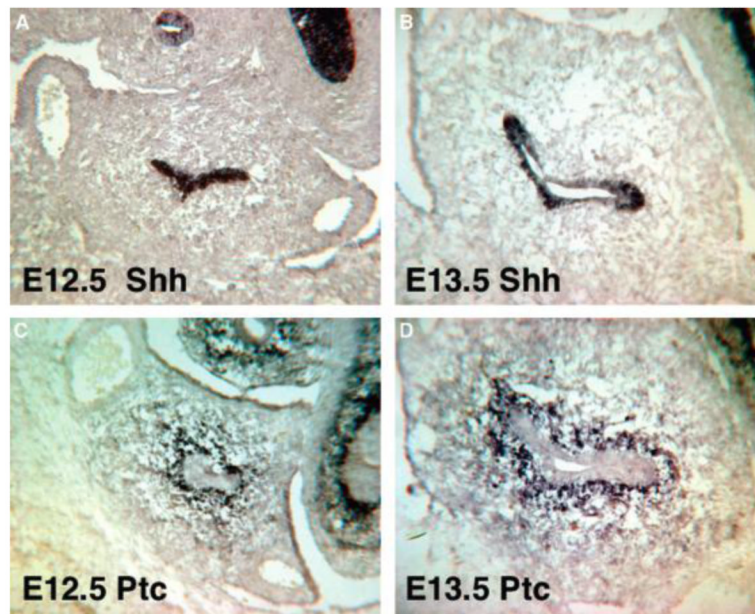
Figure 5. Shh and Ptc expression in the fetal bladder
In situ hybridization of the embryonic bladder. Sonic hedgehog (Shh) (A and B), Patched (Ptc) (C and D). E12.5 bladder (A and C), E13.5 bladder (B and D). Final magnification 100x. Reproduced with permission from Shiroyanagi et al., Differentiation 2007. [7]
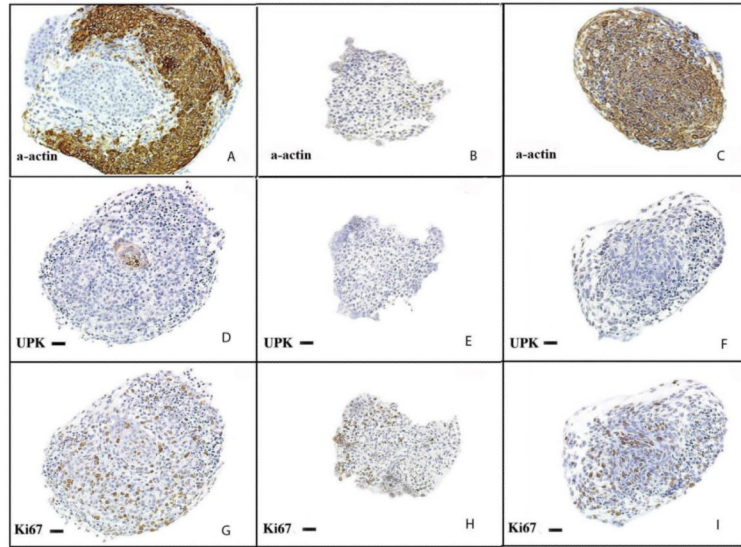
Figure 6. Urothelium and Shh regulates BLM smooth muscle differentiation
Immunohistostaining of explants after 72 hours of incubation in serum free DMEM (20X).
(A, D, G = intact bladders incubated without Shh) (A) Intact bladders incubated without Shh
demonstrated strong α-actin staining of smooth muscle. (D) The urothelium, which stained
for UPK, is seen at the center of the section (G) Diffuse expression of Ki67 was detected,
indicating specimen viability. (B, E, H = BLM incubated in Shh-free media) (B) Neither
smooth muscle nor (E) urothelium were detected. (H) K67 expression was detected,
indicating viability. (C, F, I = BLM cultured with 480nM of Shh) (C) α-actin was expressed
homogenously throughout the explant, indicating robust smooth muscle differentiation. (F)
Uroplakin was not detected, demonstrating an absence of urothelial contamination of the
specimen. (I) Ki67 expression was present. α-actin=smooth muscle α-actin,
UPK=uroplakin, Ki67=nuclear proliferation marker. Final magnification 100x. Reproduced
with permission from Cao et al., Differentiation 2010. [42]
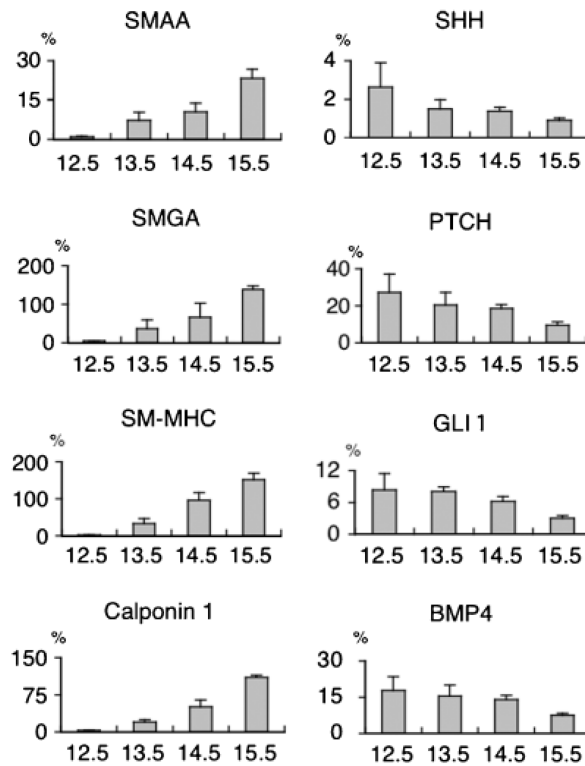
Figure 7. mRNA expression levels of Shh-pathway and smooth muscle genes in the fetal bladder
Percent RNA gene expression normalized to GAPDH mRNA by real-time reverse
transcription polymerase chain reaction (RT-PCR) in embryonic bladders from E12.5 to
E15.5 mice. (Y-axis: % mRNA gene expression against GAPDH by real-time RT-PCR).
Reproduced with permission from Shiroyanagi et al., Differentiation 2007. [7]
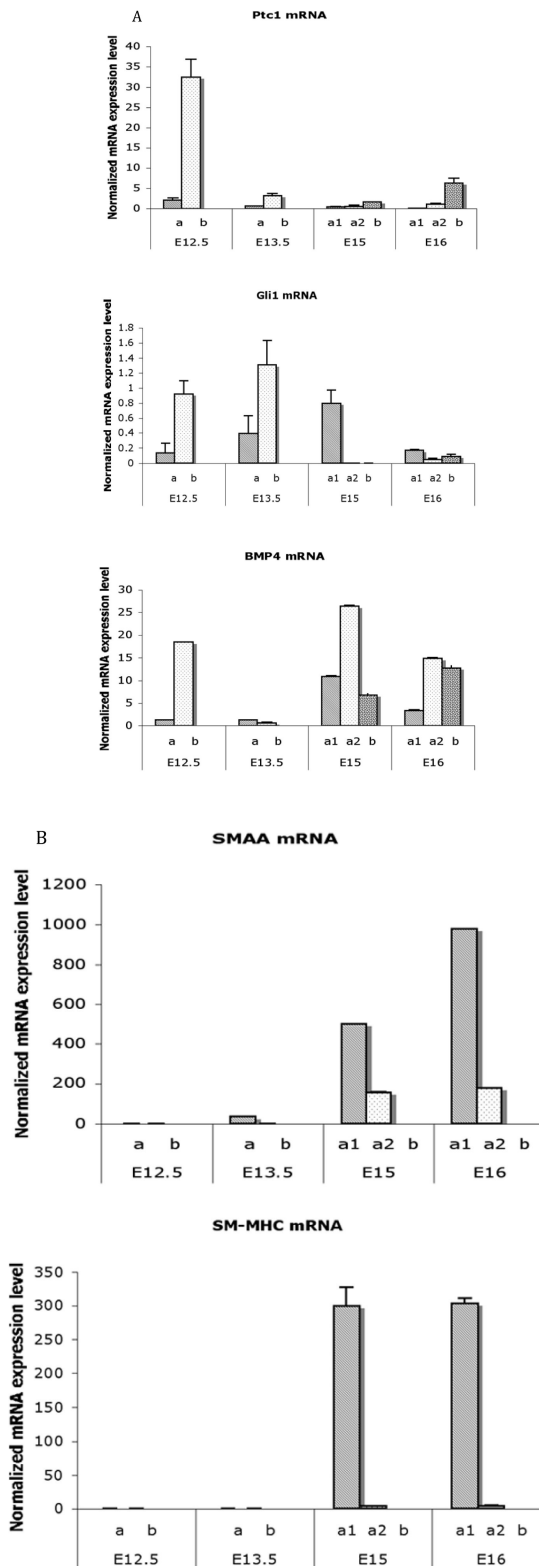
Figure 8. Differential spatial expression of Shh-pathway and smooth muscle genes in the fetal bladder

A) Real time RT-PCR analysis of mRNA encoding Ptc1, Gli1 and Bmp4 in laser-captured mesenchymal components from each gestational stage. Expression of Ptc1, Gli1 and Bmp4 was restricted to a thin layer of mesenchymal cells in the submucosa immediately adjacent to the epithelium. Expression of both Ptc1 and Bmp4 genes was significantly decreased in all mesenchymal cells in the E13.5 bladder. Ptc1 remained at a low level in contrast to Bmp4 whose expression was increased at E15 and E16. Gli1 gene expression was up regulated in the submucosa at E13.5. At E15 the expression changed to the smooth muscle layer (Mann-Whitney test, $p < 0.001$). a = serosal zone; a1 = smooth muscle layer; a2 = intermediate zone; b = submucosal zone.

B Real time RT-PCR analysis of mRNA encoding SMAA and SM-MHC in laser captured mesenchymal components from each gestational stage. Expression of SMAA mRNA was detectable in the peripheral cellular population at E13.5 and increased to significantly high levels in smooth muscle cells at E15 and E16. SM-MHC was detectable temporal slightly later than SMAA and increasing in expression at E15 and E16 (Mann-Whitney test, $p < 0.001$). a = serosal zone. a1 = smooth muscle layer; a2 = intermediate zone; b = submucosal zone. Reproduced with permission from Liu et al., Int J Dev Biol., 2010. [47]

Table 1

Role of Shh and BMP4 in different organs

Organ	Shh expression in epithelium		BMP4 expression in mesenchyme	Reference
	Important for mesenchymal growth	Important for smooth muscle differentiation		
Bladder	Yes	Yes	Yes	[7, 26, 47, 69]
Ureter	Yes	Yes	Yes	[37]
Kidney	Yes	Yes	Yes	[26, 37]
Prostate	Yes	?	Yes[a]	[70-74]
External genitalia	Yes	N/A	Yes	[25, 69, 75, 76]
Vagina	?	?	?	
Lung	Yes	Yes	Yes	[77-79]
Gut	Yes	Yes	Yes	[20, 46, 78, 80-82]
Uterus	Yes[b]	No	No	[83]
Pancreas	No[c]	N/A	Yes[d]	[84-86]
Tooth	Yes	N/A	Yes	[36, 87, 88]
Somite	Yes	N/A	Yes	[89, 90]
Mammary gland	No[e]	N/A	Yes	[91-93]

[a]Unlike Shh, BMP4 inhibits prostatic duct budding and branching.

[b]Progesterone induces Ihh expression in the adult mouse.

[c]Shh suppression is necessary for the initiation of pancreas development but tightly regulated expression is necessary for later organogenesis.

[d]BMP4 expression by the septum transversum mesenchyme is necessary to suppress pancreas differentiation from the endoderm.

[e]Ihh, rather than Shh, appears to be the most important signal necessary for mammary gland development

Table 2

Smooth muscle development-related gene expression profiles in the mouse embryonic mesenchyme

Bladder mesenchyme	E12.5	E13.5		E15	E16
Peripheral zone		SMAA TGF-β1 Gli1	*Outer zone*	Gli1 Bmp4 SRF TGF-β1 SRF SMAA SM-MHC	SMAA Bmp4
			Inner zone	SMAA Bmp4	SMAA Bmp4
Subepithelium	Ptc1 Gli1 Bmp4	Gli1		TGF-β1 Bmp4	Ptc1 Bmp4

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