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Antagonism of BMP4 Signaling Disrupts Smooth Muscle Investment of the Ureter and Ureteropelvic Junction

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Abstract

Purpose—Congenital ureteropelvic junction obstruction has been associated with aberrant ureteral smooth muscle organization. Recent evidence has shown that BMP4 may be involved in ureteral morphogenesis. We determined whether the disruption of BMP4 signaling results in abnormal smooth muscle investment of the ureter and ureteropelvic junction.

Materials and Methods—We used a *Cre* mediated *Bmp4* knockout system to conditionally excise the *Bmp4* gene in developing mouse embryos. Kidney rudiments were isolated from embryos at varying gestational ages from WT and conditional knockout mice. Metanephric kidney explants were cultured in the presence or absence of the BMP antagonist Noggin. Agarose beads pre-incubated with Gremlin, another BMP antagonist, were used for localized disruption of BMP signaling. Frozen sections and whole metanephric explants were then analyzed by immunofluorescence.

Results—*Bmp4* gene excision resulted in a dose dependent loss of ureteral smooth muscle. Antagonism of BMP signaling inhibited ureteral smooth muscle investment in a dose dependent manner and was paralleled by a dose dependent decrease in the immediate downstream targets of BMP signaling, phosphorylated Smad1, 5 and 8. Localized antagonism of BMP resulted in the focal disruption of ureteral smooth muscle investment.

Conclusions—We report that decreased BMP signaling, whether by the loss of BMP4 in vivo or direct antagonism in vitro, results in a gradual reduction of the normal, well organized coat of smooth muscle surrounding the ureter. Our results also suggest that this occurs via a direct Smad dependent pathway. This raises the possibility that abnormalities in BMP4 signaling may have a role in the development of congenital ureteropelvic junction obstruction.

Keywords

ureter; ureteral obstruction; abnormalities; bone morphogenetic protein 4; gene expression

Congenital UPJ obstruction is the most common cause of urinary tract dilatation in children, occurring in 1/1,000 to 2,000 newborns.¹ Recent studies indicate that congenital obstructions may in part be due to aberrant ureteral smooth muscle organization, resulting in impaired peristalsis.^{2–4} Additionally, surgical specimens of obstructed human UPJ segments

have demonstrated deficient or maloriented smooth muscle fibers,⁵ abnormal collagen deposition,⁶ increased myocyte apoptosis,⁷ abnormal innervation⁸ and altered distribution of interstitial cells of Cajal.⁹

Ureteral peristalsis depends on the acquisition of a functional smooth muscle investment. However, the signaling pathways that mediate this process in the developing embryo remain poorly understood.² We recently reported that ureteral smooth muscle derives from a mesenchymal population located in the most caudal aspect of the embryo and paracrine factors secreted by this tissue surrounding the nascent ureter are essential for urothelial and smooth muscle differentiation.¹⁰ Specifically we found that BMP4, a member of the transforming growth factor- β family, is expressed by the periureteral mesenchyma and is essential for ureteral morphogenesis.¹⁰ We further noted that ectopic BMP4 signaling converts tubules normally fated to form the intrarenal collecting system into tubules, showing a ureteral phenotype.¹⁰

In the current study we further analyzed the role of BMP signaling in mediating ureteral smooth muscle formation because abnormalities in this process may contribute to congenital obstructions between kidney and ureter. Using murine metanephric kidney explants we noted that BMP4 signaling antagonism inhibited smooth muscle differentiation in the mesenchymal cell population surrounding the nascent ureter. We further observed that soluble BMP antagonists inhibited smooth muscle differentiation in a dose dependent manner, as paralleled by decreased phosphorylation of Smad1, 5 and 8, which are downstream targets of BMP signaling.¹¹ Finally, we found that localized antagonism of BMP4 signaling can result in focal defects in the ureteral smooth muscle coat, similar to those seen in congenital UPJ obstruction. Collectively our results suggest that the abnormal smooth muscle architecture observed in obstructed UPJ segments may in part be due to deficient BMP4 signaling during embryogenesis.

MATERIALS AND METHODS

Animals

Timed pregnant Swiss Webster female mice (Taconic Farms, Germantown, New York) were housed at the Cornell Animal Facility and treated according to research animal resource center guidelines. Mice homozygous for the Bmp4 conditional allele created by the introduction of Cre recombinase (loxP) sites into the Bmp4 locus¹² were also used. Our procedure to conditionally excise the Bmp4 gene has been described previously.¹⁰ Briefly, Bmp4^{flox/flox} females were mated with Cre/Esr1 heterozygous male mice¹³ (Jackson Laboratories, Bar Harbor, Maine). Bmp4^{flox/+} male offspring carrying the Cre recombinase transgene were selected and mated with Bmp4^{flox/flox} females to generate Bmp4^{flox/flox}; Cre/Esr1 embryos. Complete Bmp4 excision was confirmed using in situ hybridization. As reported previously, BMP4 is required for embryonic viability.¹² Bmp4^{flox/flox} embryos carrying the Cre transgene died in utero approximately 24 hours after timed pregnant dams were injected with 5 mg tamoxifen (Sigma®).¹³

Metanephric Explant Cultures

Kidney rudiments were isolated from mouse embryos at the gestational stages noted. Appearance of a vaginal plug was designated as E0.5. Our explant culture system has been described previously.¹⁴ Recombinant BMP4, Noggin and Gremlin were used (R & D Systems®).

Immunofluorescence

Frozen sections and kidney explants were used for immunofluorescent analysis using previously described protocols.¹⁴ Specimens were analyzed using a C4742-95 digital camera (Hamamatsu, Shizuoka, Japan) and a MetaMorph® Image capturing system. The primary and secondary antibodies used were Cy3 conjugated mouse monoclonal SMA (Sigma) (1:400), E-cadherin (R & D Systems) (1:200) and donkey anti-goat (1:200) as the secondary antibody, phosphorylated Smad1, 5 and 8 (Cell Signaling Technologies®) (1:100), and donkey anti-rabbit (1:200) as the secondary antibody, and DAPI (Sigma, 1:1,000).

RESULTS

***Bmp4* Gene Dose Correlated With Ureteral Smooth Muscle Coat Thickness**

We used a transgenic approach to delete BMP4 signaling in the developing urinary tract. Female mice harboring floxed *Bmp4* alleles were mated with males carrying a transgene encoding a fusion protein of Cre recombinase and *Esr1* (*Cre/Esr1*). As described previously, maternal administration of the synthetic estrogen tamoxifen results in Cre mediated excision of the floxed *Bmp4* alleles.^{10,13} Timed pregnant mice were injected with tamoxifen at E12 and embryos were harvested 6 hours after injection.¹³ When assayed for the expression of E-cadherin and SMA, control embryos harboring floxed *Bmp4* alleles in the absence of the *Cre* transgene (*Bmp4^{lox/lox}*) demonstrated E-cadherin expression by the epithelium of the ureter and the intrarenal collecting tubules as well as a thick, well organized circumferential coat of SMA positive cells surrounding the ureter (fig. 1, A). Mice heterozygous for the floxed *Bmp4* allele carrying the *Cre* transgene (*Bmp4^{lox/+}, Cre/Esr1*) demonstrated a less robust coat of SMA positive cells investing the ureter (fig. 1, B). This was confirmed quantitatively since the width of the smooth muscle coat surrounding the ureter was decreased in heterozygous *Bmp4^{lox/+}, Cre/Esr1* mice compared to that in controls (32 vs 37 μ m, $p = 0.002$, fig. 2). Mice homozygous for the floxed *Bmp4* alleles carrying the *Cre* transgene (*Bmp4^{lox/lox}, Cre/Esr1*) had minimal, scattered SMA positive cells that did not form a well organized coat around the ureter (fig. 1, C). Cre mediated *Bmp4* excision was documented by polymerase chain reaction and confirmed by in situ hybridization. These data suggest that the *Bmp4* gene dose may have a role in determining the width of the ureteral smooth muscle coat.

Antagonism of BMP Signaling Inhibited Ureteral Smooth Muscle Investment in a Dose Dependent Manner

To better delineate the relationship between the *Bmp4* gene dose and ureteral smooth muscle investment we used the soluble BMP antagonist Noggin¹⁵ to titrate the level of BMP

signaling in the developing ureter in vitro. Kidney rudiments were isolated at E12.5 and cultured in the presence of 1, 5 and 10 $\mu\text{g/ml}$ recombinant Noggin for 5 days. Rudiments were assayed for E-cadherin and SMA expression. Controls demonstrated a thick, well organized coat of SMA positive cells surrounding the ureter (fig. 3, A, E and I). Cultures supplemented with 1 $\mu\text{g/ml}$ Noggin had SMA positive cells surrounding the ureter but smooth muscle coat thickness was decreased (fig. 3, B, F and J). At 5 $\mu\text{g/ml}$ Noggin even fewer SMA expressing cells were found surrounding the ureter (fig. 3, C, G and K). Finally, at 10 $\mu\text{g/ml}$ Noggin few scattered SMA positive cells could be seen but they did not form a well organized circumferential coat (fig. 3, D, H and L). Collectively these results indicate that the number and organization of myocytes present in the ureteral smooth muscle coat are regulated in part by the level of BMP signaling.

Phosphorylated Smad1, 5 and 8 Expression Paralleled Smooth Muscle Investment Loss in a Dose Dependent Manner

Smad1, 5 and 8, which are the immediate downstream targets of BMP receptors, have a vital role in BMP signal transduction.¹¹ Therefore, we determined whether the decrease in ureteral smooth muscle investment due to BMP4 antagonism would induce a dose dependent decrease in Smad1, 5 and 8 phosphorylation. Rudiments were isolated at E12.5, cultured in the presence of recombinant BMP4 or Noggin (1, 5 and 10 $\mu\text{g/ml}$) for 5 days and then assayed for the expression of E-cadherin, and phosphorylated Smad1, 5 and 8. Control cultures demonstrated abundant phosphorylated Smad1, 5 and 8 expression in the mesenchyma surrounding the ureter and medullary interstitium with minimal to no expression in the ureteral epithelium (fig. 4, A and F). When cultures were supplemented with 250 ng/ml BMP4 (fig. 4, B and G), an increase in phosphorylated Smad1, 5 and 8 expression was seen. In contrast, cultures supplemented with Noggin demonstrated a dose dependent decrease in phosphorylated Smad1, 5 and 8 expression (fig. 4, C to E and H to J). At 1 $\mu\text{g/ml}$ Noggin phosphorylated Smad1, 5 and 8 activity was decreased compared to that in controls but it was again seen in the mesenchyma surrounding the ureter but not in the ureteral epithelium (fig. 4, C and H). Importantly the number of cells present in the periureteral mesenchyma was similar under all experimental conditions, as determined by nuclear DAPI staining (fig. 4, K to M). Thus, the decreased levels of phosphorylated Smad1, 5 and 8 were not due to a decrease in the overall number of cells in the mesenchyma surrounding the ureter, but rather to a decrease in the number of cells responding to BMP signaling.

Localized Antagonism of BMP Signaling Resulted in Focal Disruption of Ureteral Smooth Muscle Investment

Congenital UPJ obstruction in humans is characterized by a focal rather than a global disruption in ureteral smooth muscle.⁶ Therefore, we tested whether localized perturbation of BMP signaling mimics the focal character of UPJ obstruction. Kidney rudiments were isolated at E12.5 and cultured for 5 days in the presence or absence of agarose beads pre-incubated with the BMP antagonist Gremlin,¹⁵ as described by Vainio et al.¹⁶ Gremlin was used because it has higher specific activity than recombinant Noggin and, therefore, it is better suited for focal inhibition. Specimens with implanted beads were assayed for E-cadherin and SMA expression. Controls demonstrated a thick, well organized coat of SMA

positive cells investing the ureter (fig. 5, A). Cultures in which agarose beads were pre-incubated in PBS showed a thick coat of SMA positive cells, similar to that seen in controls (fig. 5, B). In contrast, Gremlin coated beads induced focal disruption of the thick, well organized coat of SMA positive cells seen in controls (fig. 5, C).

DISCUSSION

Previous studies of congenitally obstructed UPJ segments have demonstrated an aperistaltic segment with varying levels of absent, deficient or maloriented smooth muscle fibers.^{5,6} To our knowledge the mechanism by which this develops is unknown. Our findings using a Cre mediated *Bmp4* conditional knockout demonstrate that the thickness of the smooth muscle coat surrounding the ureter is related to the *Bmp4* gene dose. To better delineate the relationship between the level of BMP signaling and ureteral smooth muscle morphology we cultured metanephric kidney explants in increasing doses of the BMP antagonist Noggin. We found a dose dependent decrease in smooth muscle investment of the ureter at increasing Noggin doses. Additionally, we noted that the downstream targets of BMP signaling, phosphorylated Smad1, 5 and 8, similarly decreased in a dose dependent manner with increasing BMP signaling antagonism and this expression occurred predominantly in the mesenchyma surrounding the ureter but not in the ureteral epithelium. Therefore, our results suggest that BMP4 may induce mesenchymal smooth muscle differentiation via a direct Smad dependent pathway. Finally, we found that localized antagonism of BMP signaling with Gremlin resulted in deficient and maloriented SMA positive cells surrounding the ureter in a manner similar to that observed in previous investigations of obstructed UPJ segments.^{5,6} Our results in conjunction with those of previous studies⁴ further implicate BMP signaling in normal smooth muscle investment of the ureter and raise the possibility that a disruption of BMP4 signaling during ureteral development may have a role in the pathogenesis of congenital UPJ obstruction.

BMP signaling is mediated by types I and II BMP receptors as well as by their downstream targets Smad1, 5 and 8. Phosphorylated Smad1, 5 and 8 form a complex with Smad4 and are then translocated to the nucleus to affect transcription.¹¹ Interestingly Oxburgh et al reported that targeted deletion of Smad4 in the epithelium of the nascent ureter did not grossly affect ureteral epithelial differentiation in vivo.¹⁷ However, it is not clear what effect the deletion of Smad4 would have on the mesenchymal population surrounding the nascent ureter and whether this would result in abnormal ureteral smooth muscle investment. Our results demonstrate that loss of BMP4 is associated with a dose dependent decrease in SMA positive cells investing the ureter as well as in a parallel decrease in phosphorylated Smad1, 5 and 8 expression in the surrounding mesenchyma. Therefore, targeted deletion of Smad4 in the epithelium of the nascent ureter may have no effect on ureteral smooth muscle development if BMP4 acts via a direct pathway to induce the surrounding mesenchyma to differentiate into smooth muscle. While our data suggest that the role of BMP4 in normal smooth muscle investment of the ureter occurs via a direct Smad dependent pathway, further functional studies of Smad4 deletion are required to elucidate the mechanism by which BMP4 regulates ureteral smooth muscle development. Furthermore, Foletta et al recently noted in an experimental model of pulmonary hypertension that the downstream effects of BMP4 activation of the type II BMP receptor are mediated by Smad independent pathways,

in which the cytoskeletal regulator LIMK1 mediates smooth muscle proliferation.¹⁸ Therefore, further studies are necessary to investigate whether BMP4 mediated smooth muscle investment of the ureter may be regulated by Smad independent pathways, such as LIMK1 signal transduction.

Recently Hoshino et al generated a *Gata2* hypomorphic mutant mouse model to determine whether the loss of GATA-2 activity would result in decreased *Bmp4* expression in the intermediate mesoderm at the time of murine UB emergence.¹⁹ Phenotypically these mice demonstrated hydro-ureteronephrosis in a manner similar to that seen in human congenital anomalies of the kidney and ureter. Our data complement these findings and suggest that GATA-2 may regulate *Bmp4* transcription and disruption of this signaling pathway may contribute to congenital anomalies of the kidney and ureter, including but not limited to UPJ obstruction.

A limitation of our study is that Noggin is a soluble antagonist against all BMPs, not only BMP4.^{10,11,15,17} In this family of signaling proteins BMP7 and BMP4 are the major BMPs involved in renal and ureteral development.^{11,15} Therefore, it is important to address whether the disruption of ureteral smooth muscle development that we observed is due to antagonism of BMP4 and/or BMP7. We have previously reported that BMP7 is not expressed in the mesenchyma surrounding the nascent ureter at the time point (E11.5) required for normal smooth muscle investment of the ureter.¹⁰ Using in situ hybridization we observed that of all BMPs only *Bmp4* mRNA is expressed in the peri-ureteral mesenchyma at the initiation of murine ureteral smooth muscle investment.¹⁰ Finally, we also noted that ectopic BMP4 but not ectopic BMP7 is sufficient to induce ureteral morphogenesis in the proximal UB zones that are normally fated to become intrarenal collecting tubules.¹⁰ Therefore, while Noggin could exert its effects by antagonizing any combination of BMPs, including but not limited to BMP4 and BMP7, we believe that our findings taken together with our previous studies and our conditional *Bmp4* knockout model provide initial evidence for the role of BMP4 in ureteral smooth muscle development.

The ureter and the intrarenal collecting tubules are derived from the same epithelial primordium, called the UB. The UB has a proximal domain from which the intrarenal collecting tubules are derived and a distal domain that becomes the ureter. We have previously reported that spatially restricted *Bmp4* expression in the mesenchyma surrounding the distal but not the proximal UB is necessary for ureteral morphogenesis.¹⁰ However, to our knowledge the mechanism by which BMP4 signaling is restricted to the distal but not proximal UB is not understood. In the current study we noted that localized antagonism of BMP4 with agarose beads incubated in Gremlin resulted in focal disruption of ureteral smooth muscle investment.

Previously Michos et al observed that *Gremlin-1* is expressed in vivo by the metanephric mesenchyma surrounding the proximal UB and Gremlin mediated antagonism of BMP signaling is essential to induce metanephric kidney development.²⁰ This raises the possibility that endogenous Gremlin signaling may be spatially restricted to the mesenchyma surrounding the proximal UB, while BMP4 expression is restricted to the distal UB. Since the UPJ arises from the border between the proximal and the distal UB, it is possible that

congenital UPJ obstruction may be a result of focal aberrant Gremlin expression in the mesenchyma surrounding the affected ureteral segment. To address this question further characterization of Gremlin expression in the metanephric mesenchyma during UB differentiation is required.

We report that decreased BMP4 signaling, whether by direct antagonism in vitro or by BMP4 loss in vivo, results in a gradual loss of ureteral smooth muscle investment. Our results also suggest that this may occur via a direct Smad dependent pathway. Therefore, it is possible that congenital UPJ obstruction may in part be due to absent or insufficient BMP4 signaling at the obstruction site, resulting in a segment with abnormal smooth muscle function. To our knowledge whether this defect is due to errors in mesenchymal migration, to ectopic Gremlin signaling or insufficient BMP4 signaling at the obstruction site, or to downstream defects in the signaling pathways mediating UB differentiation is not known. Nevertheless, our findings further elucidate the complex mechanisms underlying ureteral smooth muscle investment and, therefore, they may provide insight into the pathophysiology of congenital UPJ obstruction.

CONCLUSIONS

We report that decreased BMP4 signaling, whether by loss of BMP4 in vivo or by direct antagonism in vitro, results in a gradual decrease in the well organized coat of smooth muscle that normally surrounds the ureter. Our results suggest that this may occur via a direct Smad dependent pathway. This raises the possibility that abnormalities in BMP4 signaling may have a role in the development of congenital UPJ obstruction.

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Abbreviations and Acronyms

BMP	bone morphogenetic protein
DAPI	(4,6-diamidino-2-phenylindole)
E	embryonic day
Esr1	estrogen receptor 1
SMA	α -smooth muscle actin
UB	ureteral bud
UPJ	ureteropelvic junction

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Figure 1.

Bmp4 gene dose correlated with ureteral smooth muscle coat thickness. Cre-flox system was used to conditionally excise *Bmp4* gene. Kidney rudiments from E12.5 embryos were cultured for 4 days with 4-OH tamoxifen and assessed for expression of E-cadherin (green areas) and SMA (red areas). WT rudiments without *Cre* recombinase transgene (*Bmp4*^{flox/flox}) demonstrated thick, well organized coat of SMA positive cells surrounding ureter (arrow) (A). Mice heterozygous for floxed *Bmp4* allele and carrying *Cre* recombinase transgene (*Bmp4*^{flox/+}; *Cre-Esr1*) showed SMA positive cells surrounding ureter but smooth muscle coat was less robust than in WT mice (B). Mice homozygous for floxed *Bmp4* allele and carrying *Cre* recombinase transgene (*Bmp4*^{flox/flox}; *Cre-Esr1*) demonstrated few, disorganized SMA positive cells surrounding ureter (C).

Figure 2.

Smooth muscle coat width was decreased in heterozygous *Bmp4*^{flox/+}; *Cre-Esr1* mice compared to WT *Bmp4*^{flox/flox} mice. Six 20× high power fields were analyzed per embryo with 10 muscle coat width measurements made from each high power field. Measurement of smooth muscle coat width and data analysis were performed in blinded fashion. Median smooth muscle coat width was 32 and 37 μm in heterozygous and WT mice, respectively (Student's 2-tailed t test p = 0.002).

Figure 3.

Antagonism of BMP signaling with Noggin inhibited ureteral smooth muscle investment in dose dependent manner. Whole mount kidney rudiments from E12.5 embryos were cultured for 5 days in absence or presence of recombinant Noggin. Control cultures revealed E-cadherin (green areas) expressed by epithelium of renal tubules and ureter (A, E and I). SMA positive cells (red areas, arrow) were expressed by mesenchyma surrounding ureter, forming thick, well organized coat of smooth muscle. At increasing doses of Noggin, including 1 (B, F and J), 5 (C, G and K) and 10 $\mu\text{g/ml}$ (D, H and L) thick, well organized coat of SMA positive cells surrounding ureter decreased in dose dependent manner. At 10 $\mu\text{g/ml}$ there were few scattered SMA positive cells, which did not form well organized coat around ureter.

Figure 4.

Phosphorylated Smad1, 5 and 8 expression decreased in dose dependent manner with increasing inhibition of BMP signaling. Whole mount kidney rudiments from E12.5 embryos were cultured for 5 days in presence of recombinant BMP4 or Noggin. In control cultures E-cadherin (red areas) was expressed by epithelium of renal tubules and ureter. Phosphorylated Smad1, 5 and 8 (green areas) were visible in mesenchyma surrounding ureter and medullary interstitium (arrows) (A and F). In cultures supplemented with BMP4 there was increased phosphorylated Smad1, 5 and 8 expression (B and G). In contrast, cultures supplemented with 1 $\mu\text{g/ml}$ Noggin showed decreased phosphorylated Smad1, 5 and 8 expression compared to controls (C and H). At increasing Noggin doses, including 5 (D and I) and 10 $\mu\text{g/ml}$ (E and J), phosphorylated Smad1, 5 and 8 expression continued to decrease and was virtually absent at 10 $\mu\text{g/ml}$ Noggin. In cultures supplemented with Noggin nuclear DAPI staining was similar at all doses (arrows), demonstrating that dose dependent decrease in phosphorylated Smad1, 5 and 8 expression was not due to decrease in number of cells surrounding ureter (K to M).

Figure 5.

Localized antagonism of BMP signaling focally disrupted ureteral smooth muscle investment. Kidney rudiments from E12.5 embryos were cultured for 5 days in presence of agarose beads pre-incubated in known BMP antagonist Gremlin. Specimens were assessed for expression of E-cadherin (green areas) and SMA (red areas). Control cultures demonstrated thick, well organized coat of SMA positive cells (arrow) surrounding ureter (A). Cultures in which agarose beads (arrow) were pre-incubated in PBS showed that bead alone did not locally disrupt ureteral investment by SMA positive cells (B). Beads pre-incubated (arrow) in 2.5 $\mu\text{g}/\mu\text{l}$ Gremlin locally disrupted normal smooth muscle investment of ureter, demonstrating few disorganized SMA positive cells surrounding ureter adjacent to bead (C).