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Regulation of BMP7 expression during kidney development

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SUMMARY

Members of the *Bone Morphogenetic Protein (BMP)* family exhibit overlapping and dynamic expression patterns throughout embryogenesis. However, little is known about the upstream regulators of these important signaling molecules. There is some evidence that BMP signaling may be autoregulative as demonstrated for *BMP4* during tooth development. Analysis of *BMP7* expression during kidney development, in conjunction with studies analyzing the effect of recombinant BMP7 on isolated kidney mesenchyme, suggest that a similar mechanism may operate for *BMP7*. We have generated a β-gal-expressing reporter allele at the *BMP7* locus to closely monitor expression of *BMP7* during embryonic kidney development. In contrast to other studies, our analysis of *BMP7/lacZ* homozygous mutant embryos, shows that *BMP7* expression is not subject to autoregulation in any tissue. In addition, we have used this reporter allele to analyze the expression of *BMP7* in response to several known survival factors (EGF, bFGF) and inducers of metanephric mesenchyme, including the ureteric bud, spinal cord and LiCl. These studies show that treatment of isolated mesenchyme with EGF or bFGF allows survival of the mesenchyme but neither factor is sufficient to maintain *BMP7* expression in this population of cells. Rather, *BMP7* expression in the mesenchyme is contingent on an inductive signal. Thus, the reporter allele provides a convenient marker for the induced mesenchyme. Interestingly LiCl has been shown to activate the Wnt signaling pathway, suggesting that *BMP7* expression in the mesenchyme is regulated by a Wnt signal. Treatment of whole kidneys with sodium chlorate to disrupt proteoglycan synthesis results in the loss of *BMP7* expression in the mesenchyme whereas expression in the epithelial components of the kidney are unaffected. Heterologous recombinations of ureteric bud with either limb or lung mesenchyme demonstrate that expression of *BMP7* is maintained in this epithelial structure. Taken together, these data indicate that *BMP7* expression in the epithelial components of the kidney is not dependent on cell-cell or cell-ECM interactions with the metanephric mesenchyme. By contrast, *BMP7* expression in the metanephric mesenchyme is dependent on proteoglycans and possibly Wnt signaling.

Key words: BMPs, Kidney, Mouse, Cell signaling

INTRODUCTION

Development of the metanephric kidney depends on a program of reciprocal inductive interactions between two mesodermal derivatives, the ureteric bud and the metanephric mesenchyme (Saxen, 1987). At approximately 10.75 days post coitum (d.p.c.), the ureteric bud, an outgrowth of the Wolffian duct, contacts the metanephric blastema signaling these cells to condense and aggregate. Subsequently, this induced population undergoes a mesenchymal to epithelial transition to form a comma shaped node which elongates to form an S-shaped tubule. Further morphogenesis and differentiation of this S-shaped tubule results in the formation of the glomerulus and the distal and proximal tubule elements of the mature nephron. Concomitantly, signals provided by the mesenchyme are required for growth and branching of the ureteric bud that ultimately forms the collecting duct system.

Recent experiments demonstrate that Bone Morphogenetic Protein 7 (BMP7), a member of the *60A* sub-group of TGF-β molecules, plays an essential role during ontogeny of the mammalian eye and kidney (Dudley et al., 1995; Luo et al., 1995). Although *BMP7* mRNA is expressed in many organizing regions of the early embryo such as the notochord, zone of polarizing activity (ZPA) and apical ectodermal ridge (AER) of the limb, defects in *BMP7* deficient mice are restricted largely to the developing eye and kidney. Thus at birth, homozygous mutant animals exhibit renal hypoplasia and eye defects ranging from microphthalmia to anophthalmia. In the developing kidney, *BMP7* is expressed initially in the ureteric bud. As development proceeds, expression is next observed in the metanephric mesenchyme and then in the early tubules derived from the mesenchyme (Lyons et al., 1995; Dudley et al., 1995; Dudley and Robertson, 1997). At later stages, *BMP7* expression becomes restricted to the podocytes of the mature glomerulus. Analysis of *BMP7* null embryos reveals that the early inductive tissue interactions appear largely intact in the absence of BMP7 signaling. However, *BMP7* deficient kidneys show a gradual cessation of nephrogenesis, associated with a reduction in branching of the ureteric bud and the loss of metanephric mesenchyme via

apoptosis (Dudley et al., 1995; Luo et al., 1995; Dudley and Robertson, 1997). Thus, BMP7 activities are essential for continuous growth and morphogenesis during the later stages of kidney development.

Little is known about potential factors responsible for regulating *BMP7* expression in the metanephric mesenchyme and ureter. In vitro studies reveal that *BMP7* is upregulated in MDCK cells following treatment with PMA, an activator of protein kinase C (Ishibashi et al., 1993). In *Drosophila*, there is considerable genetic evidence that *decapentaplegic (dpp*), a homologue of *BMP2/4*, is regulated by the hedgehog (hh) pathway (Heberlein et al., 1993; Ingham and Fietz, 1995; Vincent and Lawrence, 1994). In vertebrates, three *hh* homologues have been identified, namely *sonic (shh), indian (ihh*) and *desert (dhh*) (Echelard et al., 1993). Interestingly, several members of the *BMP* family are expressed in cells adjacent to, or overlapping the area of *hh*-expressing cells throughout development (Bitgood and McMahon, 1995). Furthermore, in chick, ectopic activation of shh in the anterior limb mesenchyme leads to the induction of *BMP-2* (Laufer et al., 1994), suggesting that the signaling interaction between hh and BMP has been conserved through evolution. Many sites of *BMP7* expression in the mouse, including the notochord and ZPA of the limb (Lyons et al., 1995), display an expression pattern complimentary to that of *hh* gene family members (Echelard et al., 1993; Bitgood and McMahon, 1995). In the kidney, shh is expressed in the ureter, however not until 14.5 d.p.c. (Bitgood and McMahon, 1995). Extensive analysis of hh expression patterns via in situ hybridization analysis and RT-PCR suggest that hh signaling pathways are unlikely candidates for activating *BMP7* expression during the early stages of kidney development.

In the kidney *BMP7* is expressed in both the inducing and the responding tissues (Dudley et al., 1995). This raises the possibility that *BMP7* expression may be autoregulative as has been shown for *BMP4* during tooth development (Vainio et al., 1993). To address this issue, and to explore the dynamic regulation of *BMP7* expression during development, we have generated a mouse line carrying a *lacZ*-expressing allele of *BMP7*. Additionally, insertion of the *lacZ* cassette disrupts the coding sequence and thus generates a novel loss-of-function mutation at the *BMP7* locus. Analysis of *BMP7/lacZ* expression patterns in homozygous mutant embryos shows that *BMP7* expression is not activated or maintained through an autoregulative mechanism. However, maintenance of *BMP7* expression in the mesenchyme is contingent on factors involved in the induction of mesenchymal cells. By contrast, we demonstrate that *BMP7* expression in the ureter is autonomous and not influenced by factors produced by the mesenchyme.

MATERIALS AND METHODS

Derivation of mutant mice

To generate the targeting vector, a 1.6 kb *Eag*I-*Hin*dIII fragment containing exon 1 of the *BMP7* locus was isolated. A polylinker (5′ *Ngo*MI-*Eco*RI-*Spe*I-*Nco*I-3′) was inserted into exon 1 between *Ngo*MI and *Nco*I. The modified *Eag*I-*Hin*dIII insert was flanked on the 5′ end with a 3.5 kb *Bam*HI-*Eag*I fragment and on the 3′ end with a 4.5 kb *Hin*dIII-*Xho*I fragment obtained from a previously described vector (Dudley et al., 1995). A HSVtk cassette was blunt-end ligated into the *Xho*I site. A cDNA encoding *lacZ* and neomycin resistance (*βgeo*) was removed from a plasmid containing an *IRES-βgeo* sequence (gift from Austin Smith) and modified to include a *Hin*dIII site 3′ to the IRES motif and 5′ to the translational start site. Following digestion with *Hin*dIII and *Sal*I, the fragment encoding *βgeo* was recovered, flanked with *Spe*I sites and inserted into the *Spe*I site of the modified targeting vector. The vector was linearized with *Not*I and electroporated into CCE embryonic stem (ES) cells. Correctly targeted ES cell clones were injected into C57BL/6J host blastocysts to generate germline chimeras as described (Bradley, 1987). Male chimeras were bred to C57BL/6J or MF1 females to ascertain germ line transmission. Germ line chimeras from the 6H ES clone were also mated to 129/Sv//Ev females to generate mutants on an inbred background.

Genotyping procedures

Targeted ES cells and F_1 progeny heterozygous for the *lacZ* allele, were identified by Southern blotting of 10 μg of genomic DNA which was hybridized with a 5′ flanking external probe as described previously (Dudley et al., 1995). The wild-type band detected by this probe is 7 kb and the correctly targeted mutant band is 3.5 kb. Subsequent progeny and embryos were genotyped by either PCR (Dudley et al., 1995) or by staining with X-gal as described below.

β-gal staining and in situ hybridization

For β-gal staining, embryos were fixed in X-gal fix buffer (phosphate-buffered saline, 5 mM EGTA, 2 mM $MgCl{\cdot}6H_2O$, 0.2% NP40, 0.2 mM deoxycholate, 1% formaldehyde, 0.2% gluteraldehyde) followed by three washes in X-gal wash buffer (phosphate-buffered saline, 5 mM EGTA, 2 mM $MgCl{\cdot}6H_2O$, 0.2% NP40, 0.2 mM deoxycholate). Embryos were stained overnight at 37°C in X-gal stain solution (X-gal wash buffer containing 5 mM K_3Fe, 5 mM K_4Fe, 0.5 mg/ml X-gal), followed by three washes in X-gal wash buffer and post-fixed in 4% paraformaldehyde for 2 hours. Embryos were again washed three times in X-gal wash buffer and then either stored as whole mounts or processed for sectioning. In situ hybridization analysis was performed using standard protocols (Jones et al., 1991). Probes specific for *Pax-2* (Dressler et al., 1990), *WT-1* (Kreidberg et al., 1993) *Wnt-4* (Stark et al., 1994) and *BF-2* (Haitini et al., 1996) were used as described.

Explant treatments

Kidneys were isolated from 11.5 d.p.c. embryos and dissected in L-15 medium supplemented with 1% Cosmic Calf (Hyclone) serum (dissection medium). To obtain isolated mesenchyme, kidneys were treated with 0.25% trypsin for 6 minutes on ice, then washed in dissection medium and the mesenchyme teased away from the ureteric bud using a 26 G needle. Explants were grown in 5% CO_2 at 37°C on 0.1 μm filters (Corning) in F12/DMEM 1:1 (Gibco) supplemented with 10% defined fetal bovine serum (Hyclone). In some experiments, the medium was supplemented with one of the following: 100 ng/ml EGF (Sigma, cat # E-2645), 50 ng/ml bFGF (Sigma, cat # F-0291), 15 mM LiCl (Sigma, cat # L-0505), or 30 mM $NaClO_3$ (Sigma, cat # S-3171). Explants were then processed for either β-gal staining or for in situ hybridization.

Recombinant BMP7 and BMP2 proteins were obtained from Creative Biomolecules (Framingham, MA). Activity of recombinant proteins were verified using the C3H 10T1/2 cell assay (Ruppert et al., 1996). Affi-gel blue beads were prepared according to manufacturers directions (Biorad), and loaded with protein in a 20 μl volume of 20 mM sodium phosphate (pH 7.2) containing either BSA, BMP7 or BMP2 at 100 ng/ml and incubated at 37°C for 1 hour. Prior to use beads were briefly washed in culture medium.

RESULTS

Generation of *BMP7/lacZ* mutant mice

To examine regulation of *BMP7* expression, a novel reporter

allele of *BMP7* was generated by gene targeting. Introduction of the *lacZ* reporter gene into the first coding exon of BMP7 resulted in deletion of 263 bases including the endogenous translational start site (Fig. 1A; Dudley et al., 1995), while preserving the endogenous transcriptional regulatory elements, to create a novel null allele. Two independent, correctly targeted ES cell clones, were injected into blastocysts to generate germline chimeras. Heterozygous progeny were backcrossed for two generations onto either the C57Bl/6J or MF1 backgrounds. To establish an inbred line, 129/Sv//Ev females were mated to germline chimeras. While *BMP7/lacZ* heterozygotes showed no overt defects, *BMP7/lacZ* homozygous mutants present with the same eye and kidney defects observed in the original $BMP7^{m1Rob}$ mutants (Dudley et al., 1995). Additionally, compound homozygotes carrying the *lacZ* and the original $BMP7^{m1Rob}$ alleles display microphthalmia or anophthalmia and hypoplastic kidneys, providing additional confirmation that the *BMP7/lacZ* allele creates a null mutation at the *BMP7* locus (Fig. 1B).

Embryos from 6.5 d.p.c. through 11.5 d.p.c. were analyzed to determine if the pattern of β-gal staining faithfully reflects the *BMP7* mRNA expression pattern (Lyons et al., 1995; Dudley et al., 1995). In contrast to previous in situ hybridization data that detected *BMP7* mRNA at 6.5 d.p.c. (Arkell and Beddington, 1997), expression of the *BMP7/lacZ* allele is not observed until 7.5 d.p.c. in whole-mount X-gal stained embryos. Consistent with previous findings, expression is confined to the allantois and the axial mesoderm at this stage (Fig. 2A). Strong expression is detected in 8.5 d.p.c. embryos throughout the node, notochord and head mesenchyme as previously described (Fig. 2B; Lyons et al., 1995; Arkell and Beddington, 1997). Gross inspection at 10.5 d.p.c., reveals β-gal staining in a variety of tissues including the telencephalon, notochord, dorsal root ganglion, otic vesicle, branchial arches, heart, limb buds, and surface ectoderm (Fig. 2C). To obtain a more detailed picture of the expression pattern of the *lacZ* allele, 11.5 d.p.c. embryos were stained in X-gal and then sectioned. *BMP7/lacZ*-positive domains correlate with previously identified *BMP7* mRNA-expressing tissues (Fig. 2D-I). Expression in the telencephalon is restricted to the lamina terminalis and overlying surface ectoderm (Fig. 2D). In the hindbrain, expression is seen only in the thin roof of the mylencephalon (data not shown). The *BMP7/lacZ* allele is strongly expressed in the mesonephric tubules (Fig. 2E). Expression in the eye is observed in the pigmented layer of the retina, the optic nerve and mesenchyme associated with the eye (Fig. 2F). In the developing nose, expression is restricted to the lateral nasal process and is completely absent in the medial nasal process. β-gal activity is also detected in the atrial and ventricular chambers of the heart but absent from the thoracic body wall (Fig. 2G). Other areas of expression include the notochord and dorsal root ganglia. Collectively this analysis confirmed that the *lacZ* allele is expressed appropriately. Moreover this study underscores the sensitivity of the reporter allele by allowing single cell resolution of *BMP7* expression. The precise cellular resolution offered by the *lacZ* expression system has revealed an interesting expression pattern in the developing limb bud. The *BMP7/lacZ* allele is strongly expressed in the AER and associated limb mesenchyme in the posterior portion of the limb bud (Fig. 2H). However, in more anterior sections this mesenchymal expression is not observed. In addition, asymmetric expression is observed in the lateral mesoderm surrounding the somites (Fig. 2I), with expression restricted to the medial cells adjacent to the somites. In a few tissues previously identified as expressing *BMP7* such as the endodermal and mesodermal layers of the visceral yolk sac and optic vesicle (8.5 d.p.c.), β-gal staining was not observed. However, proper staining is observed in the eye by 9.5 d.p.c.

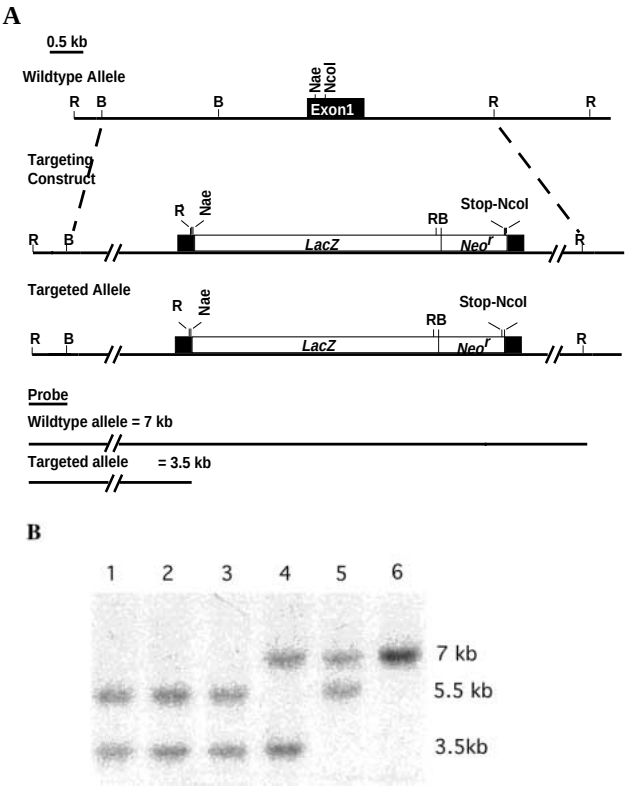

Fig. 1. Generation of a β-gal-expressing *BMP7* reporter allele. (A) Schematic representation of the wild-type locus, the targeting vector, and the predicted modified locus after homologous recombination. R, *Eco*RI; B, *Bam*HI. (B) Southern blot analysis of mid-gestation stage embryos obtained from heterozygous intercrosses. Genomic DNA was digested with *Eco*RI and hybridized with the 5′ flanking probe. This probe detects a 7 kb fragment derived from the wild-type allele, a 5.5 kb fragment derived from the original $BMP7^{m1Rob}$ allele and a 3.5 kb fragment derived from the *BMP7/lacZ* allele. Compound homozygotes (lane 1-3), *BMP7/lacZ* heterozygote (lane 4), $BMP7^{m1Rob}$ heterozygote (lane 5), and wild type (lane 6).

BMP7 expression is not autoregulative

BMP7 is expressed in the ureteric bud as it evaginates from the Wolffian duct. A few hours later, *BMP7* expression is strongly detected in the ureter and more weakly throughout the metanephric blastema. This sequential activation of *BMP7* expression in closely opposed tissues suggests that *BMP7* expression in the mesenchyme is regulated through a paracrine signaling mechanism. Consequently, if BMP7 activity in the ureteric bud is necessary for expression in the mesenchyme, then the *BMP7/lacZ* allele should not be activated in the mesenchyme of null embryos. In *BMP7/lacZ* heterozygotes, strong staining is observed in the Wolffian duct at 10.5 d.p.c. before the ureteric bud has emerged (data not shown). By 11.5 d.p.c., the ureteric bud has contacted the mesenchyme and

strong β-gal staining is observed in both the ureteric bud and throughout the surrounding mesenchyme (Fig. 3A). This pattern of staining in mutant embryos is indistinguishable from that in heterozygotes (Fig. 3B). Two days later the ureter is observed to have undergone several rounds of branching. At this stage pre-tubular aggregates are observed near the tips of the branching ureter. Expression of the *lacZ* allele is detected in the ureteric epithelium, the condensed mesenchyme around the tip of the ureter and in the pre-tubular aggregates. Identical results were obtained in both heterozygous and *BMP7* deficient kidneys (Fig. 3C,D). At 16.5 d.p.c. the ureter has undergone a significant amount of branching and more differentiated nephrons are observed in the medulla region of the kidney. In the cortex, new tubules are still being induced. β-gal staining reveals that the *BMP7/lacZ* allele is strongly expressed in the ureteric epithelium located in the periphery of the kidney, but is down regulated in the medullar regions (Fig. 3E). At this stage, β-gal activity is detected in the condensed mesenchyme but not in the stromal mesenchyme. In mutant embryos, β-gal staining in the collecting ducts and renal tubules is unaffected. However, in contrast to the earlier stages, expression is absent in the peripheral mesenchyme (Fig. 3F). This loss of *BMP7* expression in the cortical mesenchyme correlates with the cessation of nephrogenesis and apoptosis previously described in the *BMP7* mutants (Dudley et al., 1995; Luo et al., 1995). However, it is unclear whether loss of β-gal activity is due to absence of signals that maintain *BMP7* expression or is

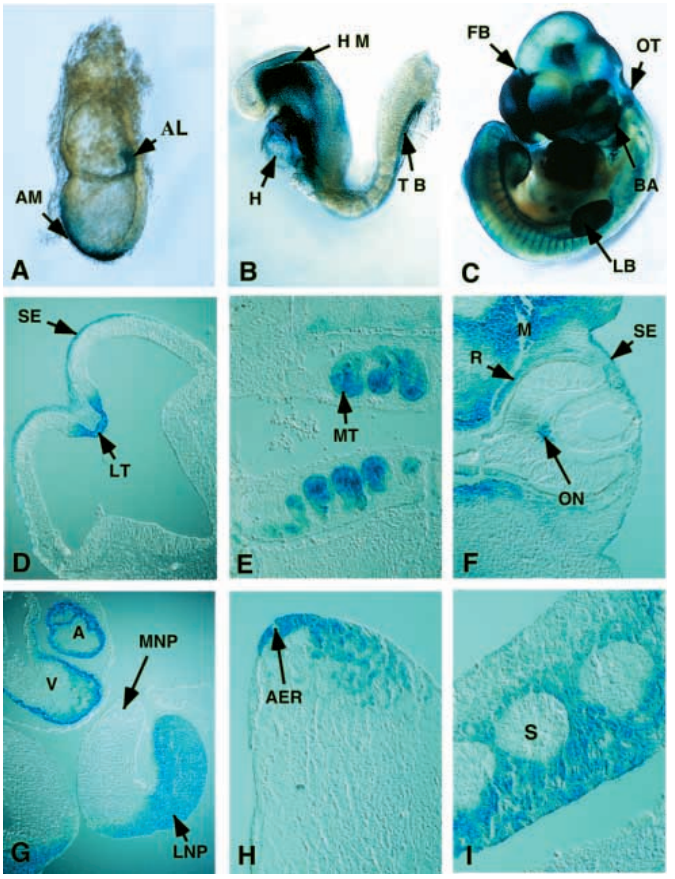

Fig. 2. Expression pattern of *BMP7/lacZ* allele during early embryogenesis is derived from crossing a heterozygous male with wild-type females. Embryos stained as whole-mounts for β-gal expression at 7.5, 8.5 and 10.5 d.p.c. (A-C) Expression at 7.5 d.p.c. is detected in the axial mesoderm (AM) and allantois (AL) (A). At 8.5 d.p.c. expression is seen in the head mesenchyme (HM), notochord and tailbud (TB), the remnant of the node; H, heart (B). By 10.5 d.p.c. BMP7 expression is upregulated in the surface ectoderm and strong β-gal staining is observed in the heart, branchial arches (BA), limb buds (LB), otic vesicles (OT) and forebrain (FB) (C). 11.5 d.p.c. embryos were isolated, stained as whole mounts for β-gal and then sectioned. β-gal staining patterns are shown in transverse sections through the embryo at different levels. In the telencephalon staining is observed in the lamina terminalis (LT) and overlying surface ectoderm (SE) (D). Strong staining is seen in the mesonephric tubules (MT) (E). In the eye, staining is seen in the retinal pigmented epithelium (R), the optic nerve (ON), surface ectoderm (SE) and mesenchyme associated with the eye (M) (F). In the heart, expression is detected in both the atrial (A) and ventricular chambers (V) (G). Interestingly, staining is restricted to the lateral nasal process (LNP) and is absent in the medial process (MNP). Sections through the anterior-posterior axis of the developing forelimb bud, shows robust β-gal staining is present in the AER and underlying mesenchyme (H). A caudal section through the embryo reveals that somites (S) do not express *BMP7*, but axial mesoderm lying lateral to the somites displays strong β-gal activity (I).

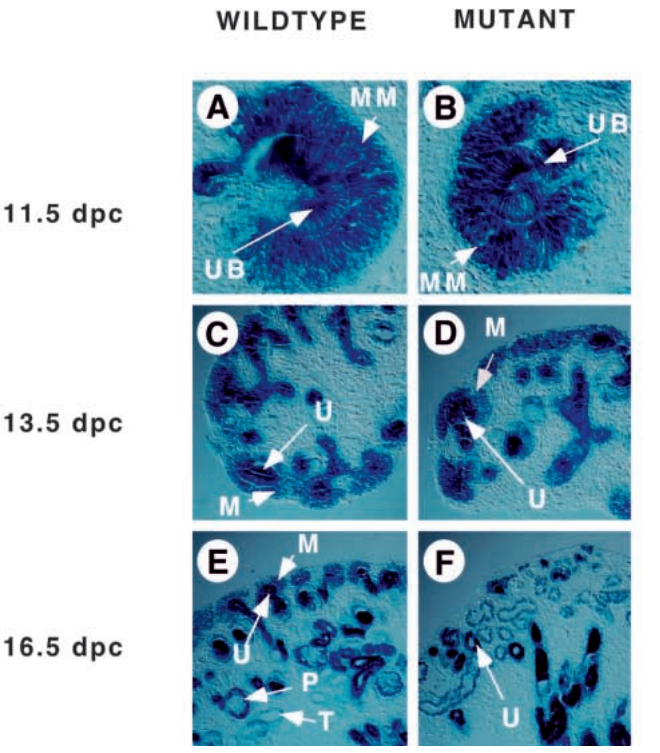

Fig. 3. Expression of the *BMP7/lacZ* allele in the developing kidney. Whole embryos were isolated at 11.5 d.p.c. or whole kidneys were isolated from 13.5 and 16.5 d.p.c. embryos, stained in X-gal and then sectioned. *BMP7/lacZ* heterozygotes (A,C,E), *BMP7/lacZ* homozygotes (B,D,F). β-gal staining is observed in the ureteric bud and surrounding mesenchyme at 11.5 d.p.c. At 13.5 d.p.c. staining is observed in the ureter and condensed mesenchyme associated with the tips of the ureter. This staining pattern is identical in both heterozygotes and nulls up until 13.5 d.p.c. However, at 16.5 d.p.c. mesenchymal expression in the periphery of the kidney is lost in the *BMP7/lacZ* null embryos. MM, metanephric mesenchyme; UB, ureteric bud; U, ureter; M, mesenchyme; P, podocyte; T, tubule.

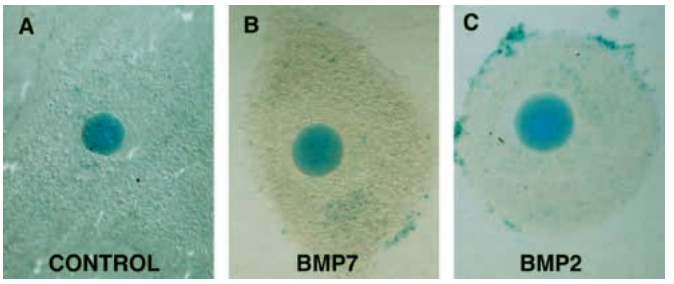

Fig. 4. *BMP7* expression is not autoregulative. Kidneys were isolated from 11.5 d.p.c. embryos and the mesenchyme isolated by trypsin digestion. Isolated mesenchymes were then treated with protein soaked beads containing either BSA (A), BMP7 (B), or BMP2 (C). Both BMP7 and BMP2 rescue survival of the mesenchyme but neither is capable of activating the *BMP7/lacZ* allele.

indicative of a loss of condensed mesenchyme cells. Overall, these findings suggest that neither activation nor maintenance of BMP7 expression in the metanephric mesenchyme is dependent on an autoregulative mechanism of BMP7 signaling acting in either an autocrine or paracrine manner. Additionally, the pattern of β-gal staining was examined in other tissues that normally express *BMP7*. No differences in expression were observed between heterozygotes and mutants in any of these tissues (data not shown). Thus it is unlikely that BMP7 signaling is autoregulatory in vivo.

It was recently shown that addition of recombinant BMP7 to isolated metanephric kidney mesenchyme results in upregulation of *BMP7* in the condensed mesenchyme (Vukicevic et al., 1996). However, our analysis of the *lacZ* allele, combined with the limited and late defects observed in *BMP7* deficient mice, do not support an autoregulative mode of signaling for BMP7 in kidney development. It is possible that BMP7 signaling is not required for the maintenance of *BMP7* expression in vivo, but may be capable of functioning through such an autoregulative mechanism. Therefore, we assessed the ability of beads soaked in BMP7 to maintain *lacZ* expression in isolated mesenchyme derived from *BMP7/lacZ* heterozygotes. At 11.5 d.p.c. the entire population of metanephric mesenchyme cells surrounding the ureteric bud express *BMP7* (Fig. 3A,B). Therefore, we first determined how long isolated mesenchyme continues to express *BMP7* in the absence of the ureter. Metanephric mesenchyme was harvested from 11.5 d.p.c. *BMP7/lacZ* heterozygous embryos and cultured in isolation on Nucleopore filter rafts. Explants were fixed at various times after culture ranging from 2 to 48 hours and stained in X-gal. β-gal activity gradually decreases in mesenchyme cultures and is absent by 48 hours (Figs 5A, 6A and data not shown). Addition of 75 μM cyclohexamide to these cultures to inhibit new transcription did not affect the β-gal staining pattern, suggesting that residual staining is most likely due to perdurance of the β-gal protein. Therefore in all subsequent experiments, explants were cultured for at least 48 hours prior to analysis to ensure that any staining observed was due to active transcription of the *BMP7* reporter allele.

Isolated mesenchyme cultured in the absence of an inducing factor does not survive (Saxen, 1987). Similarly, when mesenchyme explants were cultured with a bead loaded with BSA, the cells gradually die, display a very loose morphology and fail to maintain expression of the *BMP7/lacZ* allele (Fig. 4A). In contrast, when isolated mesenchyme was cultured with either a BMP7 or BMP2 soaked bead, the entire population of mesenchymal cells adopts a distinctive mounded appearance (Fig. 4B,C), and only a few scattered cells predominantly at the edge of the explant are positive for β-gal staining. These results indicate that BMP7 signaling promotes the survival of the mesenchymal cells, but it is not able to upregulate its own expression in these cells. Moreover, in situ hybridization results show that *Pax-2*, a marker of mesenchymal cell induction, is not expressed in BMP-treated explants (data not shown). Thus, BMP7 and BMP2 seem likely to act as survival factors for the mesenchyme, however, this signal alone is not sufficient to maintain *BMP7* expression.

BMP7 expression requires an inductive signal

The bead experiments suggest that BMP7 signaling is sufficient to allow survival of the mesenchyme cells but is not capable of promoting *BMP7* expression. Thus, it is likely that a signal from the ureter is required to maintain *BMP7* expression in the mesenchyme. To further evaluate factors potentially capable of maintaining expression of the *BMP7/lacZ* allele, mesenchyme was isolated from 11.5 d.p.c. heterozygous *BMP7/lacZ* mice and cultured under various conditions. Treatment of isolated mesenchyme with epidermal

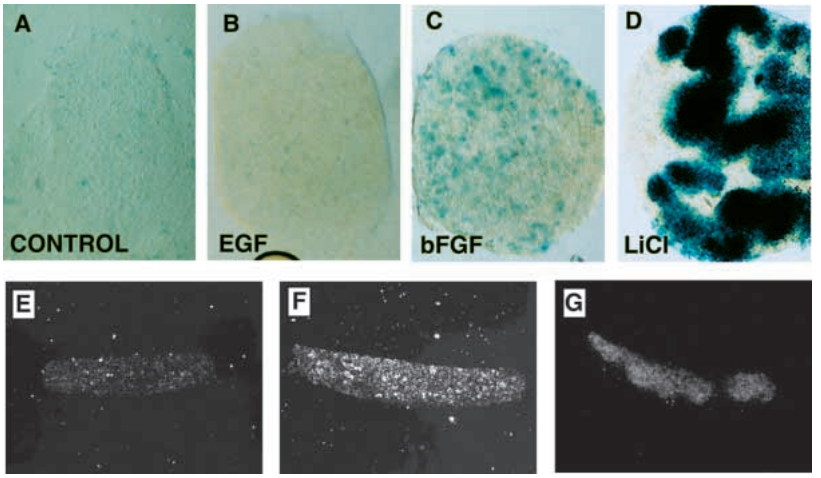

Fig. 5. *BMP7* expression requires an inductive signal. Mesenchyme was isolated from 11.5 d.p.c. embryos and cultured in the absence or presence of EGF, bFGF, or LiCl for 48 hours and then stained in X-Gal. Isolated mesenchyme in the absence of induction dies (A). When isolated mesenchyme is grown in the presence of EGF (B) or bFGF (C) the cells survive but upregulation of *BMP7* expression is not observed, although bFGF does maintain a population of cells that are β-gal positive. However, when grown in the presence of 15 mM LiCl (D), mesenchymal condensations are observed that strongly express *BMP7*. In situ hybridization analysis shows that *Pax-2*, a marker of induced mesenchyme, is downregulated by treatment with EGF (E), while treatment with bFGF shows slightly higher levels of *Pax-2* (F). In contrast, treatment with LiCl, a known inducer of the mesenchyme, results in strong expression of *Pax-2* in condensed mesenchyme (G).

growth factor (EGF) has been shown to promote survival of stromal cells at the expense of mesenchymal cells (Weller et al., 1991; Coles et al., 1993). In contrast, treatment of the mesenchyme with basic fibroblast growth factor (bFGF) is sufficient to maintain survival of the mesenchymal cells as demonstrated by expression of two mesenchymal markers, *WT-1* and *c-met* (Perantoni et al., 1995; Karavanova et al., 1996). However, neither condensation of the mesenchyme nor tubulogenesis is observed with either of these treatments. Thus EGF and bFGF have been defined as survival factors but not inducers of the metanephric mesenchyme. Isolated mesenchyme treated with EGF shows markedly improved cell survival versus controls, however, no β-gal staining is observed (Fig. 5A,B). In situ hybridization analysis reveals that *Pax-2* expression is down-regulated in EGF-treated cultures while *BF-2* is upregulated (data not shown; Fig. 5E), consistent with previous findings that EGF promotes survival of the stromal precursor cells. Treatment with bFGF also maintains survival of the mesenchyme, but in contrast to EGF treatment, low levels of β-gal staining are observed (Fig. 5C). Accordingly, *Pax-2* is expressed in these cultures (Fig. 5F), although at a much reduced level relative to spinal cord induced cultures (data not shown). These results support the conclusion that survival of the mesenchyme is not sufficient to maintain expression of BMP7.

We next tested whether signals from the spinal cord, a strong inducer of condensation and tubulogenesis in the metanephric mesenchyme (Saxen, 1987), can activate the *BMP7/lacZ* allele in isolated mesenchyme. Mesenchyme was isolated from 11.5 d.p.c. *BMP7/lacZ* heterozygous embryos and cultured transfilter with spinal cord for 48 hours. It has previously been demonstrated that filters with a pore size below 0.05 μm greatly inhibits the inductive ability of the spinal cord (Saxen, 1987). Therefore, mesenchyme was cultured with spinal cord separated by either a 0.015 μm or 1.0 μm filter. Isolated mesenchyme explants cultured in the absence of spinal cord do not express *lacZ* and transfilter cultures using a 0.015 μm filter show very little staining (Fig. 6A,C). However, *lacZ* is highly expressed in condensed regions when the mesenchyme is cultured with spinal cord across a 1.0 μm filter (Fig. 6B). These results strongly suggest that maintenance of *BMP7* expression in the mesenchyme is correlated with mesenchyme induction.

LiCl is also capable of inducing condensation and aggregation in isolated mesenchyme cells (Davies and Garrod, 1995). However, unlike co-culture with spinal cord, tubulogenesis does not proceed in lithium-treated mesenchyme. As *BMP7* is normally expressed in condensed mesenchyme we were interested to see if treatment of isolated mesenchyme with LiCl results in activation of the *BMP7/lacZ* allele. Mesenchyme was isolated from 11.5 d.p.c. *BMP7/lacZ* heterozygotes and cultured in the presence of 15 mM LiCl. After 48 hours, many condensations were apparent within the mesenchyme. These condensations strongly express *BMP7* (Fig. 5D) resembling that seen with spinal cord co-culture. In situ hybridization results show that *Pax-2* is also strongly expressed in the condensed mesenchyme (Fig. 5G). Surprisingly, unlike in spinal cord induced mesenchyme explants, *Wnt-4* is not expressed in these LiCl induced cultures (data not shown). The bead experiments described above demonstrate that BMP7 alone is not capable of maintaining BMP7 expression in the mesenchyme. However it is possible that both an inductive factor and BMP7 are required for the maintenance of BMP7 expression and that neither alone is sufficent. To address this, BMP7 null mesenchyme was treated with LiCl. These mesenchyme cultures also respond to LiCl and result in the upregulation of the *lacZ* allele. Taken together, these results establish that an inductive signal capable of inducing the initial condensation of the mesenchyme is sufficient to maintain BMP7 expression in the mesenchyme even in the absence of BMP7 activity.

BMP7 expression in the mesenchyme requires proteoglycans

Proteoglycans have been shown to play an important role in gene expression and embryonic development (Lin and Bissell, 1993; Llyod et al., 1993) and are likely responsible for binding signaling molecules and presenting them to cognate receptors on the cell surface (Schubert, 1992; Adams and Watt, 1993). In the kidney, proteoglycans are present in both the ureter and the induced mesenchyme and are required for ureteric branching and tubule induction (Vainio et al., 1989, 1992). Treatment of kidney explants with sodium chlorate ($NaClO_3$) inhibits proteoglycan synthesis and arrests branching of the ureteric bud (Davies et al., 1995). However, nephrogenesis still occurs in the mesenchyme near the tips of the ureter. Previously it was shown that the mesenchyme-specific marker *GDNF* and the ureter-specific marker *Wnt11* are down-regulated in these cultures (Kispert et al., 1996; Sainio et al., 1997). We were interested in determining what role the ECM may play in the regulation of *BMP7*. Intact kidneys from 11.5 d.p.c. *BMP7/lacZ* heterozygous embryos were cultured with or without 30 mM $NaClO_3$ for 24 or 48 hours (Fig. 7). After 24 hours in culture, untreated rudiments exhibit branched ureters. As expected, *BMP7* expression in the mesenchyme is localized specifically around the tips of the branching duct. In chlorate-treated cultures, branching of the ureter is arrested at the T-stage. Compared to controls, expression of *BMP7* in the mesenchyme is more diffuse and not specifically associated with the tips. After 48 hours in culture, the control explants have undergone a significant amount of branching with *BMP7* expression in the mesenchyme associated with the growing tips of the ureter. In treated cultures, branching of the ureter remains inhibited after 48 hours. However at this stage, *BMP7* expression in the mesenchyme is completely lost, although expression of *BMP7* in the ureter and pre-tubular aggregates is unaffected. In an attempt to rescue mesenchymal expression in these treated explants, chlorate was removed from the medium after 48 hours and the explants cultured for an additional 24 hours. Mesenchymal expression of *BMP7/lacZ* was not reinitiated. Interestingly, in rescued cultures or cultures that were grown for 72 hours in chlorate-containing medium, branching of the ureter remained arrested, however, newly formed aggregates were found to express the *BMP7/lacZ* allele (Fig. 7F). Collectively these data suggest that expression of *BMP7* in the condensed mesenchyme requires proteoglycans. In contrast, expression in the pre-tubular aggregates or in the epithelial components is not dependent on proteoglycans.

BMP7 expression in the ureter is autonomous

Chlorate experiments presented above suggest that expression of the *BMP7/lacZ* allele in the epithelial components of the kidney occurs independently of signals derived from the

mesenchyme. To further explore possible influences from the mesenchyme we analyzed *BMP7* expression in isolated ureteric buds and in heterologous recombination experiments. In contrast to the metanephric mesenchyme, isolated ureteric buds are able to maintain strong expression of the *BMP7/lacZ* reporter allele after 48 hours of culture (Fig. 8A). Ureteric buds isolated from heterozygous embryos at 11.5 d.p.c. were recombined with heterologous mesenchyme populations isolated from wild-type embryos and cultured for 48 hours (Fig. 8C,D). As a control, ureteric buds were combined with metanephric mesenchyme (Fig. 8B). Because these explants were cultured for 48 hours, only slight branching of the ureteric bud is seen, although strong expression of *BMP7/lacZ* in the ureter is observed. Previously it has been shown that lung mesenchyme can support branching of the ureter (Kispert et al., 1996). When lung mesenchyme is recombined with the ureteic bud we observe branching of the ureter and strong expression of the *BMP7/lacZ* reporter allele. Interestingly, as demonstrated by our expression analysis of the *BMP7/lacZ* allele, lung mesenchyme does not express *BMP7* (data not shown). Maintenance of *BMP7* expression in the ureter is also observed when ureteric buds are recombined with limb mesenchyme, although by contrast, this mesenchyme does not support branching of the ureter. These data, combined with in vivo data, suggest that expression of the *BMP7/lacZ* allele in the ureter is independent of any kidney mesenchyme specific signals.

DISCUSSION

Studies monitoring *BMP7* mRNA reveal that *BMP7* is expressed dynamically in many cell populations during embryogenesis. However, relatively little is known about the signaling pathways that regulate *BMP* expression during development. In order to carefully examine the regulation of *BMP7* expression, we have generated a β-gal-expressing allele at the *BMP7* locus. Expression of this allele faithfully recapitulates the expression pattern previously described for *BMP7* mRNA by standard in situ hybridization analysis. Moreover, this study demonstrates the utility of the *BMP7* reporter allele as a marker of induced mesenchyme. In addition, we have confirmed that this allele creates a null mutation at the *BMP7* locus and thus provides us with an in vivo approach to directly assay activation of *BMP7* expression in the absence of BMP7 protein.

BMP7 expression is not autoregulative

In *Drosophila*, transcription of *dpp* is maintained in many tissues by autoregulation (Biehs et al., 1996). A similar mechanism may, in part, regulate the expression of *BMP4*, a vertebrate homologue of *dpp*, in certain cell populations (Vainio et al., 1993). In situ hybridization results have shown that *BMP7* is first expressed in the Wolffian duct before the ureteric bud evaginates and makes contact with the mesenchyme. An attractive model is that the initial activity of *BMP7* in the ureteric bud may induce *BMP7* expression in the mesenchyme via a paracrine autoregulative mechanism. Utilizing the *BMP7/lacZ* line of mice as a direct test of gene activation, we have unambiguously shown that BMP7 does not signal in a paracrine fashion from the ureter to activate *BMP7* expression in the metanephric mesenchyme, since correct temporal and spatial activation of the *lacZ* allele occurs in *BMP7/lacZ* null embryos. Furthermore, expression in the ureter is maintained throughout kidney development suggesting that BMP7 does not function in a autocrine fashion. Taken together, these data rule out a autoregulatory mechanism of signaling for BMP7.

Therefore, while BMP7 is likely to be a key component in the signaling pathway that allows survival of the mesenchyme, its activity is not directly required to regulate its own expression. Moreover, since autoactivation of gene expression has been shown only for *BMP4*, it is possible that elements for autoregulatory control have been maintained only by the homologs of *dpp* and not by more distantly related family members. It is possible that other members of the *BMP* family are required for the activation or maintenance of BMP7 expression. Accordingly, previous analysis has shown that several members of the *BMP* family display overlapping expression domains (Dudley and Robertson, 1997). Although *BMP4* and *BMP5* are expressed in mesenchymal cells that line the ureter, no other *BMP* is expressed in the condensed mesenchyme. This may account for the loss of expression specifically in this population of cells. It will be interesting to analyze expression of the *BMP7/lacZ* allele in *BMP* double mutants.

BMP7 expression in the epithelium is not dependent on mesenchymal signals

Analysis of *BMP7* expression in the epithelial components of the kidney suggests that this expression is independent of mesenchymal signals. Thus, isolated ureteric buds or ureteric buds recombined with heterologous mesenchyme, maintain strong expression of the *BMP7/lacZ* allele in the ureteric epithelium. In addition, ureteric expression of *BMP7* is not affected in whole kidneys treated with chlorate. The expression of both the ureteric-specific genes *Wnt-11* and *c-ret* and the mesenchyme-specific gene *GDNF* are down-regulated in chlorate-treated explants (Kispert et al., 1996; Sainio et al., 1997). Here we show that similar treatment results in the loss of *BMP7* expression specifically in the mesenchyme, without affecting expression in the ureter and pre-tubular aggregates. These data demonstrate that *BMP7* expression in the ureter is not contingent on the Wnt-11 or GDNF/c-RET signaling pathways.

Although expression of *BMP7* appears to be a characteristic of many epithelial cell populations (Lyons et al., 1995; Dudley and Robertson 1997), both the ureter and the developing nephrons also display a spatially and temporally regulated loss of *BMP7* expression. Since the down-regulation of gene expression occurs during nephron differentiation, it is possible that changes in tissue interactions result in the loss of *BMP7* expression. However, we failed to find a heterologous mesenchyme cell population that either fails to support or actively down-regulates expression of *BMP7*. Collectively, these results suggest that the metanephric mesenchyme synthesizes factors important for directing the kidney-specific branching pattern of the ureteric epithelium, but these mesenchymal derived signals have no role in maintaining *BMP7* expression in the epithelium.

BMP7 expression in the mesenchyme parallels inductive events

Numerous experiments reported here reveal a strong

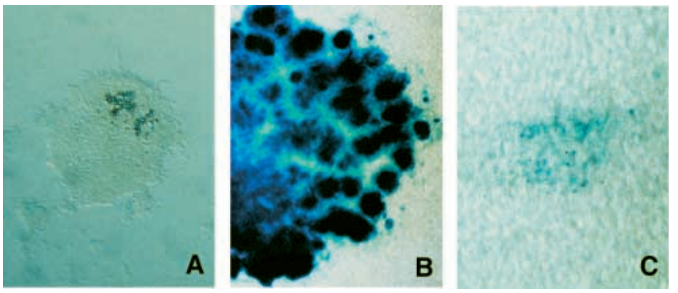

Fig. 6. *BMP7* expression is activated in the presence of spinal cord. Metanephric mesenchyme was isolated as described and placed transfilter with spinal cord. In the absence of inducing tissue the mesenchyme dies (A). When a 1.0 μm filter is used to separate the spinal cord and mesenchyme, strong induction takes place and the *BMP7/lacZ* allele is activated (B). When a 0.015 μm filter is used induction is weak or absent and very little β-gal activity is observed (C).

correlation between factors capable of inducing condensation of the mesenchyme and activation of the mesenchyme specific *BMP7* expression domain. In contrast, conditions that do not support induction do not activate or maintain *BMP7* expression. Taken together, these data suggest that *BMP7* expression in the mesenchyme requires the activity of factors capable of inducing mesenchymal condensation. This inductive factor may play a direct role in the activation of *BMP7* expression, since in LiCl treatment or spinal cord recombination experiments, the normal expression domain of *BMP7* is broader. This could be the result of more cells being in contact with the inducer, leading to a broader domain of cells that activate *BMP7* expression. Alternatively, activation of *BMP7* may lie in a signaling pathway downstream of the initial inducing activity and the increased expression domain may result from more cells initially being induced. Regardless of the identity of these key factors, results presented here, together with the distinctive kidney defects seen in *BMP7* mutant embryos, clearly indicate that BMP7 itself does not act as a inducer of mesenchymal condensation, but rather that its expression is controlled by factors that induce the initial events of condensation.

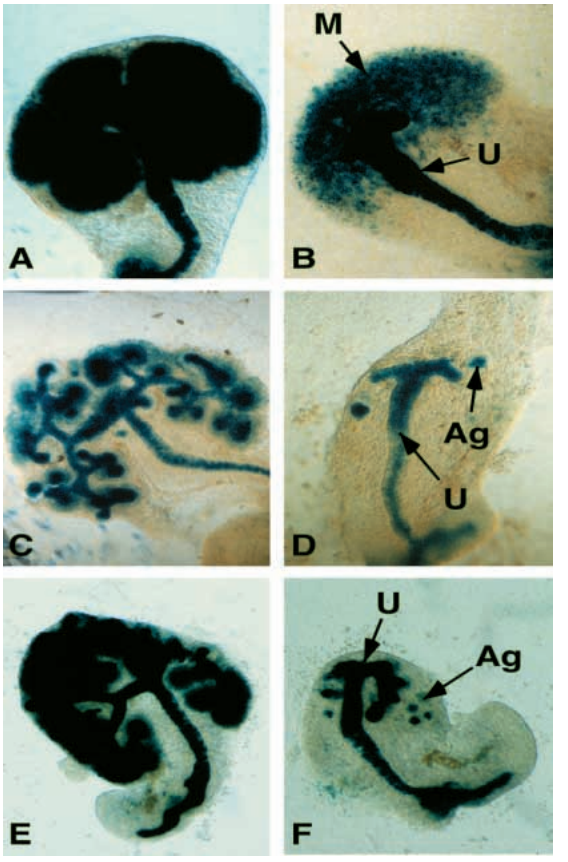

Fig. 7. *BMP7* expression in the absence of proteoglycan synthesis. Whole kidneys were isolated from 11.5 d.p.c. embryos and grown in the absence (A,C,E) or presence (B,D,F) of 30 mM $NaClO_3$. Explants were cultured for either 24 hours (A,B), 48 hours (C,D) or 72 hours (E,F) and then fixed and stained in X-gal. In treated cultures after 24 hours the *BMP7/lacZ*-positive cells are not condensed around the tips of the ureter as they are in controls. *BMP7* expression is completely lost specifically in the mesenchyme after 48 hours in the presence of $NaClO_3$ (D). In contrast, *lacZ* expression is maintained in the ureter and pre-tubular aggregates. However, nephrogenesis continues in the presence of chlorate and new condensates are formed that activate expression of the *BMP7/lacZ* allele (F). M, mesenchyme; U,ureter; Ag, aggregate.

Model of *BMP7* regulation in the kidney

Current data suggest at least two possible models for the regulation of *BMP7* expression. The first model suggests that transcription of *BMP7* is controlled by distinct mechanisms in different cell populations of the kidney. *BMP7* expression is first detected in the cells of the ureteric bud and strong β-gal staining is maintained in the growing ureter. Maintenance of this expression is not dependent on proteoglycans or any mesenchymally derived signals and thus appears to be autonomous of any cell-cell or cell-ECM interactions. The second site of *BMP7* expression is the metanephric mesenchyme. Expression in this cell population is dependent on inductive signals provided by the ureteric bud both for the initiation of expression in the blastema and maintenance of expression in the condensed mesenchyme. This signaling event continues to be responsible for activating *BMP7* expression in the mesenchyme as the ureter branches and induces more mesenchyme in the periphery. Thus, cell-cell interactions seem to be an important component for the induction and maintenance of BMP7 expression in the mesenchyme. The unique transformation of the metanephric mesenchyme to an epithelium introduces an intriguing aspect to the control of *BMP7* expression in the kidney. Once the mesenchyme has been induced to aggregate and epithelialize, this population of cells no longer requires mesenchyme specific factors in order to maintain *BMP7* expression, a situation reminiscent of what we observe in the ureteric epithelium.

The second model contains a common regulator for expression in both cell types based on the morphology of the cells. The ordered structure of the condensed mesenchyme, combined with the absence of interstitial protein (Ekblom et al., 1980; Ekblom, 1981) might be indicative of a semi-epithelial state. However, unlike the mature epithelium of the ureter, the condensed mesenchyme is dependent on signals from the ureter and on proteoglycans for the maintenance of

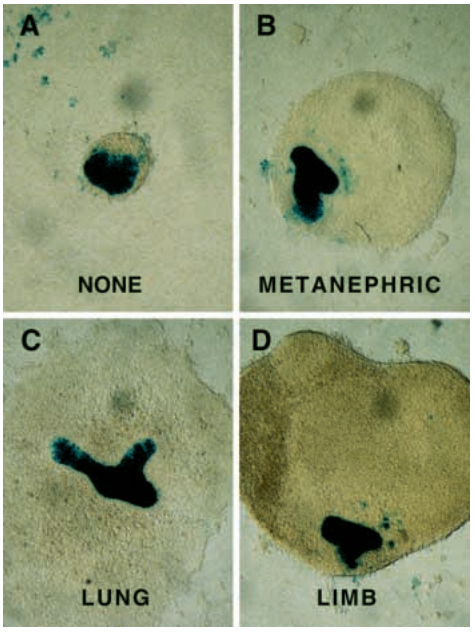

Fig. 8. *BMP7* expression in the ureter is autonomous. Ureteric buds were isolated from 11.5 d.p.c. *BMP7/lacZ* embryos and cultured either alone (A) or in recombination with either metanephric mesenchyme (B), lung mesenchyme (C), or limb mesenchyme (D) from non *lacZ* mice. Ureteric buds grown in the absence of mesenchyme do not branch but maintain *BMP7* expression after 48 hours of culture. Similarly, ureteric buds grown in the presence of kidney, lung or limb mesenchyme also express *BMP7*. However, only ureteric buds grown in kidney and lung mesenchyme support the initial stages of branching.

this type of morphology. In support of this, when mesenchyme is cultured in the absence of an inducer this ordered structure is lost and subsequently expression of *BMP7* is lost. Similarly, when whole kidneys are treated with chlorate, the mesenchyme cells are not found condensed around the tips of the ureter and eventually lose expression of BMP7. Analysis of *BMP7* expression in disaggregated ureter cells may help to further define this model as a possible mode of BMP7 regulation.

BMP7 expression linked to other signaling pathways

There are a number of candidate genes expressed in domains overlapping or adjacent to *BMP7* expression, and a subset of these loci are also regulated in parallel with the *BMP7* locus. For example, expression levels of *WT-1*, *Pax-2* and *GDNF* are all upregulated in the mesenchyme when contacted by the ureteric bud, and are maintained in the condensed mesenchyme that will undergo tubulogenesis (Lechner and Dressler, 1997). Based on the results presented here, it is likely that BMP7 in the mesenchyme is transcriptionally activated by one of these signals.

Pax-2 expression in the ureter, mesenchyme and tubules parallels that of *BMP7* during normal kidney development (Dressler et al., 1990; Dressler and Douglas, 1992). Similarly in chlorate-treated explants, expression of both *Pax-2* and *BMP7* in the ureter is unaffected (Kispert et al., 1996). Moreover, expression of both genes are lost in the mesenchymal component of the kidney upon chlorate treatment. Heterologous recombinations with lung mesenchyme also show that both *BMP7* and *Pax-2* expression are maintained in the ureter (Kispert et al., 1996). It remains to be determined if *Pax-2* expression precedes that of *BMP7* expression. However, the expression pattern of the mRNA in conjunction with the phenotypes of these mutant mice, suggest that *Pax-2* lies upstream of *BMP7* and may be responsible for regulating its expression in the both the epithelial and mesenchymal components of the kidney.

Our results clearly demonstrate that BMP7 expression in the mesenchyme requires a signal capable of inducing this population of cells. Although *Wnt-1* is not expressed in the kidney, Wnt-1-expressing cells cultured with isolated mesenchyme induce tubulogenesis (Herzlinger et al., 1994), potentially by mimicking activity of another Wnt molecule. Additionally, the spinal cord, a tissue which strongly promotes induction in isolated mesenchyme, expresses several members of the *Wnt* gene family (Parr et al., 1993). Interestingly LiCl has been shown to promote induction of isolated mesenchyme (Davies and Garrod, 1995) and recent evidence suggests that lithium functions to inhibit *GSK3-β* and thus activation of the Wnt signaling pathway (Klein and Melton, 1996). We find that the *BMP7/lacZ* allele is activated upon LiCl treatment, suggesting that activation of *BMP7* may lie downstream of a Wnt signal. Accordingly *Wnt-11* is expressed in the tips of the ureter (Kispert et al., 1996). Interestingly, expression of both *Wnt-11* in the ureter and *BMP7* expression in the mesenchyme are lost in chlorate-treated kidneys. These data are consistent with Wnt-11 functioning as an inductive signal emanating from the ureteric bud to initiate *BMP7* expression in the mesenchyme. Increasing the intracellular levels of PKC also lowers the activity of GSK3-β and thus similarly leads to activation of a Wnt signal (Cook et al., 1996). If this model of decreasing GSK3-β activity is correct in the context of kidney development, then treatment of mesenchyme with activators of PKC should also lead to up-regulation of *BMP7*. Consistent with this, it has been shown that treatment of MDCK cells with PMA, an activator of PKC, initially leads to upregulation of *BMP7* expression (Ishibashi et al., 1993). However, isolated mesenchyme treated with PMA fails to form condensations or aggregates (Davies and Garrod, 1995), and PMA cannot promote expression of the reporter allele (data not shown). Thus, LiCl and PMA may not activate the same signaling pathways in the metanephric mesenchyme. Regardless, our studies are consistent with lithium functioning to artificially activate a Wnt signaling pathway in a ligand-independent fashion to promote condensation and aggregation of the mesenchyme and possibly activation of *BMP7* expression. This may reveal a link between regulation of the Wnt and BMP signaling pathways. It will be interesting to explore possible genetic interactions between *BMP7* and other potential regulators of its expression such as members of the *Wnt* and *Pax* gene families utilizing the *BMP7/lacZ* line of mice.

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