Int. J. Dev. Biol. 46: 525-533 (2002)

BMPs and BMP receptors in mouse metanephric development: *in vivo* and *in vitro* studies

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ABSTRACT BMPs have recently emerged as likely regulators of development of the permanent kidney (metanephros). Transcripts for BMPs and their receptors have been localised in the developing metanephros. *In vitro,* BMPs 2, 4 and 7 have direct or indirect roles in regulation of ureteric branching morphogenesis and branch formation. *In vivo,* renal phenotypes have been reported in BMP7 homozygous null mutant mice and BMP4 heterozygous null mutant mice. In the present study, *in vivo* and *in vitro* roles of BMPs and BMP receptors in metanephric development were further analysed. Stereology and histology were used to analyse kidneys from mice heterozygous for mutations in either BMP2, BMPR-IA or ActR-IA. Roles of BMPs 2 and 4 in mouse metanephric development *in vitro* were analysed by culturing whole metanephroi in the presence of BMP2, BMP4, the BMP inhibitor noggin, and BMP4 plus noggin. Ureteric branching morphogenesis and nephrogenesis were analysed. By qualitative histology, kidneys from BMP2, BMPR-IA and ActR-IA heterozygous null mutant mice were found to be the same as those from wild type mice. The kidneys of the heterozygous mice contained the normal complement of nephrons. *In vitro,* high concentrations of BMP4 inhibited branching of the ureteric epithelium and changed its morphology, while nephrogenesis was inhibited by 50%. A range of concentrations of BMP2 did not alter ureteric or mesenchyme morphology, or the number of glomeruli formed. Noggin did not alter metanephric development *in vitro,* but did block the effect of BMP4. The experiments described in this study have shown that BMP4 has distinct roles from BMP2 in metanephric development.

KEY WORDS: *BMPs, noggin, metanephros, kidney, branching*

Introduction

Bone morphogenetic proteins (BMPs) comprise the largest subfamily of the transforming growth factor-β (TGF-β) superfamily of secreted proteins. Like most members of the TGF-β superfamily, BMPs signal through complexes of type I and type II receptors. Upon ligand binding, type I receptors become phosphorylated and in turn phosphorylate, and thus activate, downstream signaling molecules including SMAD (a term derived from fusion of *Drosophila* Mad and *C. elegans* genes Sma 2, 3 and 4). BMPs have important roles in the development of many tissues and organs, in particular those that develop through epithelial-mesenchymal interactions. The permanent kidney, the metanephros, is an organ whose normal development partly relies on such epithelial-mesenchymal interactions.

Evidence from expression studies and *in vitro* and *in vivo* studies suggests some BMPs have important roles in metanephric development. The expression of BMP ligands and BMP receptors has been detected in the developing mammalian metanephros (Dudley and Robertson, 1997; Martinez *et al.*, 2001). BMP7 homozygous null mutant mice die *post partum* from renal failure and closer examination of their kidneys shows evidence of disrupted nephrogenesis due to extensive apoptosis of the metanephric mesenchyme (Luo *et al.*, 1995; Dudley *et al.*, 1995). Unfortunately, many other BMP or BMP receptor homozygous null mutant mice have provided limited insights into the roles of these molecules in metanephric development because more often than not they die early in embryogenesis, prior to metanephric development. On the other hand, most heterozygous null mice for BMP ligands or BMP receptors do not show overt renal phenotypes, although BMP4 heterozygous null mutant mice have an incompletely penetrant renal phenotype which is dependent on the genetic background of the mice (Dunn *et al.*, 1997). Briefly, when renal abnormalities are present in BMP4 heterozygous mice, they consist of cystic kidneys with hydronephrosis without dilation of the ureter. The kidney cortex present is atrophic with multiple cysts affecting glomeruli and tubules. Like BMP4 homozygous null mutant

Abbreviations used in this paper: BMP, bone morphogenetic protein; BSA, bovine serum albumin.

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0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

mice, BMP2 homozygous null mutant mice also die prior to metanephric development (Zhang and Bradley, 1996). Unlike BMP4 heterozygous null mutant mice, BMP2 heterozygotes do not have significant defects in the kidney despite the similarity of the two ligands (Zhang and Bradley, 1996). Mice lacking functional BMP type I receptors ActR-IA (Gu *et al*., 1999; Mishina *et al*., 1999) or BMPR-IA (Mishina *et al*., 1995) die prior to metanephric development, and no overt phenotype is reported in the heterozygous null mutants (Gu *et al*., 1999; Mishina *et al*., 1999; Mishina *et al*., 1995).

A number of *in vitro* studies investigating the roles of BMPs in metanephric development have been described. Obara-Ishihara *et al,* (1999) showed that BMP4 was required for the formation of the nephric duct from the intermediate mesoderm and for the prevention of ectopic budding from the Wolffian duct. Exogenous BMP4 altered the ureteric branching pattern (Miyazaki *et al*., 2000), and culture of kidney rudiments in the presence of BMP4 revealed that the posterior part of the metanephros was most severely affected (Raatikainen-Ahokas *et al*., 2000).

In metanephric organ culture, BMP2 at a range of doses inhibits overall metanephric growth. BMP2 also inhibits the number and length of tubular structures formed by mIMCD-3 cells (Piscione *et al*., 1997). The inhibitory effects of BMP2 on branching morphogenesis of mIMCD-3 cells was inhibited by hepatocyte growth factor (HGF), suggesting that BMP2 and HGF control parallel pathways downstream of the receptors, possibly at the level of transcriptional or post-transcriptional events (Gupta *et al*., 2000). Using the same models of branching morphogenesis, BMP7 has been shown to have a stimulatory effect at low doses whereas high doses are inhibitory to growth and branching (Piscione *et al*., 1997; Gupta *et al*., 2000). It has recently been proposed that the dose-dependent effects of BMP7 on branching morphogenesis of ureteric epithelium and mIMCD-3 cells are mediated via SMAD-dependent and independent pathways (Piscione *et al*., 2001).

The aims of the present study were to determine if the kidneys of mice heterozygous for mutations in BMP2, BMPR-IA or ActR-IA develop normally. Qualitative histological examination was performed and total nephron number, an index of the overall efficiency of kidney development, was determined. In addition, the effects of exogenous BMP2, BMP4 and noggin (a BMP inhibitor) on the *in vitro* development of mouse metanephroi were analyzed.

Results

Histology of WT and Het Kidneys

Photomicrographs of kidneys from wild-type (WT), BMP2, BMPR-IA and ActR-IA heterozygous (+/-) mice are shown in Fig. 1. No abnormalities were evident in the kidneys of heterozygous animals.

Kidney Weight and Volume

Kidney weights and volumes are shown in Table 1. Kidneys from BMP2 +/- mice weighed approximately 16% less than WT kidneys ($p<0.05$). Kidneys from BMPR-IA and ActR-IA +/- mice were the same weight as WT kidneys. While kidney weight was reduced in BMP2 heterozygous mice, kidney volume was not. The data indicate that kidney weight was reduced by about 16% ($p=0.04$) and kidney volume by 10% ($p=0.22$). This indicates that the difference in kidney size is probably of the order of 10-15% and possibly of marginal biological importance. Volumes of BMPR-1A and ActR-1A +/- kidneys were similar to WT kidneys.

Nephron Number and Size In Vivo

There was no difference in nephron number between the four groups (Table 1). The kidneys of all mice contained between about 11,700 and 12,700 nephrons. Similarly, there was no difference in mean glomerular or renal corpuscle volumes between the groups.

Effect of Exogenous BMP2 on Mouse Metanephric Development In Vitro

The weight of the mice whose metanephroi were used in BMP2 *in vitro* experiments was similar in all groups. After metanephroi were cultured in increasing concentrations of BMP2 for 4 days, their projected area was similar to that of control metanephroi (Fig. 2A). Phase contrast photomicrographs of metanephroi from these groups are shown in Fig. 3 and are similar. Metanephroi cultured in the presence of 0.2, 5 and 10 nM BMP2 demonstrated normal histology in terms of ureteric duct branch morphology, S and comma-shaped body formation, glomerular development and interstitium distribution (Fig. 4 C,E,G). The qualitative appearance of ureteric duct branching in metanephroi cultured for 3 days in the presence of BMP2 at a range of concentrations was comparable to that of metanephroi grown in control media (Fig. 5B). The gross

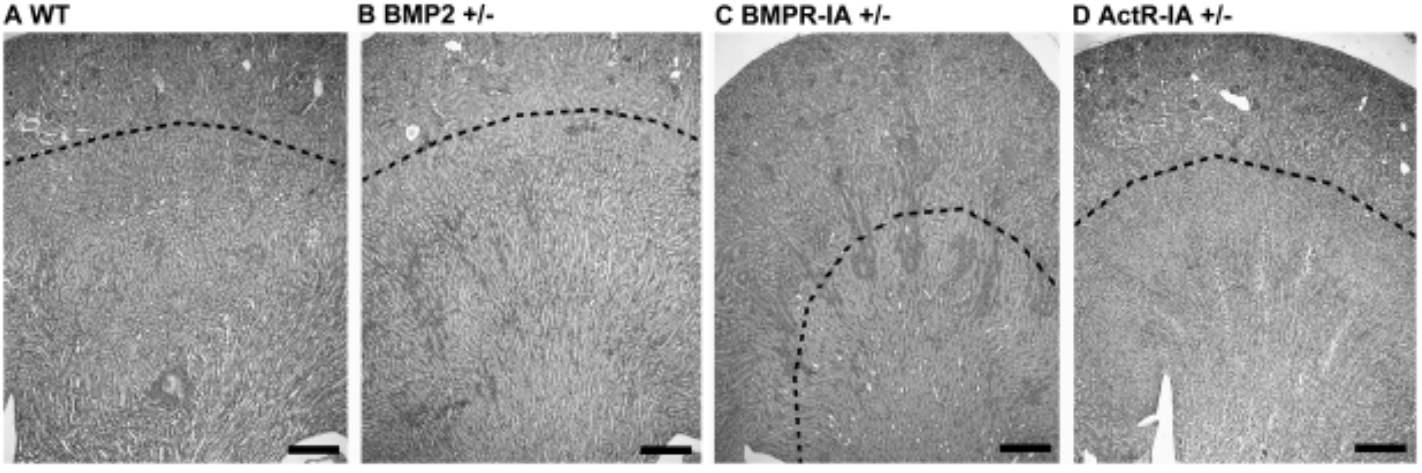


Fig. 1. Representative photomicrographs of wild type and heterozygous kidneys. *Low power images of H + E stained sections showing gross kidney architecture of* **(A-D)** *WT, BMP2, BMPR-IA and ActR-IA +/- kidneys respectively. The overall structure of the cortex (above), cortico-medullary region and the medulla (below) are the same in all kidneys. The cortico-medullary boundary is indicated with a dashed line. Bars, 250μm.*

TABLE 1

KIDNEY WEIGHTS AND STEREOLOGICAL ESTIMATES

	GENOTYPE			
	WT (10)	**BMPR-IA +/- (10)**	**ActR-IA +/- (10)**	**BMP2 +/- (8)**
Kidney weight (mg)	65.7 ± 5.8	62.9 ± 8.4	61.4 ± 6.3	55.5 ± 5.1*
Kidney volume (mm^3)	51.63 ± 8.23	46.70 ± 5.67	49.87 ± 4.96	46.47 ± 5.93
Glom. number	12321 ± 2460	12702 ± 1365	12423 ± 1721	11703 ± 719
Glom. Vol. ($mm^3 x\ 10^{-5}$)	7.24 ± 2.00	6.40 ± 1.43	8.33 ± 1.11	6.56 ± 1.35
Corp. Vol. ($mm^3 x\ 10^{-5}$)	10.32 ± 2.37	8.56 ± 2.11	10.35 ± 1.51	8.71 ± 1.59

Kidney weights and stereological estimates for kidney volume, total glomerular number, average glomerular volume and average renal corpuscle volume. The number of kidneys analysed in each group is shown in parentheses. Values are mean ±SD.

length and thickness of the ureteric duct branches appeared similar to that of control metanephroi (Fig. 5A). Semi-quantitative analysis of ureteric duct branching found that the number of branch tips in metanephroi cultured with all concentrations of BMP2 was not significantly different from metanephroi grown in control media (Fig. 2E). The number of glomeruli formed in metanephroi cultured with 0.2, 5 and 10 nM BMP2 was similar to that in control metanephroi (Fig. 2C).

Stereological analysis was used to estimate the absolute and relative volumes of ureteric epithelium, nephrons and mesenchyme/interstitium following culture in the presence of exogenous BMP2 for 4 days. No differences in absolute or relative volumes were identified between control and BMP2 cultures (Fig. 6).

Effect of Exogenous BMP4 on Mouse Metanephric Development In Vitro

The weights of embryos used to isolate metanephroi for BMP4 culture experiments were similar in all groups. When metanephroi were grown in the presence of 1 nM BMP4 for 4 days, the projected area was similar to that of control metanephroi, but when BMP4 concentrations increased to 5 and 10 nM, the projected areas of the metanephroi were significantly less than that of control metanephroi ($p<0.001$ in both cases) (Fig. 2B*).* Phase contrast microscopy revealed no detectable difference in the gross morphology of metanephroi cultured in the presence of 1 nM BMP4 (Fig. 3F), but metanephroi cultured with 5 and 10 nM BMP4 were clearly different to control metanephroi (Fig. 3 G,H). In these metanephroi there was a loss of definition of the metanephric perimeter. Some ureteric duct branches were seen to grow with a reduced incidence of branching and glomeruli were not clearly visible.

The histology of metanephroi cultured in the presence of 1 nM BMP4 (Fig. 4D) was normal. However, metanephroi cultured in the presence of 5 and 10 nM (Fig. 4 F,H) exogenous BMP4 often exhibited grossly dilated ureteric branches. There was a reduction in the number of S- and comma-shaped bodies and glomeruli, and the overall size of the metanephroi was reduced.

Anti-calbindin immunofluorescence revealed that ureteric branching and growth in metanephroi cultured in the presence of 1 nM BMP4 was similar to that in control metanephroi (data not shown). However, metanephroi cultured in media containing 5 nM

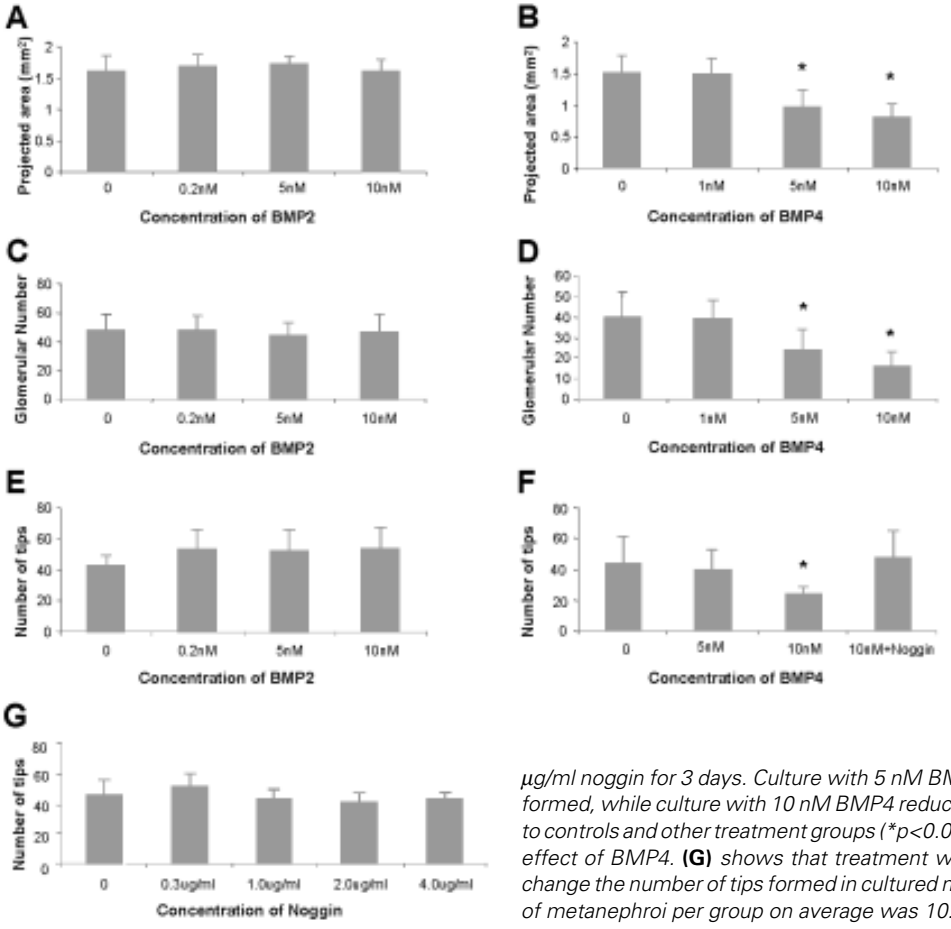


Fig. 2. Projected areas, glomerular number and number of branch tips formed in cultured metanephroi. *After 4 days in culture, projected areas of metanephroi were determined (A and B).* **(A)** *shows no difference in the projected areas of metanephroi grown in control media versus media supplemented with various concentrations of BMP2.* **(B)** *shows a significant reduction in projected area of metanephroi cultured in the presence of 5 and 10 nM BMP4 (*p<0.001).* **(C)** *shows the number of glomeruli formed in control metanephroi and metanephroi cultured in the presence of 0.2, 5 or 10 nM BMP2 for 4 days. The number of glomeruli formed is similar in all groups.* **(D)** *shows that when metanephroi were cultured in the presence of 5 or 10 nM BMP4 for 4 days, the number of glomeruli that formed was approximately halved compared with control metanephroi (*p<0.001 in both cases).* **(E)** *shows the numbers of branch tips formed in metanephroi grown in control media (BSA), or media supplemented with 0.2, 5 or 10 nM BMP2 for 3 days. Values are similar in the four groups.* **(F)** *shows the numbers of branch tips formed in metanephroi grown in control media (BSA), or media supplemented with 5 or 10 nM BMP4 or 10 nM BMP4 plus 1.0 μg/ml noggin for 3 days. Culture with 5 nM BMP4 did not significantly alter the number of tips formed, while culture with 10 nM BMP4 reduced the number of tips formed by 50% compared to controls and other treatment groups (*p<0.001). The addition of 1.0 μg/ml noggin blocked this effect of BMP4.* **(G)** *shows that treatment with a range of concentrations of noggin did not change the number of tips formed in cultured metanephroi. Values are mean ± SD. The number of metanephroi per group on average was 10. Control cultures contained BSA.*

(data not shown) and 10 nM BMP4 had abnormal ureteric branches (Fig. 5C). The number of branches was reduced, those branches present appeared longer, the overall symmetry of the branched "tree" was lost, and some of the branch tips had a "pointy" appearance. The number of branch tips in metanephroi cultured with 5 nM BMP4 was the same as in control metanephroi (Fig. 2F). In contrast, treatment with 10 nM BMP4 reduced the number of branch tips by approximately 50% ($p<0.001$) (Fig. 2F).

Using rhodamine-PNA histochemistry, it was determined that the number of glomeruli which formed in metanephroi cultured with 1 nM BMP4 was similar to the number in control metanephroi (Fig. 2D). However, the number of glomeruli formed in metanephroi cultured with 5 and 10 nM BMP4 was approximately half that of the number formed in control metanephroi ($p<0.001$ in both cases) (Figs. 2D, 5F).

***Effect of Exogenous Noggin on Mouse Metanephric Development* In Vitro**

As shown by phase contrast microscopy (Fig. 3 I-M), addition of a range of concentrations of noggin to cultured metanephroi did not visibly alter morphology after 3 days. Anti-calbindin immunofluorescence (Fig. 5D) showed normal appearance of ureteric duct branches and the number of ureteric duct tips formed in metanephroi treated with noggin was the same as in control metanephroi.

***Effect of Exogenous BMP4 plus Noggin on Mouse Metanephric Development* In Vitro**

When metanephroi were cultured with 10 nM BMP4 plus 1 μg/ml noggin, the normal morphology of metanephroi was largely restored (Fig. 3 N,O). Anti-calbindin immunofluorescence showed grossly normal ureteric branching in metanephroi cultured with BMP4 plus noggin. The number of ureteric duct tips in these metanephroi was the same as in control metanephroi and significantly different from that in metanephroi cultured with 10 nM BMP4 alone (Fig. 2F).

Discussion

In this study we used loss of function models (*in vivo* and *in vitro*) and a gain of function model (*in vitro*) to investigate the roles of BMPs and BMP receptors in metanephric development. Previous findings from expression studies, *in vitro* studies and *in vivo* studies had suggested important roles for certain BMP ligands and receptors in metanephric development. However, it is not possible to ascertain the roles of BMP2, BMPR-IA and ActR-IA in metanephric development from the analysis of homozygous null (-/-) embryos because they die prior to the commencement of metanephric development. Therefore, in the present study kidney structure was analysed in BMP2, BMPR-IA and ActR-IA heterozygous null mice. This approach was chosen because it is well established that BMP4 +/- null mice display renal phenotypes, suggesting the BMP4 haploinsuffiency can influence renal development. The qualitative and quantitative microscopic analyses performed showed that at p20-22, when nephrogenesis in mice is complete, there was no detectable morphological abnormality in any of the heterozygous kidneys. Importantly, total nephron number in the three groups of heterozygous mice was similar to that in wildtype mice. The only difference observed was the 16% reduction in kidney weight in the BMP2 heterozygous mice. Body weights were unfortunately not available for these mice, but reduced body weight of BMP2 heterozygous mice has not previously been reported. It is unlikely, therefore, that this reduction in kidney weight reflects reduced body weight. Taken together, these *in vivo* results suggest that loss of one BMPR-1A or ActR-1A allele does not compromise renal development, whereas loss of one BMP2 allele may have a minor effect on renal mass.

The major findings from the present *in vitro* studies were: (1) incubation of metanephroi in the presence of 0.2, 5 and 10 nM

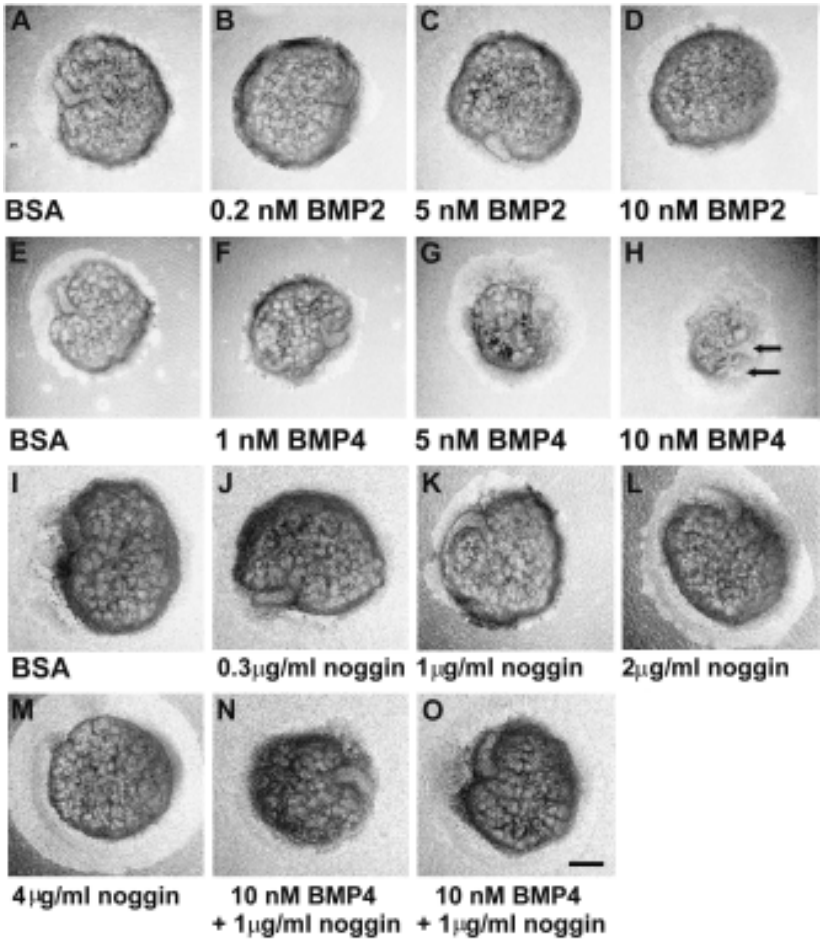
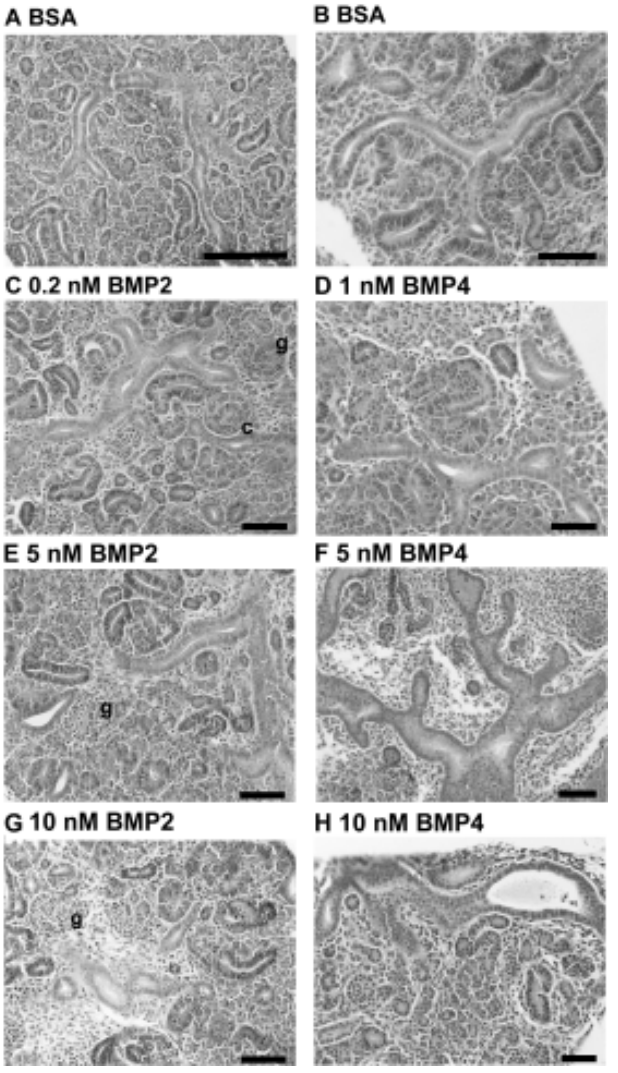


Fig. 3. Phase contrast photomicrographs of metanephroi cultured in control media and media supplemented with BMP2, BMP4, noggin or BMP4 plus noggin. (A-D) *show metanephroi cultured for 4 days in control media (BSA) or media supplemented with 0.2, 5 or 10 nM BMP2, respectively.* **(E-H)** *show metanephroi cultured for 4 days in control media (BSA) or media supplemented with 1, 5 or 10 nM BMP4, respectively. Note the reduced size of metanephroi in G and H and the reduced ureteric branching (arrows).* **(I-M)** *show metanephroi cultured for 3 days in control media (BSA), and media supplemented with 0.3 μg/ml, 1 μg/ml, 2 μg/ml, or 4 μg/ml noggin.* **(N,O)** *show metanephroi cultured for 3 days with 10 nM BMP4 plus 1 μg/ml noggin. Unlike metanephroi cultured with BMP4 alone, these metanephroi appear similar to control metanephroi. Bar, 250μm.*



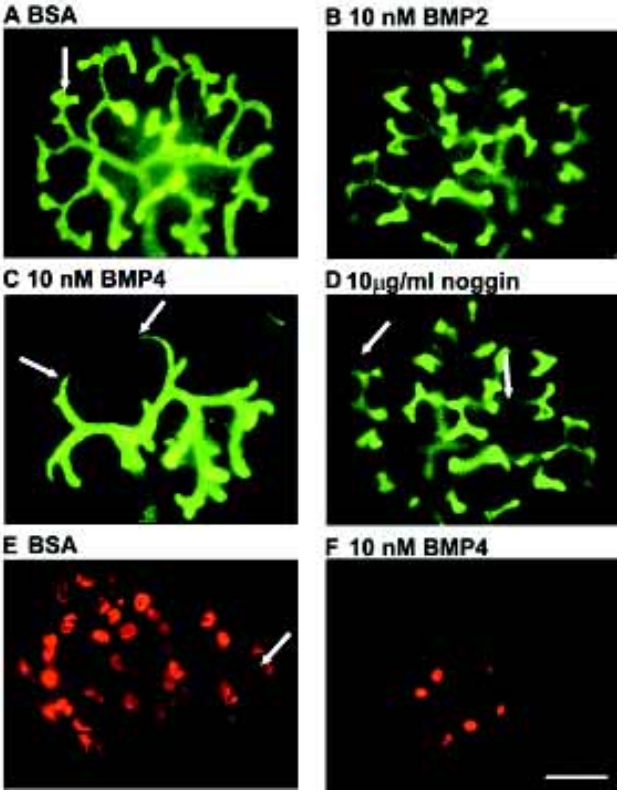


Fig. 4. (Left) Photomicrographs of H + E stained sections of metanephroi cultured in the presence of exogenous BMP2 or BMP4 for 4 days. (A,B) *show sections of metanephroi cultured in control media (with BSA). Normal* in vitro *metanephric development is evident, including ureteric branching morphogenesis and nephron formation.* **(C,E,G)** *show sections of metanephroi cultured in the presence of 0.2, 5 and 10 nM exogenous BMP2, respectively. Normal metanephric histology is seen. Glomeruli (g) and comma-shaped bodies (c) are shown.* **(D,F,H)** *show metanephroi cultured in the presence of 1, 5 and 10 nM BMP4, respectively. Normal development was observed in metanephroi cultured with 1 nM BMP4. However, metanephroi cultured with 5 and 10 nM BMP4 showed thickened ureteric epithelium which was frequently seen to be abnormally dilated. Bars, 100 μm.*

Fig. 5. (Right) Whole mount photomicrographs of BMP2, BMP4 or noggin-treated metanephroi immunostained for calbindin and histochemically stained for PNA. *After 3 days of culture metanephroi were fixed and immunostained for calbindin. Observe the branching pattern in metanephroi cultured in control media (BSA)* **(A)** *and the similar branching pattern in metanephroi cultured in 10 nM BMP2* **(B)***, and 10 μg/ml noggin* **(D)***. In contrast, metanephroi cultured in the presence of 10 nM BMP4* **(C)** *demonstrated abnormal ureteric branching. The overall symmetry of the ureteric "tree" was altered, branching events diminished, and the tips of some branches appeared thin and pointy (arrows) instead of rounded as seen in control metanephroi (arrow) (A). After 4 days of culture, some metanephroi were stained with rhodamine-PNA for detection of glomeruli (E,F).* **(E)** *shows a metanephros incubated in control media (BSA) and* **(F)** *shows a metanephros incubated with 10 nM BMP4. Note the marked reduction in the number of nephrons after culture with 10 nM BMP4. Bar, 250μm.*

BMP2 did not alter *in vitro* metanephric development; (2) incubation in the presence of 5 and 10 nM BMP4 led to asymmetric and reduced ureteric branching, the development of some dilated ureteric branches, and reduced the number of glomeruli formed; (3) the effects of BMP4 on metanephric development were blocked by noggin; and (4) incubation in the presence of varying concentrations of exogenous noggin did not alter normal metanephric development.

The present findings regarding BMP2 and metanephric development *in vitro* are in contrast with those of Piscione *et al.*, (1997) who found that culture of metanephroi in the presence of nanomolar concentrations of exogenous BMP2, added to the culture medium, marginally inhibited overall metanephric growth. When micromolar concentrations of BMP2 were presented to cultured metanephroi on an agarose bead, reduced branching of the ureteric epithelium was obvious. The most likely explanation for the difference in results between the present study and those of Piscione *et al.* (1997) lies in the concentrations of BMP2 used and the mode of delivery. In the present study, BMP2 was added directly to the culture medium, therefore resulting in a uniform concentration throughout the explanted metanephros. When Piscione *et al.* (1997) conducted similar experiments, the observed effect was barely identifiable, and in our hands not reproducible despite the use of quantitative techniques.

Piscione *et al.* (1997), however, also delivered much higher concentrations of BMP2 on an agarose bead, thereby establishing a concentration gradient of BMP2. BMPs have been shown to have concentration-dependent effects on ectoderm, with different concentrations of BMPs or BMP inhibitors resulting in the formation of either epidermis, cement gland or neural tissue (Knecht and Harland, 1997; Wilson *et al.*, 1997). More recently, markers of different regions of the developing brain have been shown to be regulated by thresholds of sensitivity to BMP signaling (Barth *et al.*, 1999).

The present findings clearly indicate that culture in the presence of 5 and 10 nM BMP4 dramatically alters metanephric development. The metanephroi that developed contained fewer ureteric branches, many of these branches had an abnormal shape, and the number of glomeruli that formed was reduced. Moreover, ureteric branching was more inhibited on one side of the kidney than the other. These findings are in agreement with those of Raatikainen-Ahokas *et al.* (2001) who showed BMP4 to be inhibitory to posterior ureteric branch formation in cultured metanephroi. Interestingly, Miyazaki *et al.* (2000) recently reported that BMP4 and FGF2 together promoted an increase in the Pax2-negative cells of the metanephric mesenchyme suggesting that in the presence of FGF2, BMP4 influences differentiation of metanephric mesenchyme. Like the present study, Miyazaki *et al.* (2000) reported that growth of the ureteric duct was stunted in the presence of 10 nM BMP4 and although branching events were observed, these would have occurred prior to the time of explant.

Branching of the ureteric epithelium is dependent on signals from the metanephric mesenchyme, and epithelialisation of mesenchyme cells is dependent on signals from cells at the tips of the ureteric duct. It has been suggested that BMP4 induces apoptosis of neural crest cells (Graham *et al.*, 1994), and developing heart (Zhao and Rivkees, 2000), and Raatikainen-Ahokas *et al.* (2000) observed increased apoptosis in mesenchymal cells of metanephroi cultured with exogenous BMP4. The decreased growth and branching of the ureteric epithelium in the presence of BMP4 may thus reflect a decrease in growth promoting signals from mesenchymal cells. The reduction in branching would in turn lead to reduced signaling to the metanephric mesenchyme and reduced nephrogenesis. We and others have shown that at E12.5 and E14.5, BMPR-1A and BMPR-1B are expressed in the tips and body of the branching ureter as well as mesenchymal condensates, vesicles and comma-shaped bodies (Martinez, *et al.*, 2001; Miyazaki *et al.*, 2000). The expression pattern of BMPR-II is similar although this receptor is not expressed in the body of the ureter. At E17.5, transcripts for all of these BMP receptors are expressed in the nephrogenic zone and some developing tubules.

Some of the ureteric branches formed in metanephroi cultured in the presence of exogenous BMP4 were dilated. Interestingly, *in vivo* overexpression of BMP4 in the developing lung results in mesenchymal cell death, inhibition of epithelial cell proliferation and grossly dilated terminal buds (Bellusci *et al.*, 1996). Bellusci *et al.* (1996) proposed a model of lung branching morphogenesis in which BMP4, Shh and Wnt-2 interact. The hypothesis suggests that Shh secreted by distal lung tip epithelial cells induces the expression of BMP4/Wnt-2 by surrounding mesenchyme, which in turn activates expression of BMP4 by the distal epithelium, thereby establishing an autoregulatory circuit.

If the above hypothesis can be applied to the developing metanephros, then BMP4 may autoregulate itself so that the concentration is not too high or too low. However, in metanephric cultures with exogenous BMP4 present, the autoregulation may be disturbed, thereby resulting in apoptosis of the mesenchyme. This loss of mesenchyme results in a reduction in the signals which regulate ureteric branching. Another possibility is that the excess BMP4 is activating receptors in the tips and body of the ureter, and is hence inhibiting branch initiation. The dilatations may be a consequence of loss of mesenchyme and thus mesenchymal-derived signals which regulate the integrity and shape of the ureter.

One obvious issue to be addressed is the different metanephric phenotypes observed following culture with BMP2 and BMP4. The high level of sequence and amino acid identity between these two BMPs has been well reported so it would be reasonable to expect similar phenotypes following metanephric culture. One potential explanation could be the difference in SMADs recruited to transduce BMP signals to the nucleus. BMP2 and BMP4 have different affinities for different receptors, and although there is no evidence, it is possible that different receptors preferentially phosphorylate different SMAD molecules. Therefore, depending on which BMP ligand is bound, SMAD signaling may be different, thus altering the transcription in the nucleus and eventually the outcome for the cell.

Incubation in the presence of a range of concentrations of noggin failed to alter metanephric structure. Noggin was confirmed to be active by its ability to block the effects caused by exogenous BMP4. A possible explanation for the lack of phenotype in metanephroi cultured with noggin may again concern the local concentration of noggin. It may have been a more fruitful study had the

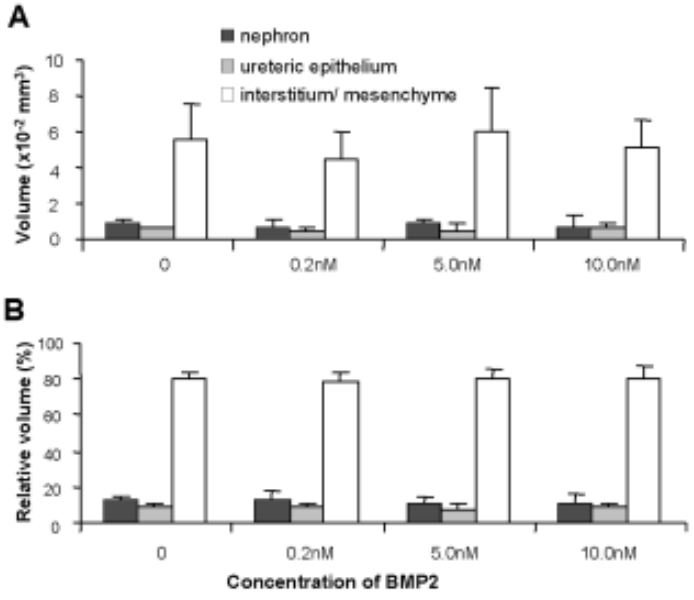


Fig. 6. Histograms showing the absolute and relative volumes of ureteric epithelium, nephron epithelia and mesenchyme/interstitium in metanephroi cultured in the presence of BMP2. (A) *shows absolute volumes of ureteric epithelium, nephron epithelia and mesenchyme/interstitium in metanephroi cultured in control media (BSA), or media supplemented with 0.2, 5 and 10 nM BMP2. The volume of all compartments was the same in all groups.* **(B)** *shows the relative volumes of ureteric epithelium, nephron epithelia and mesenchyme/interstitium in metanephroi from the four groups. There was no difference in relative volumes of any compartment between the four groups. Values are mean ± SD.*

noggin been administered to the explanted metanephroi on a bead. Administration of noggin on a bead can be hypothesised to result in increased branching of the ureteric epithelium. As discussed above, BMP4 induces apoptosis, regulates budding and may be autoregulated. Taking this information, the data generated in the present study, and findings from earlier reports (Miyazaki *et al.*, 2000; Raatikainen-Ahokas *et al.*, 2000), it can reasonably be expected that addition of noggin on a bead may increase ureteric branching and/or result in excessive mesenchymal proliferation. This would result in an increase in signals emanating from the mesenchyme assuming it was normally and fully differentiated. This could lead to an increase in nephrogenesis.

Materials and Methods

In Vivo Studies

Kidney Dissections. Heterozygous null male mice for BMP2, BMPR-IA or ActR-IA (Zhang and Bradley. 1996; Mishina *et al.*, 1999; Mishina *et al.*, 1995) were mated with wild type females and genotypes of offspring were confirmed by Southern blot analysis. All mice were originally generated in a 129SvEv background. The BMP2 mice were congenic for C57BL6/J (backcrossed 8 times), the BMPR-1A mice were also congenic for C57BL6/J (backcrossed 12 times), and the ActR-1A mice were a mixture of 129SvEv and C57BL6/J. At postnatal days 20-22, kidneys from heterozygous and wild type mice were removed, weighed and immersion-fixed in 10% neutral buffered formalin. Experiments were conducted in strict accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.*

Stereology. Following immersion fixation, right kidneys were cut transversely into approximate halves and rinsed in water for 1 hour. Tissue was processed for embedding in Technovit glycolmethacrylate resin (Kulzer and Co. Gmbh, Friedrichsdorf, Germany). Resin was allowed to polymerise for at least 2 days. Blocks were sectioned at 20 μm using a Leica RM 2165 Supercut microtome with glass knives until no tissue remained in the block. Average section thickness (t) was confirmed to be 20 μm by measuring the block thickness before and after cutting and dividing by the number of sections cut. Every 10th and 11th section was collected onto glass slides, the first being chosen randomly in the interval of 1 to 10. Sections were dried overnight at 60^{o}C and then stained with haematoxylin and eosin (H + E).

Estimating Kidney Volume. Kidney volume was estimated using the Cavalieri principle (Bertram *et al*,. 1992; Gundersen *et al.*, 1988; Gundersen and Jensen, 1987). Briefly, every 10th glycolmethacrylate section was projected onto a Fuji Minicopy Reader at a magnification of 24.25X. An orthogonal grid with an area per grid point (a(p)) of 0.68 mm^2 (corrected for magnification) was placed over the screen. The number of grid points overlying all kidney sections (P_s) as well as complete (not grazing sections) kidney sections (P_f) was determined. Kidney volume (V_{kid}) was estimated using:

$$V_{kid} = 10 \times t \times a(p) \times P_s$$

Estimating Glomerular Number. To estimate the total number of glomeruli, and therefore nephrons in kidneys, glomeruli were counted using physical disectors (Sterio, 1984) in a known fraction of the kidney: the physical disector/fractionator combination (Bertram *et al*,. 1992; Bertram, 1995; Nyengaard and Bendtsen, 1992; Bertram, 2001). Tenth and 11th sections were placed side-by-side on two Olympus BX-50 light microscopes modified for projection. Corresponding fields of the 10th and 11th sections were located and projected at a final magnification of 362X onto orthogonal grids with an a(p) of 0.003 mm^2 (adjusted for magnification). The grid onto which the 10th section was projected incorporated an unbiased counting frame (Gundersen, 1977). The microscope projecting the 10th section was fitted with an automated stage (Autoscan Systems Pty. Ltd., Australia) which allowed fields to be sampled at predetermined intervals. Glomeruli were counted according to the disector principle - they were counted if they were sampled by the unbiased counting frame in the 10th section and if they were not present in the corresponding part of the 11th section (Q^-). To double the efficiency of counting, glomeruli sampled by an unbiased counting frame in the 11th section that were absent in the 10th section were also counted. At the same time, points of the grid covering kidney, renal corpuscles and glomeruli were counted in order to estimate volumes of these structures. Total glomerular number ($N_{glom,kid}$) was estimated using:

$$N_{glom,kid} = 10 \times (P_s/P_f) \times [1/(2f_a)] \times Q^-$$

where 10 is the inverse of the fraction of sections sampled, P_s and P_f were described above, $1/(2f_a)$ gives the fraction of the total section area used to count glomeruli and f_a is determined by

$$[P_{kid} \times a(p) \text{ of phys. Disector}]/[P_f \times a(p) \text{ of microfiche}]$$

and Q^- indicates the total number of glomeruli actually counted (approximately 100 to 200 with an average of 130).

Estimating Mean Glomerular and Renal Corpuscle Volume. Mean glomerular (V_{glom}) and mean renal corpuscle volume (V_{corp}) were estimated using:

$V_{glom} = V_{v(glom,kid)}/N_{v(glom,kid)}$, and

$V_{corp} = V_{v\ (corp,kid)}/N_{v(glom,kid)}$, respectively

where $V_{v(glom,kid)}$ and $V_{v\ (corp,kid)}$ were estimated by dividing the number of points overlying glomeruli and renal corpuscles, respectively by the number of points overlying kidney sections, and $N_{v(glom,kid)}$ was calculated by dividing $N_{glom,kid}$ by V_{kid}.

Histology. Left kidneys were processed for embedding in paraffin, sectioned at 4 μm and stained with H + E, periodic acid-Schiffs (PAS), Masson's trichrome or methenamine-silver. Sections were examined by a renal pathologist, Professor John Dowling (Department of Anatomical Pathology, Alfred Hospital, Prahran, Victoria, Australia).

In Vitro Studies

Animals and Collection of Metanephroi. Balb-c mice were mated overnight. The detection of a vaginal plug in the morning determined a mouse as being pregnant at E0. Metanephroi were dissected from embryos at E12.5. Briefly, pregnant mothers were sacrificed at the desired stage of gestation by cervical dislocation. The uterine horn, typically containing between 3-8 embryos, was dissected and placed in sterile phosphate buffered saline (PBS), embryos were removed from the uterus, weighed and decapitated before being dissected as required using a dissecting microscope. Only kidneys from embryos weighing between 0.08-0.10 g were taken for experimentation to minimise the variability in experimental data.

Upon dissection, metanephroi were washed twice in pre-warmed DMEM/Ham's F12 media (Trace Biosciences, Australia) supplemented with 12.9 μg/ml L-glutamine (Trace Biosciences, Castle Hill, NSW, Australia), 100 μg/ml penicillin/ 100 U/ml streptomycin (Trace Biosciences) and 5 mg/ml transferrin (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia). Care was taken to ensure that the two kidneys from one embryo were not allocated to the same treatment group.

Culturing of Metanephroi. After washing, metanephroi were placed on inserts with a membrane pore size of 3.0 μm (Transwell, Corning Costar Corporation, Cambridge, Massachusetts, USA) in wells containing 350 μl of DMEM/Hams F12 supplemented media as described above. Metanephroi were cultured in the presence of rhBMP2 (R&D Systems, MN, USA), rhBMP4 (R&D Systems) and rmnoggin/Fc chimera (R&D Systems), which were all reconstituted in 0.1% cell culture grade Bovine serum albumin (BSA) (Sigma Chemical Company) in sterile PBS.

Metanephroi were cultured for either 3 or 4 days, with daily media changes and phase contrast photomicrographs being taken. The projected area (A) of metanephroi on the day of fixation was determined by counting points (p) on a 1 cm x 1 cm orthogonal grid with an a(p) of 0.00943mm^2 which overlaid the phase contrast image of the metanephros, using the following formula:

$$A = a(p) \times \Sigma p$$

At the conclusion of the culture period, metanephroi were fixed and processed for examination via either routine histology, immunofluorescence or histochemistry.

Histological Analysis. After 4 days of culture, metanephroi on their inserts were fixed in 10% buffered formalin, processed for embedding in paraffin wax, sectioned at 4 μm and stained with H + E.

Whole-Mount Calbindin Immunohistochemistry. After 3 days of culture, metanephroi to be immunostained with calbindin were fixed in ice-cold methanol, washed twice briefly in PBS. For detection of ureteric epithelium, metanephroi were incubated with monoclonal mouse anti-calbindin D-28K antibody (Sigma Chemical Company) at a dilution of 1:200, at 37ºC for 2 hours. Two 30 minute washes in PBS were performed prior to incubation with goat anti-mouse Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Eugene, Oregon, USA) at a dilution of 1:100, either at 4ºC overnight or at 37ºC for 2 hours. One last wash of PBS was performed to remove excess secondary antibody. Staining was visualised on an Olympus Provis fluorescence microscope (Olympus, Australia) and the number of ureteric branch tips counted.

Whole-Mount Rhodamine Conjugated Peanut Agglutinin (PNA) Lectin Histochemistry. To determine the number of glomeruli formed *in vitro*, metanephroi were stained with rhodamine-PNA. After 4 days of culture, metanephroi were fixed in 10% buffered formalin for 30 minutes, washed for 10 minutes in PBS, incubated at room temperature for 1 hour in 50 mM NH_4Cl, and permeabilised with 0.1 % saponin (BDH Laboratory Supplies, Poole, England) in PBS for 1 hour. Tissue was incubated at room temperature for 30 minutes in 2% H_2O_2 in methanol, followed by a 30 minute wash in 0.1% saponin in PBS, a 2 hour digest in 0.1 U/ml neuraminidase (Sigma Chemical Co.) at 37ºC, and 2X 30 minute washes in 0.2% saponin in PBS. Metanephroi were incubated in 50 μg/ml rhodamine-conjugated PNA (Vector Laboratories Inc., CA, USA) with 0.3% Triton in PBS and 100 nM $MgCl_2$, $MnCl_2$ and $CaCl_2$ ions for 3-4 hours at room temperature. Excess PNA was removed by extensive washing over 3 days in 0.1% saponin in PBS. Glomeruli were identified on the basis of PNA staining as well as cell morphology as sometimes the podocytes of newly-formed glomeruli were immature and were not strongly stained by the rhodamine-PNA. Glomeruli were counted using an Olympus Provis microscope.

Estimation of Metanephric Compartment Volumes using Stereology and Biotinylated *Dolichos biflorus* (DBA) stained Sections. Metanephroi cultured in the presence of BMP2 were also analyzed using stereology. To analyse these metanephroi more thoroughly, stereology was used on DBA-stained sections to estimate the absolute and relative volumes of metanephric compartments (ureteric epithelium, mesenchyme/interstitium and nephron epithelia). Metanephroi were fixed in 10% buffered formalin and sections were prepared as described previously. Serial sections were dewaxed, rehydrated through graded alcohols and washed in PBS for 5 minutes. Non-specific binding sites were blocked with 2% BSA in 0.3% Triton X100 in PBS for 30 minutes. Sections were incubated with 20 μg/ml biotinylated DBA (Sigma-Aldrich Pty. Ltd., Castle Hill, Australia) at room temperature for 3-4 hours and subsequently washed twice for 5 minutes in PBS. Binding of biotinylated DBA was visualised using the *Elite* streptavidin/biotin amplification ABC kit (Vector Laboratories Inc.). Colour development took place with 10 mg/ml diaminobenzedene (DAB) (DAKO Corporation, Carpinteria, CA, USA) with 0.01% H_2O_2/PBS under coverslips for 30-60 seconds. Sections were counterstained with haematoxylin.

Stereology of Metanephric Compartments. Starting with a random section in the interval of one to three, every 2nd or 3rd section (n) was chosen for stereological analysis, aiming for a total of 12-15 sections from each metanephros to be analysed. Using an Olympus BX50 microscope (Olympus, Australia) modified for projection, the sampled sections were projected onto a bench, at a final magnification of 148, with the field of view being overlaid with a 2 cm x 2 cm orthogonal grid with an a(p) of 0.0183mm^2. Grid points either overlaid (hit) DBA-stained ureteric duct epithelium, unstained epithelial structures (nephron components), or undifferentiated interstitium / mesenchyme. The total number of grid points overlying these compartments (P_{ue}, P_N, P_{mi}) was used to calculate their respective volumes (V) according to the Cavalieri Principle (Bertram *et al.*, 1992; Gundersen *et al.*, 1988; Gundersen and Jensen, 1987). This was done using the following formula (for ureteric epithelium):

$$V_{ue} = n \times t \times a(p) \times P_{ue}$$

where t was section thickness. Percent volumes of the three compartments were calculated.

Statistical Analysis. *In vivo* data were analysed using a two-way ANOVA followed by the Tukey post-hoc test. Software used to analyse data was Sigma Stat Version 2.0 software. *In vitro* data were analysed using Minitab for Windows Release 10.51. Xtra. Data were analysed using a one-way ANOVA followed by the post-hoc Tukey Test. A probability of 0.05 or less was considered to indicate statistical significance.

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