**Alk3 controls nephron number and androgen production via lineage-specific effects in intermediate mesoderm**

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**SUMMARY**
The mammalian kidney and male reproductive system are both derived from the intermediate mesoderm. The spatial and temporal expression of bone morphogenetic protein (BMP) 2 and BMP4 and their cognate receptor, activin like kinase 3 (ALK3), suggests a functional role for BMP-ALK3 signaling during formation of intermediate mesoderm-derivative organs. Here, we define cell autonomous functions for Alk3 in the kidney and male gonad in mice with CRE-mediated Alk3 inactivation targeted to intermediate mesoderm progenitors (Alk3<sup>Cre<sup>−<sub>−</sub></sup></sup>). Alk3-deficient mice exhibit simple renal hypoplasia characterized by decreases in both kidney size and nephron number but normal tissue architecture. These defects are preceded by a decreased contribution of Alk3-deleted cells to the metanephric blastema and reduced expression of Osr1 and SIX2, which mark nephron progenitor cells. Mutant mice are also characterized by defects in intermediate mesoderm-derived genital tissues with fewer mesonephric tubules and testicular Leydig cells, epithelial vacuolization in the postnatal corpus epididymis, and decreased serum testosterone levels and reduced fertility. Analysis of ALK3-dependent signaling effectors revealed lineage-specific reduction of mesonephric tubules and testicular Leydig cells, epithelial vacuolization in the postnatal corpus epididymis, and decreased serum testosterone levels and reduced fertility. Analysis of ALK3-dependent signaling effectors revealed lineage-specific reduction of phosho-p38 MAPK in metanephric mesenchyme and phosho-SMAD1/5/8 in the testis. Together, these results demonstrate a requirement for Alk3 in distinct progenitor cell populations derived from the intermediate mesoderm.

**KEY WORDS: Alk3, Intermediate Mesoderm, Nephron, Mouse**

**INTRODUCTION**
The intermediate mesoderm gives rise to two distinct organ systems within the urogenital tract: the mesonephric kidneys, and the gonads and their associated duct structures (Saxen, 1987; Joseph et al., 2009). The intermediate mesoderm is specified, in part, by bone morphogenetic proteins (Dressler, 2009; Preger-Ben Noon et al., 2009). After gastrulation, a renal progenitor cell population, marked by expression of odd-skipped-related (Osr) 1, arises from the intermediate mesoderm along an anterior-posterior axis. The demonstration that overexpression of a constitutive active activin-like-kinase (ALK) 3 receptor in the chick intermediate mesoderm generates dose-dependent regulation of Osr1 expression (James and Schultheiss, 2005) suggested a functional role for ALK3. However, the function of endogenous BMP signaling during specification of the intermediate mesoderm is undefined.

Murine urogenital development commences at embryonic day (E) 8.0 with the formation of intermediate mesoderm-derived paired epithelial tubes, the nephric ducts, in the urogenital ridge bilateral to the midline. Initially, a transient kidney-like structure, the pronephros, is induced by the nephric duct only to degenerate completely (Saxen et al., 1986; Saxen and Sariola, 1987; Kuure et al., 2000). Coincident with their caudal migration at E9.5, the nephric ducts each induce formation of the mesonephric tubules from the intermediate mesoderm (Kuure et al., 2000). Ultimately, mesonephric cells only contribute to the testis. By E10.5, a caudal region of the nephric duct forms an epithelial outgrowth termed the ureteric bud in response to signals from the intermediate mesoderm-derived metanephric blastema. Permanent metanephric kidney development commences with invasion of the blastema by the ureteric bud.

Kidney organogenesis is dependent on reciprocal signaling between the ureteric bud and what is now the metanephric mesenchyme (Grobstein, 1956; Saxen and Sariola, 1987). A subset of cells in the metanephric mesenchyme express Osr1, which is required for the differentiation of the derivative renal stromal and nephrogenic lineages (Mugford et al., 2008). Metanephric mesenchyme-derived signals trigger elongation and repetitive branching of the ureteric bud to form the collecting system. In turn, ureteric-derived signals induce aggregation of mesenchymal nephrogenic progenitor cells around ureteric bud tips. Nephrogenic cells either replenish the progenitor pool or undergo a mesenchyme-to-epithelial transition and form a succession of intermediate structures culminating in formation of the mature nephron (Vainio and Lin, 2002). The number of nephrons formed during nephrogenesis is an important determinant of human health. Reduced nephron endowment results in pathological states ranging from childhood renal failure to adult-onset hypertension, depending on the severity of nephron deficiency (Schedl, 2007; Cain et al., 2010).

Male genital tissues arise from intermediate mesoderm-derived mesonephric and nephric duct cells. The nephric duct contributes to formation of the epididymis and seminal vesicle (Dyche, 1979; Joseph et al., 2009). Mesonephric cells contribute to testicular androgen-producing Leydig cells and peritubular myoid cells (Buehr et al., 1993; Merchant-Larios et al., 1993). Formation of testicular seminiferous cords is dependent on migration of mesonephric endothelial cells (Combes et al., 2009). By contrast,
testicular Sertoli and germ cells are derived from coelomic epithelium and the epiblast, respectively (Lawson and Hage, 1994; Karl and Capel, 1998). The molecular mechanisms that control specification of intermediate mesoderm-derived progenitor cell populations into testicular cell populations are undefined.

BMPs bind to heteromeric complexes composed of type I (confers ligand specificity) and type II serine-threonine kinase receptors. The mode of ligand binding to the receptor complex dictates activation of distinct signaling effectors. BMP ligand, binding to pre-formed receptor complexes, activates phosphorylation of cytoplasmic SMAD 1/5/8 proteins (ten Dijke et al., 1994), while de novo formation of ligand-receptor complexes activates MAPKs, including p38 and ERK (Nohe et al., 2004). BMP2 and BMP4 are expressed as early as E7.5 in the intermediate mesoderm. Bmp4+/- mice die during gastrulation from defective posterior mesoderm formation (Winner et al., 1995; Zhang and Bradley, 1996). Although analysis of deficient mice demonstrates that both Bmp2 and Bmp4 control primordial germ cell number, embryonic lethality in these and Alk3-deficient mice before other types of testicular cells are specified, has limited a more complete analysis of BMP function during testis development (Lawson et al., 1999; Ying and Zhao, 2001). The demonstration that knockdown of BMP expression results in defective pronephric formation in zebrafish combined with Alk3 expression in the murine metanephric mesenchyme suggests a possible role for BMP signaling in murine nephrogenesis (Kishimoto et al., 1997; Dick et al., 2000).

Here, we investigated the functions of BMP-ALK3 signaling in mice in which Alk3 deficiency is targeted to intermediate mesoderm progenitors and renal and genital derivatives. Our results demonstrate cell-autonomous functions for Alk3 in the metanephric blastema and the testis. Renal embryogenesis in mutants is characterized by decreased participation of Alk3-deficient cells in formation of the metanephric blastema and derivative nephrons, resulting in a decrease in the nephrogenic progenitor cell population and simple renal hypoplasia. In addition, targeted Alk3 deficiency results in perturbed formation of intermediate mesoderm-derived testicular cells and the corpus epididymis and decreased fertility. Analysis of Alk3-dependent signaling effectors revealed a lineage-specific reduction of phospho-p38 MAPK and phospho-SMAD1/5/8 in the metanephric mesenchyme and testis, respectively.

MATERIALS AND METHODS

Generation of mice model

Alk3+/- (CD1.C57BL/6) and Rarb2-Cre mice (CD1) (kindly provided by Cathy Mendelsohn, Columbia University, New York, USA) were intercrossed. Rarb2-Cre; Alk3lox/lox mice were generated using Rarb2-Cre; Alk3+/- and Alk3lox/lox (C57BL6) mice. Mice were maintained on a genetic background and genotyped by PCR (Mishina et al., 2002). Alk3 heterozygotes (Alk3+/-; Rarb2-Cre; Alk3lox/lox) were phenotypically identical to Alk3 wild type (Alk3lox/lox) and both were used as controls. ROSA26-Cre reporter mice (R26b) were purchased from Jackson Laboratories (stock number 003310). Male gender was determined by PCR for the SRY locus (Lambert et al., 2000). Mice were housed in the Laboratory Animal Housing facility, Hospital for Sick Children, Toronto. The presence of a copulatory plug in the morning was considered as E0.5.

β-Galactosidase staining

Frozen sections were fixed at 37°C for 15 minutes in lacZ fix (25% glueraldehyde, 100 mM EGTA, 1 M MgCl2, 0.1 sodium phosphate) and then incubated in lacZ stain (25 mg/ml X-gal, potassium ferrocyanide, potassium ferricyanide) solution at 37°C overnight in the dark. Embryos were fixed in lacZ fix, washed in wash (0.1 M sodium phosphate buffer, 2% nonidet-P40) solution and incubated in lacZ stain at 37°C overnight.

Kidney, testes and seminal vesicles were fixed in 10% buffered formalin at RT and paraffin embedded. Nephron number was estimated by counting glomeruli in three tissue sections (4 μm) extending from the renal cortex through the papilla for each genotype. Cross-sectional area was measured by AxioVision 4.6.3-SP1 (Zeiss) software.

Immunohistochemistry was performed using 4 μm sections washed in xylene and re-hydrated in ethanol. Sections were sequentially treated with 3% H2O2, heated in a pressure cooker in 0.1 M sodium citrate/citric acid buffer (pH 6.0), blocked in a 30% BSA/goat serum solution and incubated with primary antibodies overnight at 4°C. Sections were incubated with anti-rabbit secondary (Vectorstain, Vector Labs), followed by ABC (Vector Labs) and visualized by AEC (Zymed). Primary antibodies: anti-P-SMAD1/5/8 (1:150, Cell Signaling), anti-phospho-ERK1/2 (1:250, Cell Signaling) and anti-phospho-p38 MAPK (1:150, Cell Signaling).

For immunofluorescent microscopy, 4 μm sections were heated in a pressure cooker in 0.1 M sodium citrate buffer (pH 6.0). Sections were blocked as above and incubated with primary antibody overnight at 4°C. Tissue was incubated with either Alexa 488/568 goat-anti-mouse/anti-rabbit secondary antibody (1:500, Molecular Probes) and DAPI for 1 hour at room temperature, mounted in VectaShield (Vector) and visualized on an AxioVert 200M (Zeiss) microscope. Primary antibodies: anti-CITED1 (1:200, NeoMarkers), anti-CYTOCHROME P450 (1:200, Abcam), anti-INS1/3 (1:200, Phoenix Pharmaceuticals), anti-NCAM (1:150, Sigma), anti-PAX2 (1:200, Covance), anti-SALL1 (1:200, Abcam), anti-SIX2 (1:500, Proteintech), anti-SMA (1:250, Sigma) and anti-WT1 (1:1000, Santa Cruz).

For immunoblotting, 30 E13.5 kidneys were harvested per genotype. Primary antibodies: phospho-SMAD1/5/8 and total SMAD1 (1:250, Cell Signaling), and total p-38 MAPK (1:150, Cell Signaling).

In situ hybridization

Embryonic tissue was harvested at E12.5 and fixed in 4% PFA. Tissue was paraffin-embedded and sectioned (4 μm). RNA in situ hybridization was performed as described (Hartwig et al., 2008). Probe hybridization was visualized by BM Purple AP substrate (Roche). An Alk3 exon 2-specific probe was used to assess Alk3 deletion (Hebert et al., 2002).

In vitro culture conditions

E12.5 kidneys were cultured on 0.4 μm pore filter membranes (Becton Dickinson) in DMEM-F12 (Gibco) and apo-transferrin (Sigma) for 24 hours at 37°C in a CO2 incubator. E11.5 urogenital ridges were cultured in the same manner for 4 hours. Tissue was fixed in methanol at −20°C and treated with 0.1% Triton-X 100. Tissue was blocked with 30% BSA and goat serum, and incubated with primary antibodies for 1 hour at 37°C. Primary antibodies: anti-CALBINDIN-D28k (1:200, Sigma), anti-WT1 (1:100, Sigma) and anti-PAX2 (1:100, Covance). Explants were incubated with Alexa 488 goat anti-rabbit/Alexa 568 goat anti-mouse secondary antibody for 1 hour at 37°C and visualized on an AxioVert 200M (Zeiss) microscope.

Cell proliferation and apoptosis assays

Renal cell proliferation at E12.5 was assessed by incorporation of 5-bromo-2’-deoxyuridine (BrdU, Roche). Pregnant mice were administered BrdU via the peritoneum (100 mg/g of body weight) 2 hours prior to sacrifice. Tissue was processed as described (Cain et al., 2009). Apoptosis was assayed in E11.5 urogenital ridges and E12.5 kidneys using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) on paraffin sections following the manufacturer’s instructions (Promega). E10.5 and E11.5 embryos, apoptosis and proliferation were assessed by anti-activated caspase 3 (1:250, Cell Signaling) and anti-Ki67 (1:150, Dako), respectively. E11.5 mesonephric tubule proliferation was assessed by anti-PCNA antibody (1:500, Cell Signaling).

Serum testosterone assay

Total blood was collected by cardiac puncture from P30 male mice. Blood was separated into serum and submitted for serum testosterone analysis by tritium-based radioimmunoassay (IDEXX Labs).
Statistical analysis
Statistical analysis of nephron number and kidney area was performed by one-way ANOVA (GraphPad Prism, Version 5.0c). Kidney area was determined by measuring the outline of sagittal sections generated through the kidney midline using ImageJ software (n=3). Statistical analysis of apoptosis, proliferation, ureteric bud branch tips, Leydig cells and testosterone levels was performed using Student’s t-test (Excel). The number of Leydig cells at E18.5 were quantitated by counting INSL3-positive cells in four randomly selected fields (three tests per genotype). At P30, Leydig cells were quantitated by counting CYTOCHROME P450-positive cells in 10 randomly selected fields, in three nonadjacent sections per testis, three animals per group, as previously reported (Hu et al., 2010).

RESULTS
Genetic model of Alk3 deficiency in the intermediate mesoderm
Alk3 is expressed as early as E9.5 in the mesonephric epithelium and mesenchyme, the metanephric kidney and the gonad (Dewulf et al., 1995; Mishina et al., 1995; Hartwig et al., 2008). As homozygous Alk3 deficiency results in lethality before the onset of urogenital development, Alk3 function during formation of intermediate mesoderm-derived tissues is undefined (Mishina et al., 1995). Therefore, we generated mice with conditional inactivation of Alk3 in intermediate mesoderm progenitors (Alk3IMP null) using an Alk3loxP allele and Cre recombinase directed by the retinoic acid receptor β isoform 2 (Rarb2) promoter (Fig. 1A-F). The spatial domain of Cre recombinase expression under control of the Rarb2 promoter was identiﬁed using the ROSA26 (R26R) reporter strain (Soriano, 1999). Consistent with a previous report (Kobayashi et al., 2005), lacZ expression marked the region of intermediate mesoderm that forms the metanephric blastema (Fig. 1A; see Fig. S1A in the supplementary material) and which contributes to renal mesenchyme cell aggregates, developing epithelial nephrogenic structures and nephrogenic tubules in Rarb2-Cre;Rosa26 (R26R) embryos (Fig. 1B,C). lacZ expression was also observed in mesonephric tubules (Fig. 1D) and mesonephric-derived peritubular myoid and interstitial cells (Fig. 1E), but not in testicular endothelial, Sertoli or germ cells (Fig. 1E). Furthermore, lacZ marked the intermediate mesoderm-derived epididymis and mesonephric-derived interstitial cells between testicular seminiferous cords (Fig. 1E,F). Consistent with this pattern of expression, in Alk3IMP null mice, Alk3 mRNA expression was not detected in uninduced metanephric mesenchyme cells, but was maintained in the ureteric bud (compare Fig. 1G with 1H), and was absent in mesonephric epithelium but was unaltered in the bi-potential gonad, prior to invasion by mesonephric cells (compare Fig. 1I with 1J). Thus, Rarb2-Cre targets Alk3 deletion to intermediate mesoderm-derived nephrogenic precursors in the metanephric blastema and the epididymis, and in the mesonephros and derivative myoid and interstitial cells.

Alk3 deficiency in intermediate mesoderm progenitors results in renal hypoplasia
Alk3IMP null mice were recovered in the expected Mendelian ratio (152/698 recovered pups, 22%). The requirement for Alk3 during kidney development was assessed by analyzing renal structure at P30, a stage after completion of nephrogenesis. Although renal architecture was normal, fewer glomeruli were observed in mutants, suggesting a defect in nephrogenesis (compare Fig. 2B with 2A). However, as 23% (5/22) of Alk3IMP null mice died in the perinatal period, it was possible that the P30 data were not indicative of the entire spectrum of mutants. Therefore, subsequent analyses were performed at E18.5, prior to the demise of mutants (Fig. 2C-I). At E18.5, renal histoarchitecture appeared normal but kidney area was reduced by 39% in Alk3IMP null mice compared with wild type and by 35% compared with Alk33+/- littermates (Fig. 2C-E). As embryo size was equivalent among wild-type, Alk33+/- and Alk3IMP null mice, the decrease in kidney area was not due to a generalized decrease in somatic growth (data not shown). Quantification of nephrons, identified by WT-1 expression, revealed a significant decrease in Alk3IMP null but not Alk33+/- mice.

Fig. 1. Targeted deletion of Alk3 to intermediate mesoderm progenitors. (A-F) lacZ expression as a marker of Rarb2-Cre activity. (A) Expression of lacZ in the E10.5 metanephric blastema (arrowhead). (B) At E12.5, lacZ was expressed in mesenchyme aggregates (arrow, inset) and developing nephrons (black arrowheads), but was absent from ureteric cells (red arrowhead). (C) At E18.5, all nephrogenic structures expressed lacZ, including tubules (arrow) and podocytes (yellow arrowhead). lacZ was not expressed in collecting ducts (red arrowheads). (D) In E11.5 urogenital ridges, lacZ marked the metanephric blastema (arrow) and mesonephros (red arrowhead), but not in the nephric duct (black arrowhead). (E) At E15.5, lacZ marked interstitial (red arrowhead) and peritubular myoid (arrow), but not endothelial (black arrowhead) and germ cells (asterisk). (F) At P30, lacZ was noted in interstitial cells (arrow), ductus epididymis (yellow arrowhead) and the epididymis (red arrowhead). (G) At E12.5, Alk3 mRNA (blue) was observed in control ureteric cells (red arrowhead, red line, inset) and the metanephric mesenchyme (arrow). (H) In Alk3IMP null mice, Alk3 mRNA was not detected in metanephric mesenchyme cells (arrow), but was present in ureteric cells (red arrowhead; red line, inset). (I, J) At E12.5, Alk3 mRNA was expressed in gonads (arrow) and mesonephric tubules (arrowheads, inset) of controls (I), but was markedly reduced in mesonephric tubules (outline) of Alk3IMP null mice (arrowhead, inset) (J).

DEVELOPMENT
Thus, **Alk3** deficiency in the metanephric mesenchyme causes simple renal hypoplasia defined as decreased kidney size and nephron number but normal tissue patterning.

Analysis of renal area in **Alk3** null mice at progressively early stages of embryogenesis revealed that renal size was persistently decreased in mutants starting at E12.5 (Fig. 3A). Next, we analyzed the expression of genes required for nephrogenesis. **Gdnf** is expressed in the metanephric mesenchyme and signals via a GDNF-RET-WNT11 regulatory loop to control ureteric branching. Ureteric bud tips induce nephron formation, thereby controlling nephron number (Dziarmaga et al., 2003; Narlis et al., 2007). In **Alk3** null mice, the expression of **Gdnf** was similar to that in controls (Fig. 3B,D). Despite this, the number of ureteric branch tips in E12.5 whole-mount kidney preparations were significantly decreased in **Alk3** null mice (Fig. 3F-H). Yet, we could detect no decrease in the expression of **Ret** and **Wnt11** mRNA in mutants (see Fig. S1E,F,H,I in the supplementary material). Similarly, ureteric expression of **Wnt9b**, which is required for initiation of nephrogenesis (Carroll et al., 2005), was not disrupted in **Alk3** null tissue (see Fig. S1G,J in the supplementary material). **Wnt4** is required for formation of nephrogenic aggregates and vesicles (Stark et al., 1994). Although we observed fewer **Wnt4**-positive nephrogenic structures in **Alk3** null tissue, all intermediate nephrogenic structures present expressed **Wnt4** (Fig. 3C,E). Together, these data demonstrate decreased formation of nephrogenic precursor structures and associated ureteric branches in **Alk3** null mice but no change in the expression of genes that control nephrogenesis and branching morphogenesis.
We investigated the possibility that the deficit in nephrons in \textit{Alk3}\textsuperscript{IMP null} mice was due to defective progression of nephrogenic intermediates to mature nephrons. NCAM expression was used to mark nephrogenic intermediate structures at advancing stages of development, including pre-tubular aggregates and later nephrogenic structures (Klein et al., 1988). The ratio of immature nephrogenic structures (renal aggregates, vesicles) to total NCAM-expressing structures (including more mature comma and S-shaped bodies, renal tubules) was assessed at E16.5. Although a shift in this ratio towards more immature nephrogenic structures would reflect a delay in maturation in mutants, we observed no significant difference between \textit{Alk3}\textsuperscript{IMP null} and controls (Fig. 3I-K).

Next, we determined whether \textit{Alk3}-dependent effects on nephrogenesis were associated with altered cell proliferation and/or apoptosis. First, we analyzed these events at the stage at which nephrogenesis is initiated (E12.5). Apoptotic nuclei were identified by TUNEL assay and proliferating cells were marked by BrdU incorporation (see Fig. S2A-D in the supplementary material). Quantification of the proportion of apoptotic and proliferating cells revealed no significant difference between controls and mutants (Fig. 3L,M). The expression of \textit{Rarb2-Cre} in the intermediate mesoderm and metanephric blastema prior to the onset of renal development (Fig. 1A; see Fig. S1A in the supplementary material) suggested that a defect in intermediate mesoderm cell survival prior to nephrogenesis could cause early nephrogenic defects. Accordingly, we analyzed apoptosis and cell proliferation at a stage prior to the onset of nephrogenesis (E10.5, E11.5). At these early stages, the metanephric blastema in control and mutants was identified by expression of \textit{PAX2} (see Fig. S3A,D,G,J in the supplementary material). Proliferating and apoptotic cells were marked by \textit{Ki67} and anti-activated caspase 3, respectively (see Fig. S3A-K in the supplementary material). Quantification of apoptotic and proliferating cells as a fraction of the total number of metanephric blastema cells demonstrated no significant effect of \textit{Alk3} deficiency (see Fig. S3C,F,I,L in the supplementary material).

\textit{Alk3} acts cell autonomously to control renal progenitor gene expression

Next, we investigated the contribution of \textit{Alk3} to the population of nephrogenic precursors. Following formation of the metanephric blastema, mesenchyme cells, marked by the expression of specific genes, become progressively committed to a nephrogenic fate. Using these cell markers, we investigated whether \textit{Alk3} deficiency negatively impacted the capacity to generate nephrogenic precursors (Fig. 4, schematic). We used the \textit{R26R} allele in combination with \textit{Rarb2-Cre} to mark the cells that constitute the
metanephric blastema prior to induction by the ureteric bud. At E10.5, lacZ expression demonstrated that the metanephric blastema was 56% smaller in Alk3IMP null embryos compared with controls (Fig. 4A,B). Consistent with this finding, the number of cells expressing SALL1, which marks blastema mesenchyme cells, was markedly decreased in mutants at E11.5 (Fig. 4D). Similarly at E11.5, the size of the metanephric mesenchyme, demarcated by PAX2 expression, was 26% smaller in Alk3IMP null mice (Fig. 4E,F). Prior to the onset of nephrogenesis, Osr1 is expressed in nephrogenic and stromal cell precursor cells (Mugford et al., 2008). At E12.5, expression of Osr1 was reduced in Alk3IMP null mice (Fig. 4G,H). After E11.5, Osr1-expressing cells contribute to SIX2 expressing nephrogenic progenitors (Kobayashi et al., 2008; Mugford et al., 2008). SIX2 is required for maintenance of the self-renewing nephrogenic progenitor population (Self et al., 2006). In Alk3IMP null mice, SIX2 expression was reduced (Fig. 4J). CITED1 is expressed in non-self-renewing SIX2-positive cells undergoing nephrogenesis. At E12.5, the number of CITED1-positive cells was significantly reduced in mutants (see Fig. S4C-E in the supplementary material).

Reduced SIX2 expression suggested that the nephrogenic progenitor pool could be prematurely exhausted in Alk3IMP null mice. At P4, an assessment of nephrogenic progenitor contribution to nephrogenesis was performed using the R26R allele and examining cortical sections for lacZ expression. lacZ marked cells contributed to 99% (4547 of 4613 cells) of nephrogenic structures in controls compared with only 75% (2413 of 3251 cells) in mutants (Fig. 4K-I). The number of mutant Leydig cells was significantly reduced (*P<0.001). Data are means±s.e.m. (L, O) Seminal vesicle histology in 1-year old males. In Alk3IMP null males (O), the epithelial layer was thin and disorganized (arrowheads) compared with controls (L). (M, P) Corpus epididymis histology in 1-year old males. The epithelial cells in Alk3IMP null mice (P) demonstrated vacuolation (arrowheads) compared with the organized layer in controls (M). The mutant epididymis still contained sperm (arrows). (N) At P30, the average mean amount of serum testosterone (red bar) in Alk3IMP null males was significantly decreased (*P=0.05). Data are means±s.e.m.

**Alk3 signaling controls mesonephric tubule number and mesonephros-derived testicular cells**

The expression of Rarb2-Cre in progenitor mesonephric tubules (Fig. 1D) provided a basis to investigate the requirement for Alk3 in specification of testicular cells of intermediate mesoderm origin. Alk3 mRNA expression was reduced in Alk3IMP null mesonephric tubules, which were decreased in number compared with controls.
(Fig. 11-J). We confirmed the reduction in mesonephric tubule number by using LacZ or Pax2 staining of whole-mount E11.5 urogenital ridges (Fig. 5A,B), in which all tubules can be examined in the same plane. In Alk3impnull mice, mesonephric tubule number was significantly reduced (Fig. 5B, *P<0.02). However, this reduction was not associated with increased apoptosis or decreased proliferation of mutant mesonephric tubule cells (see Fig. S5 in the supplementary material).

In the testis, peritubular myoid and interstitial Leydig cells are postulated to arise via cell migration from the mesonephros (Merchant-Larios and Moreno-Mendoza, 1998; Nishino et al., 2001). Analysis of E18.5 testis histological sections revealed fewer seminiferous tubules and a paucity of interstitial cells in Alk3impnull mice (Fig. 5C,D), suggesting a mesonephros-derived cell defect. Peritubular myoid cells are required for seminiferous tubule integrity. Tubule integrity, assessed by location of SMA-marked peritubular myoid cells, appeared unaltered in mutants (see Fig. S5 in the supplementary material). Next, we assessed the Leydig cell population, which express Rarb2-Cre (Fig. 1E,F) and are marked by cytochrome P450 expression. Interestingly, at E18.5, fewer cytochrome P450-positive Leydig cells were present in Alk3impnull males (Fig. 5H). Mutants also contained non-cytochrome P450-positive interstitial cells, indicative of defects in Leydig cell differentiation, which is dependent in part on testosterone levels. These results were confirmed using InsL3, an independent marker of Leydig cells (Fig. 5I). Analysis of InsL3-expressing cells revealed more loosely packed Leydig cells in the mutant interstitial compartment, a significantly lower number of Leydig cells compared with control (*P=0.00), and a population of un-differentiated non-InsL3 expressing interstitial cells, consistent with our results using anti-cytochrome P450. During early postnatal life, androgen secretion from Leydig cells is required for initiation and maintenance of spermatogenesis, and sexual differentiation of the male brain for acquisition of copulatory and aggressive behaviors (Baker et al., 1996; Matsuda et al., 2008). Consistent with results in embryos, the number of Leydig cells was statistically decreased in P30 Alk3impnull males (Fig. 5G,J,K).

Decreased Leydig cell number in Alk3impnull mice suggested a possible decrease in testosterone production. Indeed, testosterone levels were significantly reduced in P30 mutant males (Fig. 5N). Mutant males also demonstrated physical and behavioral abnormalities consistent with reduced testosterone. Testosterone secretion stabilizes the nephric duct during seminal vesicle development (Hannema and Hughes, 2007). Although the seminal vesicle does not express Rarb2-Cre, (see Fig. S6 in the supplementary material), structural defects were present in Alk3impnull males (Fig. 5O; see Fig. S6 in the supplementary material). The postnatal seminal vesicle contains a pseudo-stratified epithelial cell layer, arranged in intricate folds enclosing the seminal fluid (Fig. 5L). In Alk3impnull males, the cell layer was thinner and demonstrated reduced branching morphogenesis (Fig. 5O). Testosterone is also required for copulatory behavior. Six-week old control and Alk3impnull males were caged with naturally cycling 5-week-old females for 2 months. During this period, the mutant male did not sire offspring or demonstrate mating behavior, whereas control males fathered two litters of pups each. Finally, low testosterone is associated with decreased body size (Ingman and Robertson, 2007); postnatal Alk3impnull males were on average 30% smaller than control (n=3). Together, these data demonstrate that Alk3 deficiency decreases testosterone and adversely affects testosterone-dependent morphogenesis and behavior.

**Alk3 is required for epididymis integrity** Portions of the intermediate mesoderm-derived nephric duct contribute to the epididymis (Joseph et al., 2009). Rarb2-Cre epididymis expression (Fig. 1F) allowed us to assess the requirement for Alk3 during this process. The epididymis is a highly coiled epithelial tube required for sperm maturation, and can

*Fig. 6. Expression of BMP signaling effectors.* (A) At E12.5, phospho-SMAD1/5/8 expression marked control ureteric cells (arrow) and the metanephric mesenchyme (arrowhead). (C) The phospho-SMAD1/5/8 renal expression domain was identical in Alk3impnull mice. (B) At E12.5, phospho-p38 MAPK expression was observed in control ureteric bud cells (arrow) and the metanephric mesenchyme (arrowhead). (D) By contrast, whereas phospho-p38 MAPK was expressed in Alk3impnull ureteric cells (arrow), expression was reduced in the uninduced metanephric mesenchyme (arrowhead). (E) At E30, phospho-SMAD1/5/8 is expressed in control testicular cells, including Leydig cells (arrowhead). (G) The expression of phospho-SMAD1/5/8 is reduced in Leydig cells in Alk3impnull mice (arrowhead). (F,H) At P30, p38 MAPK was expressed in an identical manner in control (f) and Alk3impnull (H) Leydig cells (arrowheads). (l) In E13.5 kidney protein lysate, phospho-p38 MAPK protein expression was reduced by 25% in Alk3impnull kidneys. Phospho-SMAD1/5/8 protein levels were comparable between mutants and controls.
be divided into three morphologically distinct domains: the caput, corpus and cauda. The finding of caput and cauda degeneration in Bmp8a–/– and compound Bmp7+/–; Bmp8a–/– mice (Zhao et al., 2001) and of corpus degeneration in Bmp4–/– mice (Hu et al., 2004) demonstrated a requirement for BMP signaling in epididymis development. We found disorganization and vacuolation of corpus epithelial cells in Alk3IMP null mice (Fig. 6P), a phenotype consistent with Bmp4–/– mice. This data suggests that Alk3 is the receptor for BMP4, but not BMP7 or BMP8a, during epididymis morphogenesis.

**Alk3IMP null mice demonstrated reduced activation of BMP intracellular signaling**

BMPs signal via distinct canonical and non-canonical intracellular signaling pathways. Although previous work in mice with Alk3 deficiency targeted to ureteric cells demonstrated that Alk3 signals via SMAD 1/5/8 (Hartwig et al., 2008), the intracellular signaling of BMP intracellular signaling is not been previously investigated. Our results indicate that Alk3 signals in an organ-specific manner in intermediate mesoderm-derived cells.

**DISCUSSION**

The intermediate mesoderm gives rise to the permanent kidney and to components of the male genital system in mice. Previous studies have implicated BMP-ALK signaling in intermediate mesoderm gene expression (James and Schultheiss, 2005). However, the physiological functions of ALK-dependent signaling have been undefined. Here, we investigated these functions by targeting Alk3 deficiency to intermediate mesoderm progenitor populations and their derivative structures using Rarb2-Cre and Alk3IMP null mice. Our results provide novel insights into nephrogenesis and testicular androgen production, and expand on a previous body of knowledge that had focused only on regulation of renal branching morphogenesis and of Mullerian duct regression, and germ cell development and migration (Jamin et al., 2002; Pellegrini et al., 2003; Hartwig et al., 2008; Dudley et al., 2010).

**The renal progenitor population is reduced in Alk3IMP null mice**

Extrinsic signals are required for commitment of the intermediate mesoderm to a renal lineage. Renal specification and formation of the first primitive kidney requires the development of the nephric duct, which can be induced to form by BMP4-soaked beads in the absence of the overlying surface ectoderm (Obara-Ishihara et al., 1999). However, the cell-autonomous requirement for BMP signaling during murine intermediate mesoderm specification had not been previously investigated. Our results indicate that Alk3 controls metanephric blastema specification from the intermediate mesoderm. The smaller metanephric blastema consists of a reduced metanephric mesenchyme population in Alk3IMP null mice. Remarkably, the adrenal gland, which is partly derived from the intermediate mesoderm, was normal in mutants, supporting a specific effect of Alk3 deficiency (see Fig. S1D in the supplementary material). Decreased expression of Osr1 in Alk3IMP null mice suggests that Alk3 functions upstream of Osr1 to control the generation of Foxd1-positive stromal and SIX2-positive nephrogenic cell populations (Fig. 7). Reduction in the SIX2-positive nephrogenic progenitor population has been recognized in other cases of reduced nephron endowment in humans and mice (Weber et al., 2008; Fogelgren et al., 2009).

BMP signaling has previously been implicated in regulation of nephron number. Deletion of the pro-BMP molecule C2v2 or podocyte-specific deletion of Bmp7 both result in reduced nephron number (Kazama et al., 2008; Ikeya et al., 2010). However, our results are unique in that they provide direct insight into the functions of BMP signaling in nephrogenic progenitor cells. Our studies suggest that Alk3 is not required for nephrogenesis after the stage at which the first phase of nephron progenitor development occurs. We found no evidence for a delay in nephrogenesis, as had been observed in cadherin 6 mutants (Mah et al., 2000). Nor was decreased nephrogenesis in Alk3IMP null mice associated with abnormal nephron differentiation, as is observed in mice deficient in Wnt4, Lim1 or Fgfr8 expression (Grieshammer et al., 2005; Kobayashi et al., 2005). In contrast to other metanephric mesenchyme targeted models of renal hypoplasia, including Myc, Mycn and Pax2 mutants, decreased nephrogenesis in Alk3IMP null mice could not attributed to reduced cell survival (Bates et al., 2000; Dziarmaga et al., 2003; Couillard and Trudel, 2009). Furthermore, the lack of effect of Alk3 deficiency on cell expression was unchanged in mutant versus control testis tissue (Fig. 6F,H). Thus, ALK3 signals in an organ-specific matter in intermediate mesoderm-derived cells.
proliferation and apoptosis is distinct from that observed in Bmp2−/−, Smad4−/− mice, which demonstrate reduced mesenchymal proliferation, and Bmp7−/− mice, which are characterized by metanephric mesenchyme cell apoptosis (Dudley and Robertson, 1997; Hartwig et al., 2005). Together, these observations suggest that Alk3 signals in the metanephric mesenchyme via molecular pathways that are distinct from those controlled by SMAD4 and BMP7.

**Alk3 signaling regulates mesonephric derived testicular populations**

Our results demonstrate a functional requirement for Alk3 in intermediate mesoderm-derived testis cells. The murine testis is formed from genital swellings ventral to the mesonephros, which are populated by migrating primordial germ cells at E10.5 (Wilhelm and Koopman, 2006). BMP signaling regulates primordial germ cell number, proliferation and migration, yet no role had been defined in testicular somatic cells (Hu et al., 2004; Puglisi et al., 2004; Dudley et al., 2007). No germ cell defects were observed in Alk3imp null mice, consistent with lack of expression of Rarb2-Cre in these cells. Indeed, abnormalities were observed only in cells derived from the Cre-expressing mesonephric progenitor cells.

The mesonephric origin of peritubular myoid cells is controversial (Nishino et al., 2001; Cool et al., 2008). Based on the mesonephric expression pattern of Rarb2-Cre, and the presence of flattened lacZ-expressing cells in the periphery of E15.5 seminiferous tubules (Fig. 1E), our data suggest that peritubular myoid cells are of mesonephric origin. However, the peritubular myoid cells do not require Alk3 signaling for differentiation, as SMA expression was normal in Alk3imp null males. Defective peritubular myoid cell differentiation was observed in Dhh−/− males (Clark et al., 2000), and was associated with the presence of germ cells in interstitial space, which was noted only in our model. Alk3 is required for Leydig cell development, explaining the paucity of interstitial cells noted in E18.5 mutant testes (Fig. 5D,H,I). The significant reduction in P30 Leydig cell number in Alk3imp null males was further supported by reduced serum testosterone.

Low testosterone levels in Alk3imp null males may have contributed to their inability to sire offspring. We postulate that low testosterone resulted in reduced copulatory behavior in Alk3 mutants. Analysis of androgen receptor knockouts have shown that testosterone itself is required for differentiation of adult Leydig cells (Hardy et al., 1990; Murphy et al., 1994; Wang et al., 2009), which are marked by the expression of steroidogenic enzymes such as cytochrome P450. Indeed, we noted non-expressing cells in E18.5 mutant tissue (Fig. 5H), which may have further contributed to reduced testosterone production. Low testosterone is also associated with seminal vesicle developmental defects (Hannema and Hughes, 2007), which were present in Alk3imp null males. The seminal fluid is required for sperm nourishment and integrity, so abnormal seminal vesicle development may contribute to Alk3imp null male infertility. Low testosterone is associated with 20-30% of cases of human male infertility (Dohle et al., 2003). Our results may indicate a role for BMP signaling in the etiology of male sexual dysfunction.

We also demonstrated that Alk3 is required for epididymis integrity in a ligand-specific manner. BMP4 is expressed through the entire epididymis epithelium (Hu et al., 2004), as is Rarb2-Cre. However, BMP7 and BMP8a/b expression are only observed in the caput and cauda (Zhao et al., 2001), and therefore do not control corpus epididymis development. The epithelial vacuolation observed in the Alk3imp null corpus epididymis (Fig. 5P) is comparable with abnormalities noted in Bmp4−/− males (Hu et al., 2004). Based on our data, we postulate that during epididymis morphogenesis, BMP4 signaling requires Alk3, whereas BMP7 and/or BMP8a/b signaling through other BMP receptors compensates for Alk3 deletion during cauda and caput formation. Alk6 is ubiquitously expressed in the epididymis and the expression pattern of Alk2 is unknown (Hu et al., 2004). Epididymis defects affect sperm motility and integrity, and may contribute to the inability of Alk3imp null males to sire offspring.

**Alk3 utilizes different downstream signaling mechanisms in a tissue-specific manner**

During renal development, Alk3 signals via SMAD proteins in ureteric cells (Hu et al., 2003; Hartwig et al., 2008). Our data demonstrated that Alk3 signals via MAPKs in the metanephric mesenchyme (Fig. 7). These results are consistent with previous findings. Analysis of a SMAD-dependent BMP signaling reporter mouse found no reporter activity in developing nephrons or progenitor cells (Blank et al., 2008). Treatment of ex vivo kidney explants with p38MAPK inhibitors attenuates nephrogenesis (Hida et al., 2002). Renal hypoplasia in mice with Bmp7 deficiency targeted to podocytes is also associated with reduced p38MAPK activity and normal SMAD expression (Kazama et al., 2008). By contrast, the pro-BMP factor Cv2 mediates metanephric mesenchyme cell adhesion and nephrogenesis through SMAD signaling (Ikeya et al., 2010). However, Cv2 appears to act downstream of BMP7, which is thought to signal via ALK2.

In contrast to the metanephric mesenchyme, our results show that Alk3 regulates SMAD, but not p38MAPK signaling in the testis (Fig. 7). Previous studies show that the BMP-dependent SMADS, Smad1 and Smad5, are required for primordial germ cell specification (Arnold et al., 2006). We demonstrated that phospho-Smad1/5/8 is ubiquitously activated in the developing testis, pointing to an additional requirement for SMAD signaling in somatic testicular cells. Consistent with this requirement, the mesonephric derived Leydig cells demonstrated reduced SMAD activity. These data demonstrate a requirement for BMP-induced SMAD activity in somatic androgen-producing cells during testis development.

In summary, we have demonstrated that Alk3 is required for specification of urogenital development from the intermediate mesoderm (Fig. 7). During renal development, Alk3 regulates formation of the metanephric blastema and controls the number of Osrl- and SIX2-expressing nephrogenic progenitor cells. Alk3 also controls nephrogenesis that uses p38 MAPK signaling. Similarly, Alk3 controls mesonephric tubule and derivative Leydig cell number through Smad1/5/8 signaling. Different BMP signaling pathways are used in the different progenitor compartments. These results not only delineate a role for BMP signaling during intermediate mesoderm specification, but also in urogenital disease.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.059030/-/DC1
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