BMP Receptor ALK3 Controls Collecting System Development

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ABSTRACT

The molecular signals that regulate growth and branching of the ureteric bud during formation of the renal collecting system are largely undefined. Members of the bone morphogenetic protein (BMP) family signal through the type I BMP receptor ALK3 to inhibit ureteric bud and collecting duct cell morphogenesis in vitro. We investigated the function of the BMP signaling pathway in vivo by generating a murine model of ALK3 deficiency restricted to the ureteric bud lineage (Alk3UB−/− mice). At the onset of branching morphogenesis, Alk3UB−/− kidneys are characterized by an abnormal primary (1°) ureteric bud branch pattern and an increased number of ureteric bud branches. However, during later stages of renal development, Alk3UB−/− kidneys have fewer ureteric bud branches and collecting ducts than wild-type kidneys. Postnatal Alk3UB−/− mice exhibit a dysplastic renal phenotype characterized by hypoplasia of the renal medulla, a decreased number of medullary collecting ducts, and abnormal expression of β-catenin and c-MYC in medullary tubules. In summary, normal kidney development requires ALK3-dependent BMP signaling, which controls ureteric bud branching.


Formation of the renal collecting system begins via branching morphogenesis, defined as growth and branching of the ureteric bud (UB) and its branches. The first branching event is invariably a symmetrical bifurcation of the UB at its tip,1 which generates two daughter branches, termed 1° UB branches because of their spatial attachment to the initial UB outgrowth. Subsequent branch generations are formed by a combination of terminal bifid and lateral branching. The molecular signals that control UB branch patterning, including the inhibitory signals that limit both position and number of 1° branches that form off the ureter trunk, remain undefined.

Members of the bone morphogenetic protein (BMP) family of secreted peptide growth factors signal through their activin-like kinase (ALK) type I receptors and downstream Smad effectors to control embryonic tissue development.2 In the embryonic kidney, two closely related BMPs, BMP2 and BMP4, are expressed in discrete, nonoverlapping domains in the mesenchyme surrounding the tips of the branching UB, and adjacent to the ureter trunk, respectively,3 whereas the BMP2/4 type I receptor ALK3 is expressed in both the UB and mesenchyme lineages.4 Exogenous treatment of embryonic kidney explants with either BMP2 or BMP4 potently inhibits branching morphogenesis in vitro,5–7 and Bmp4 inhibits ectopic UB outgrowth off the Wolffian duct in vivo.8 Similarly, ALK3 inhibits UB branching both in vitro and in transgenic mice overexpressing Alk3 in the UB.9,10 However,
the mechanisms by which BMP-ALK signaling regulates renal branching morphogenesis in vivo are undefined. Here, we used the Cre-loxP system to delete Alk3 in the UB lineage. Our results demonstrate that ALK3 deficiency disrupts UB patterning, resulting in an increased number of 1° UB branches followed by a reduced number of both UB branches and collecting ducts, and postnatal kidney malformation.

RESULTS

During renal embryogenesis, Alk3 mRNA is highly expressed in the Wolffian duct, including its caudal aspect, which is the origin of the UB (Figure 1A). After invasion of the UB into the metanephric mesenchyme, Alk3 is expressed in both the UB and mesenchyme (Figure 1, B and C). Because homozygous inactivation of Alk3 causes embryonic lethality at approximately E8.11, we used a conditional Alk3 allele (Alk3C) and induced excision of exon 212 in UB cells using HoxB7Cre-EGFP transgenic mice.13 In Alk3UB−/− kidneys, Alk3 mRNA was expressed in the nephrogenic mesenchyme and mesenchyme-derived structures but was not detected in collecting ducts (Figure 1, D through I). In Alk3UB−/− kidney tissue lysates, ALK3 protein was markedly decreased compared with Wt (wild-type) (Figure 1L). E11.5 Alk3UB−/− kidneys exhibited a marked reduction of phospho-Smad1, an intracellular BMP-ALK3 signaling effector,14 in UB cells, whereas expression was maintained in metanephric mesenchyme compared with Wt (Figure 1K versus 1J). Similarly, phospho-Smad1 protein expression was decreased in tissue lysates of Alk3UB−/− kidneys (Figure 1L). Thus, both ALK3 protein and downstream signaling activity are reduced in Alk3UB−/− kidneys.

Abnormal Ureteric Bud Branching in ALK3-Deficient Kidneys

Green fluorescent protein (GFP)-fluorescence was used to visualize UB branching in Alk3UB−/− kidneys. Branch patterns were schematized using color-coded stick figures to classify UB branches according to their spatial orientation relative to the ureter trunk (Figure 2A). At E11.5, the Wt UB exhibits the archetypal bifid 1° branch pattern (Figure 2B), characterized by a ureter trunk (red) and two 1° UB branches (cyan). At E12.5, successive bifid branching of UB tips yielded 2° (violet) and 3° (black) branches, defined by their attachment to 1° and 2° branches, respectively (Figure 2C). In contrast to Wt kidneys, a minority of heterozygous Alk3UB−/UB− kidneys (two of five kidneys) expressed in a nuclear pattern in ureteric cells in Wt tissue (arrows) (J). Phospho-Smad1 expression is markedly decreased in ureteric cells in Alk3-deficient tissue (arrow) (K). (L) Western blot analysis of E18.5 whole kidney lysates show that ALK3 protein was reduced 57% (P = 0.01), whereas phosphorylated Smad1 (phospho-Smad1) was reduced 53% in Alk3UB−/− kidneys (P = 0.01) (n = 5). Scale bars: (A) 500 μm, (B and C) 25 μm, (D and E) 500 μm, (F through I) 100 μm.
Figure 2. Primary ureteric bud branch pattern is disrupted in Alk3UB−/−/kidneys. (A) Ureteric bud branches are classified according to their spatial orientation relative to the ureter trunk. (B and C) Green fluorescent protein (GFP) fluorescence in E11.5 and E12.5 Wt kidneys demonstrates the archetypal 1° ureteric bud branch pattern consisting of a ureter trunk (red) connected to two 1° ureteric bud branches (cyan). (D through K) GFP fluorescence of ureteric bud trees at E12.5 and Dolichos biflorus (DBA) fluorescence of these ureteric bud trees after 48 h of culture (+48h), together with corresponding color-coded stick figures. (D and E) The Wt (+48h) ureteric bud tree maintains the 1° branch pattern and exhibits 2° to 6° branch generations. (F and G) Some E12.5 and I) kidneys exhibit a trifid branch pattern (inset in F) that is maintained after 48 h of culture. (H and I) Some Alk3UB−/−/kidneys, severe reductions in 3° to 7° branch number and in total ureteric bud branch number are observed. Ureteric bud trunk and tip segments are also dilated, and terminal ureteric bud ampullae formed 4 to 6 new tips. (J and K) Some Alk3UB−/−/kidneys formed new 1° ureteric bud branches at the junction of the ureter trunk and initial ureteric branches. (L) Quantitation of ureteric bud branch number demonstrates that increased 1° to 2° ureteric bud branch number is associated with decreased 4° to 7° ureteric bud branch number in Alk3UB−/−/kidneys. Scale bar (D through K) = 100 μm.

To analyze the effect of ALK3 deficiency on subsequent branching events, we cultured E12.5 Alk3UB−/−/kidneys for 48 h (+48h) in vitro (Figure 2, D through K). Quantitation of UB branch number (Figure 2, H through L) demonstrated a severe reduction in the number of higher order UB branches (4° to 7°) formed in Alk3UB−/−/ (+48h) kidneys compared with Wt (branch type versus reduction in branch number: 4° (41%), P = 0.0004; 5° (76%), P = 0.0006; 6° (92%), P = 0.001; 7° (100%), P = 0.04). Cumulatively, the total number of UB branches formed in Alk3UB−/−/kidneys was reduced by 22% (P = 0.05).

We analyzed embryonic kidneys immediately after isolation to confirm that the reductions in total UB branch number we observed were independent of organ culture conditions. Histological analysis of E13.5 Alk3UB−/−/kidneys suggested a decrease in the number of UB and mesenchyme tissue elements, together with reductions in overall kidney size (Figure 3, A through C). Next, we imaged UB branches marked by Dolichos biflorus (Figure 3, D through F) and quantitated UB branch number in replica stick figures (Figure 3, G through I). Most Alk3UB−/−/kidneys (five of seven) exhibited a moderate (approximately 40%) decrease in branch number: 4° (41%), P = 0.0004; 5° (76%), P = 0.0006; 6° (92%), P = 0.001; 7° (100%), P = 0.04). Cumulatively, the total number of UB branches formed in Alk3UB−/−/kidneys was reduced by 38% in Alk3UB−/−/kidneys compared with Wt (P = 0.002; n = 7 kidneys/group).

We next investigated cellular mechanisms that could mediate ALK3-dependent effects on UB branching. Quantitation of cell proliferation using BrdU incorporation in Alk3UB−/−/kid-
To define molecular effectors that might mediate ALK-dependent control of 1° UB branching, we examined expression of genes known to regulate UB branch patterning including *Gdnf*<sup>13</sup>, *Ret*<sup>16</sup>, *Wnt11*<sup>17</sup>, and *Sprouty 1* (*Spry1*)<sup>18</sup> in E11.5 metanephric tissue (Supplemental Figure 3). The expression patterns of *Gdnf* and *c-Ret* mRNAs in the metanephric mesenchyme and UB, respectively, were similar in *Alk3<sup>UB−/−</sup>* metanephric tissue compared with *Wt* tissue. Similarly, we detected no qualitative difference in the expression of *Wnt11* in UB branch tips or *Spry1* in the UB epithelium and metanephric mesenchyme in *Alk3*-deficient tissue. These data imply that *Alk3* dependent *Bmp* signaling acts via genes other than *Gdnf*, *Ret*, *Wnt11*, and *Spry1* to regulate early UB branch patterning.

Next, we investigated molecular mechanisms that could underlie decreased ureteric branching observed in *Alk3*-deficient kidneys. *Pax2* is a critical effector of renal branching morphogenesis,<sup>19,20</sup> *Alk3*-Deficient kidneys with a moderate branching defect exhibited a marked decrease in *Pax2* mRNA expression in the UB lineage, whereas mesenchymal expression did not appear to be affected (Figure 3, J and K). However, hypoplastic *Alk3<sup>UB−/−</sup>* kidneys with a severe branching defect exhibited a near-total loss of *Pax2* expression in both the UB and nephrogenic mesenchyme (Figure 3L). Consistent with these results, the number of nephrogenic intermediates, marked by expression of Wilms’ tumor suppressor-1 (*WT-1*) was progressively reduced in moderately and severely affected *Alk3<sup>UB−/−</sup>* mutant kidneys, respectively (Figure 3, M versus N and O). These results indicate that *Alk3* regulates *Pax2* expression and suggest that reduced UB branching in E13.5 *Alk3<sup>UB−/−</sup>* kidneys is caused, in part, by reduced *Pax2*-dependent signaling.

**Reduced Collecting Duct Number in Embryonic *Alk3<sup>UB−/−</sup>* Kidneys**

We determined the impact of decreased UB branch number on collecting duct formation in *Alk3<sup>UB−/−</sup>* kidneys at E18.5, a stage when all major nephron elements are established. Histological examination of E18.5 *Alk3<sup>UB−/−</sup>* kidneys demonstrated a paucity of medullary tissue (Figure 4, A and B), whereas expression of *Pax2*, a marker of both collecting ducts and nephrogenic zone at E18.5, was specifically reduced in the renal medulla of these mice (Figure 4, C through F). Together, these data suggested that collecting duct number was reduced in *Alk3<sup>UB−/−</sup>* kidneys. Quantitation of anti-calbindinid<sup>28K</sup>–labeled collecting ducts in serial tissue sections revealed a 39% reduction in collecting duct number in *Alk3<sup>UB−/−</sup>* kidneys compared with *Wt* (*P* = 0.0001; *n* = 6 kidneys/group) (Figure 4, G through I).

Figure 3. Histological and molecular phenotype of E13.5 *Alk3<sup>UB−/−</sup>* kidneys. (A through C) At E13.5, *Alk3<sup>UB−/−</sup>* kidneys exhibited moderate (B) to severe (C) reductions in size and number of mesenchyme- and ureteric bud–derived tissue elements. (D through F) DBA-stained E13.5 *Alk3<sup>UB−/−</sup>* kidneys and replica stick figures (G through I) *Alk3<sup>UB−/−</sup>* mutants are characterized by a variable decrease in ureteric bud branch number. Some mutants show a moderate decrease (E and H), whereas others demonstrate a severe decrease as well as dilation of ureteric bud tips (F and I). (J through L) *Pax2* expression is reduced in the ureteric bud (arrow) in E13.5 moderately affected *Alk3<sup>UB−/−</sup>* mutants, whereas in severe mutants *Pax2* is reduced in both the ureteric bud and mesenchyme. (M through O) *WT-1* expression is markedly reduced in moderately affected mutants (arrowhead) and is undetectable in severe mutants. Scale bars (A through O) = 100 μm.

Kidneys showed similar rates of proliferation in *Alk3<sup>UB−/−</sup>* UB trunks and tips compared with *Wt* kidneys at E12.5 and E13.5 (*P* = 0.66 and *P* = 0.36, respectively) (Supplemental Figures 1 and 2). *Alk3<sup>UB−/−</sup>* kidneys also displayed similar, negligible rates of apoptosis compared with *Wt* as measured by terminal deoxynucleotidyl transferase biotin dUTP nick end labeling (TUNEL) assay (Supplemental Figure 2, F and G). Thus, neither alterations in the rate of cell proliferation or apoptosis appear to play a significant role in the genesis of the *Alk3*-deficient phenotype.
Adult Alk3UB−/− Mice Exhibit Severe Medullary Hypoplasia

Postnatal Alk3UB−/− kidneys (P21) exhibited a dysplastic phenotype characterized by absence of the renal papilla, and medullary hypoplasia (Figure 5, A and B). Medullary collecting ducts, identified by calbindin28K antibody, were dilated and did not form linear arrays characteristic of collecting ducts in Wt mice (Figure 5, C and D). Phospho-Smad1 was undetectable in the nuclei of Alk3UB−/− medullary collecting duct cells (Figure 5, E and F), and phospho-Smad1 protein was reduced 52% in Alk3UB−/− whole kidney lysates (Figure 5K), confirming the loss of ALK3-dependent signaling in these kidneys. Malformed collecting ducts of Alk3UB−/− mice exhibited markers of abnormal renal epithelial tubular differentiation. Cellular expression of β-catenin was markedly increased in Alk3UB−/− kidney tissue sections (Figure 5, G and H), corresponding to a 511% increase in β-catenin protein in kidney tissue lysates (Figure 5K). Remarkably, we detected de novo MYC expression in Alk3UB−/− medullary collecting duct cell nuclei (Figure 5, I and J), and in kidney tissue lysates (Figure 5K). Thus, the dysplastic phenotype in adult Alk3UB−/− kidneys is associated with reduced ALK3-dependent Smad1 signaling, malformation of medullary collecting ducts, and abnormal activation of β-catenin and MYC.

DISCUSSION

The experiments reported here demonstrate a critical role for ALK3 during renal development. Deficiency of ALK3 in the UB lineage results in renal malformation associated with a decreased number of collecting ducts and their progenitor UB branches. Remarkably, our analysis of embryonic Alk3 kidneys demonstrates a biphasic branching defect, which manifests as an early increase in the number of 1° and 2° UB branches, but a decrease in the number of subsequent branches formed, resulting in an overall reduction in UB number. These results suggest that ALK3 normally functions to limit UB branching at the earliest stages of branching morphogenesis. Furthermore, loss of ALK3-dependent inhibition during this period interferes with the number of branches that can be subsequently formed.

We previously showed that constitutive activation of ALK3-Smad1 signaling in the UB of transgenic mice also results in decreased UB branching.10 Although both ALK3 gain- and loss-of-function mouse models exhibit a similar outcome, the mechanisms underlying these outcomes are distinct. The former represents a proof-of-principle model, establishing that constitutive overexpression of ALK3 can inhibit branching morphogenesis. Indeed, the increase in early branching observed in the present loss-of-function model is consistent with results from the overexpression model. However, the ALK3-deficient model also reveals that abnormal early patterning of the UB is deleterious to later branching events, suggesting that the subsequent decreases in UB branching and...
Collecting duct formation in Alk3UB−/− kidneys are secondary to the failure in these early patterning events.

Previous studies have demonstrated a role for BMPs and BMP inhibitors in regulating UB outgrowth from the Wolffian Duct. Bmp4+/− mice exhibit ectopic and supernumerary UB outgrowths from the Wolffian duct. Aberrant outgrowth is associated with abnormal metanephric development. Similarly, homozygous deficiency of gremlin, a BMP inhibitor, causes renal aplasia as a result of failure of UB outgrowth.21 Interestingly, E12.5 Alk3UB−/− mice do not exhibit ectopic UB outgrowths along the Wolffian duct, defects observed in Bmp4+/− kidneys. Thus, Bmp4-dependent regulation of UB outgrowth at the level of the Wolffian duct appears to be Alk3-independent and may instead be mediated through ALK6 or another ALK receptor. Our results are consistent with a role for ALK3 in transducing BMP2-dependent signals. Bmp2 transcripts are strongly detected in the mesenchymal condensates surrounding actively branching UB tips, as well as weakly in stromal cells adjacent to UB trunk segments.3 Thus, formation of supernumerary UB branches could be caused by the loss of BMP2-ALK3 inhibition of growth along UB trunk segments and terminal UB branching.

ALK3-deficient E13.5 kidney tissue exhibited variable alterations in Pax2 expression. In mice with a moderate branching defect, a specific reduction of Pax2 expression was observed in the UB lineage. These results suggest that ALK3 signaling regulates Pax2 expression in the UB, possibly at the level of transcription, thereby controlling growth and branching. In contrast, mutants with severe reductions in UB branch number exhibited a marked loss of Pax2 expression in both the UB and mesenchyme cell lineages. Because Pax2 is normally expressed in induced mesenchyme, these results suggest that a failure in UB branching in severely affected mutants disrupts mesenchyme induction in these mice, leading to a loss of induced, Pax2- and WT-1–expressing mesenchyme.

Our results, which demonstrate reduced Pax2 expression in both moderately and severely affected Alk3 mutant kidneys at E13.5, were surprising given the normal levels of Gdnf expression we observed at E11.5 in these mice and the established role of Pax2 in positively regulating Gdnf expression during kidney induction.22,23 Severely and moderately affected E13.5 Alk3 mutant kidneys are indistinguishable from each other at earlier stages of development, both groups appearing as trifid structures at E11.5. Thus, it is possible that E11.5 Alk3 mutant kidneys exhibiting normal expression patterns of Gdnf-Ret, Wnt11, and Spry1 represent the cohort of kidneys with a moderate branching defect at E13.5, together with a UB-specific absence of Pax2. In this context, Pax2-dependent expression of Gdnf in the mesenchyme of these Alk3 mutants would be unaffected because mesenchymal Pax2 expression is maintained in these mice. Indeed, complete loss of Pax2 before kidney induction has been shown to be incompatible with renal development, resulting in renal agenesis as a result of a loss of Gdnf-Ret signaling.22,23 Alternatively, all Alk3 mutants, regardless of their patterns of Pax2 expression at E13.5, may exhibit normal or near-normal Pax2 expression at E11.5, with the loss of Pax2 expression observed at E13.5 occurring subsequent to initial UB induction, thus explaining the normal expression of Gdnf-Ret at E11.5. Notably, in E16.5 Pax2+/−;Pax8+/− compound hetero-

Figure 5. Adult Alk3UB−/− kidneys exhibit medullary hypoplasia. (A and B) P21 Alk3UB−/− kidneys exhibit cortical cysts, loss of the renal papilla, and medullary hypoplasia. (C and D) Malformation of medullary collecting ducts, identified with anti-calbindin28K antibody, in Alk3UB−/− kidneys (boxed areas in A and B). (E and F) Nuclear phospho-Smad1 expression is not detected in the medulla of Alk3UB−/− kidneys. (G and H) Cytosolic β-catenin expression is increased in Alk3UB−/− kidneys compared with Wt. (I and J) De novo nuclear MYC expression is observed in Alk3UB−/− kidneys. (K) Reduced ALK3 and phospho-Smad1 protein, together with increased β-catenin and MYC proteins, are observed in P21 Alk3UB−/− whole-kidney lysates compared with Wt. Scale bars: (A and B) 1 mm, (C through F) 50 μm, (G and H) 500 μm, (I and J) 50 μm.
zygote kidneys exhibiting a partial reduction of Pax2 expression, both Gdnf and Ret expression are unaffected, suggesting that the expression of these genes is resilient against moderate reductions in Pax2, and further, that expression of these genes may be regulated by both Pax2-dependent and -independent pathways.

Postnatal Alk3-deficient kidneys are characterized by misexpression of β-catenin and MYC. These abnormalities are likely secondary events because we did not observe increased expression of either β-catenin or MYC in embryonic kidney tissue and because we have observed increased β-catenin and MYC expression in overexpression models of BMP signaling. Both β-catenin and MYC are increased by BMP2 treatment in vitro and by constitutive expression of Alk3 in vivo. Collectively, these results suggest that activation of β-catenin and MYC in dysplastic Alk3UB/−/− kidneys does not represent a Bmp-dependent disease pathway but rather a final convergent pathway in the pathogenesis of diverse models of cystic renal dysplasia.

Collectively, our results suggest that initial patterning of the UB branch tree is an important determinant of later branching events will help illuminate the mechanisms that control collecting duct development.

**CONCISE METHODS**

**Mice and Genotyping**

HoxB7-Cre-GFP;Alk3+/−, Wt, and Alk3UC/C mice were maintained on 129SvJ:C57BL/6 genetic backgrounds and genotyped by PCR.11,12

**Assessment of Ureteric Bud Branching, Cell Proliferation, and Apoptosis**

UB branches were imaged by fluorescence microscopy for expression of GFP or FITC-conjugated Dolichos biflorus agglutinin (20 μg/ml; Vector Labs, Burlingame, CA). UB branch points were defined as the intersection between three connected branches. Cell proliferation was assayed by incorporation of 5-bromo-2′-deoxyuridine (BrdU; Roche, Indianapolis, IN).5 Apoptosis was assayed using an in situ TUNEL assay.14

**Quantitation of Collecting Duct Number**

Each kidney was serially-sectioned (4 μm thick) in its entirety. The number of collecting ducts, identified by anti-calbindin28K antibodies, was counted as the mean value of that in each of ten tissue sections (five on each side of the deepest section).

**Immunohistochemistry, In Situ Hybridization, and Western Blot Analysis**

Paraffin wax-embedded sections (4 μm thick) of embryonic kidney tissue were prepared.27 Primary antibodies were directed against calbindin28K (Sigma, St. Louis, MO), phospho-Smad1 (Upstate, Charlottesville, VA), β-catenin (Upstate), and c-myc (Cell Signaling, Danvers, MA). RNA in situ hybridization was performed as described, using probes encoding Alk3, Pax2, Wt1, Gdnf, c-Ret, Wnt11, and Spry1. For immunoblotting, primary antibodies were directed against ALK3 (1:250 dilution; Santa Cruz, Santa Cruz, CA), phospho-Smad1 (1:250 dilution), β-catenin (1:1000 dilution), c-myc (1:250 dilution), and β-actin (1:20,000 dilution; Sigma). Values are presented as mean differences computed from pooled data using a two-tailed t-test (P < 0.05).

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**DISCLOSURES**

None.

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