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Role of Tbx2 in defining the territory of the pronephric nephron

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SUMMARY

Despite extensive study of the development of the nephron, which is the functional unit of the kidney, the molecular mechanisms underlying the determination of nephron size remain largely unknown. Using the *Xenopus* pronephros, we demonstrate here that Tbx2, a T-box transcriptional repressor, functions to demarcate the territory of the pronephric nephron. *Tbx2* is specifically expressed around three distinct components of the pronephric nephron: the tubule, duct and glomus. Gain of function of Tbx2 inhibits nephric mesoderm formation. Conversely, Tbx2 loss of function expands the boundary of each component of the pronephric nephron, resulting in an enlarged pronephros. BMP signals induce Tbx2 in the non-nephric mesoderm, which inhibits the expression of the nephric markers *Hey1* and *Gremlin*. Importantly, these pronephric molecules repress *Tbx2* expression by antagonizing BMP signals in the nephric mesoderm. These results suggest that the negative regulatory loops between BMP/Tbx2 and Gremlin or Hey1 are responsible for defining the territory of the pronephric nephron.

KEY WORDS: Tbx2, Pronephric nephron, *Xenopus*, BMP, Gremlin, Hey1

INTRODUCTION

One of the most fundamental questions in organogenesis is how the final shapes and sizes of organs are determined. Organ size is determined by several factors that regulate cell growth, proliferation and death (Stanger, 2008a; Stanger, 2008b). The size of the pancreas is restricted by the number of embryonic progenitor cells (Stanger et al., 2007), and Hox genes regulate the production and mobility of a morphogen to control the size of the wing and haltere in *Drosophila* (Crickmore and Mann, 2006). However, the detailed molecular mechanisms that determinate organ size are largely unknown.

The kidney is one of the main filtering and excretory organs of the body. During vertebrate kidney development, several inductive signaling pathways lead to the origination of three successive renal structures – the pronephros, mesonephros and metanephros – from the intermediate mesoderm (IM). These three levels of renal structure have a similar functional organization and development, and all three have the nephron as the basic functional unit (Brandli, 1999; Dressler, 2006). The nephron is induced from the condensed nephric mesenchyme through a mesenchymal-epithelial transition and consists of three basic components: tubule, duct and glomerulus. Although the roles of several growth factors, including BMP4, BMP7, Notch, Gdnf and Wnt4, and transcription factors, including *Pax2*, *WT1*, *Foxd1* and *Eya1*, have been identified in kidney development (Schedl, 2007; Vainio and Lin, 2002), the mechanisms of nephron size determination remain to be investigated.

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Xenopus laevis has a single large nephron as a functional kidney at the tadpole stages. Since the genetic program regulating kidney development is evolutionarily conserved in frog, fish, mouse and human (Dressler, 2006; Vize et al., 1997), the *Xenopus* pronephric nephron is the most effective model with which to investigate the mechanism of nephron development. The *Xenopus* pronephric nephron is induced from the nephric mesenchyme of the IM, which is surrounded by the non-nephric mesenchyme. The non-nephric mesenchyme contains several types of cells involved in functional kidney development, including hemangioblasts, angioblasts, hematopoietic progenitors, erythrocytes and trunk neural crest cells (Brandli, 1999). Nevertheless, the role of the non-nephric mesenchyme in nephron morphogenesis has not been widely investigated.

Tbx2 is a T-box transcription factor that can be induced by BMP signaling (Suzuki et al., 2004) and acts as a transcriptional repressor (Carreira et al., 1998). Tbx2 plays important roles in cell type specification and morphogenesis (Gibson-Brown et al., 1998; Smith, 1999). It specifies posterior digit identity (Suzuki et al., 2004) and is essential for patterning the atrioventricular canal of the heart (Harrelson et al., 2004). In addition, Tbx2 regulates regional morphogenesis in the hypothalamus by inhibiting *Shh* (Manning et al., 2006). *Tbx2* expression in the kidney has been reported in many organisms including human, mouse and *Xenopus* (Campbell et al., 1995; Chapman et al., 1996; Hayata et al., 1999), but little is known about its function in kidney development.

Here, we show that *Xenopus Tbx2* is specifically expressed around three components of the pronephric nephron – the tubule, duct and glomus – and defines the boundary of these basic structures. During pronephric nephron morphogenesis, BMP signaling induces *Tbx2* expression around the nephric mesoderm, which inhibits the expression of the nephric mesodermal markers *Gremlin* and *Hey1*. These pronephric molecules suppress *Tbx2* expression by counteracting BMP signals. Thus, these negative regulatory loops between BMP/Tbx2 and Gremlin or Hey1 function to delineate the interface between the nephric and non-nephric mesoderm, affecting the size of the pronephric nephron.

MATERIALS AND METHODS

Plasmid constructs and morpholino oligonucleotides

The complete coding regions of *Xenopus laevis Tbx2* (GenBank accession number AB032941) and dominant-negative *Tbx2* (Tbx2ΔC, comprising amino acids 1-518) were amplified by PCR and inserted into the *Bam*HI/*Xho*I sites of the pCS2+ vector. 5′UTR-Tbx2-myc and Δ5′UTR-Tbx2-myc constructs were produced by subcloning the PCR products encompassing the Tbx2 coding region and MO target site (5′UTR) or its coding region only into the pCS2+-myc vector. To produce the dexamethasone-inducible constructs, the coding region of the human glucocorticoid receptor ligand-binding domain (hGR) was fused in frame to the C-terminus of wild-type Tbx2 (Tbx2-GR) and Tbx2ΔC (Tbx2ΔC-GR) as described (Tada et al., 1997). The transactivation domain of VP16 (Sadowski et al., 1988) and the transrepression domain (EnR) of the Engrailed transcription factor (Jaynes and O'Farrell, 1991) were amplified by PCR and ligated to Tbx2ΔC to produce VP16-Tbx2ΔC and EnR-Tbx2ΔC, respectively.

Capped mRNAs were in vitro synthesized using the mMessage mMachine Kit (Ambion). Antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools. The Tbx2 MO was 5′-GGAAAGC-TGGATCTCTCATCGGTGC-3′; the Control MO was a standard MO from Gene Tools of sequence 5′-CCTCTTACCTCAGTTACAATTTATA-3′.

Embryo manipulation, dexamethasone treatment and in vitro kidney induction

In vitro fertilization, embryo microinjection and culture were performed as described previously (Sive et al., 1989). Developmental stages of embryos were determined according to the Nieuwkoop and Faber normal table of development. For targeting the pronephros, synthesized mRNA or MO was injected into one of the vegetal-ventral blastomeres of the 8-cell stage embryo. For the induction of hGR-fused constructs, 10 μM dexamethasone (DEX; Sigma) was added at the indicated time. For in vitro kidney induction, animal cap explants were dissected at stages 8-9 and cultured in Steinberg's Solution with activin A (10 ng/ml) and retinoic acid (0.1 mM). After 3 hours, the animal caps were washed twice with Steinberg's Solution and cultured for 3 days at 20°C in Steinberg's Solution.

Lineage tracing and in situ hybridization

For lineage tracing, *lacZ* mRNA (β-gal, 250 pg) was injected along with antisense MOs or mRNAs. The β-galactosidase activity was visualized with X-Gal or Red-Gal substrate (Sigma). Whole-mount in situ hybridization was performed as described (Harland, 1991). Antisense DIG-labeled probes were in vitro synthesized using the following previously described templates: *Xenopus laevis Tbx2* (Hayata et al., 1999), *Pax8* (Heller and Brandli, 1999), *Pax2* (Heller and Brandli, 1997), *Lim1* (Chan et al., 2000), *Hey1* (Pichon et al., 2002), *WT1* (Carroll and Vize, 1996), *Nephrin* (Gerth et al., 2005), *Gremlin* (Hsu et al., 1998), *XC3H-3b* (Kaneko et al., 2003) and *Vimentin* (Dent et al., 1989).

Histology, immunostaining and western blotting

For histology, the stained embryos were embedded in agarose (Sigma, A0701) and then sectioned at 50 μm using a Vibratome (Series 1000 Plus). Immunostaining of embryos was carried out using 3G8/4A6 antibodies (which are pronephric tubule and duct specific, respectively) (Vize et al., 1995). The secondary antibody was alkaline phosphatase-conjugated anti-mouse IgG+IgM (AMI4705, Biosource).

For western blotting, whole embryos were homogenized in Triton X-100 lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Equal amounts of protein were separated by 10% SDS-PAGE. Western blotting was performed according to a standard protocol with anti-Myc and anti-actin (both 1:1000; Santa Cruz) antibodies.

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted from whole embryos and tissue explants using TRI Reagent (Molecular Research Center) and treated with RNase-free DNase I (Roche Molecular Biochemicals) to remove genomic DNA. RNA was transcribed using M-MLV reverse transcriptase (Promega) at 37°C for 1 hour. PCR products were analyzed on 2% agarose gels. The number of PCR cycles for each primer set was determined empirically to maintain amplification in the linear range.

RESULTS

Tbx2 is expressed around the pronephric nephron

We first analyzed in detail the expression of *Tbx2* in the pronephric nephron of the *Xenopus* embryo; its general localization to the organ has been described previously (Hayata et al., 1999). Pronephric expression of *Tbx2* started weakly in the presumptive pronephric anlage (PA) at stage 21, which is when pronephric morphogenesis begins (Fig. 1A), and became gradually restricted to the area surrounding the PA (Fig. 1B,C). At stages 31/32, when each component of the pronephric nephron begins to be formed, increased *Tbx2* expression was visible in the non-nephric mesenchyme of the IM, which encloses the pronephric mesenchyme (Fig. 1D,E). Of note, *Tbx2* was markedly expressed in the dorsal region of the PA where the nephrostomes are induced (Fig. 1F) and around the pronephric duct (Fig. 1G). Subsequently, *Tbx2* transcripts exhibited ring-shaped expression, circling the three end tips of the nephrostomes (Fig. 1I, arrowheads). Furthermore, its expression was more markedly restricted to the non-nephric mesenchyme around the pronephric tubules and duct (Fig. 1J,K). This expression pattern of *Tbx2* contrasts with that of *Pax2*, which was expressed preferentially in the nephric mesoderm at each stage (Fig. 1L-P). Interestingly, transverse sections revealed a dramatic change in *Tbx2* expression in the medial (splanchnic) layer of the IM: *Tbx2* was absent from this mesodermal layer prior to glomus bud formation (Fig. 1F, arrow), whereas it later became detectable in the non-glomus region of this layer (Fig. 1J, arrow), but not in the glomus bud area.

Gain of Tbx2 function inhibits pronephric nephron formation

To investigate the function of Tbx2 in pronephric development, we first injected its mRNA into the presumptive pronephric region of the *Xenopus* embryo and examined the expression of pronephric markers at stage 31 or 34. Interestingly, overexpression of *Tbx2* mRNA strongly abrogated the expression of *Pax2* (a marker of the tubule and duct; 80% of embryos affected, *n*=45), *WT1* (a glomus-specific marker; 82%, *n*=45) and *Gremlin* (a duct-specific marker; 89%, *n*=45) (Fig. 2A,C,E).

To avoid effects of Tbx2 on mesoderm formation at earlier stages, we generated a stage-specific inducible Tbx2 construct in which the hormone-binding domain of the glucocorticoid receptor (GR) is fused to the C-terminus of Tbx2 (Tbx2-GR). Upon treatment with dexamethasone (DEX), this construct allows conditional activation of Tbx2 function at a specific time during early embryogenesis. With DEX treatment at stage 22, injected *Tbx2-GR* mRNA impaired the expression of *Pax2* (76%, *n*=42), *WT1* (80%, *n*=45) and *Gremlin* (83%, *n*=45) on the injected side of embryos (Fig. 2B,D,F), suggesting direct, late effects of Tbx2 on pronephros formation.

To test whether Tbx2 functions as a transcriptional repressor, as reported (Carreira et al., 1998), to regulate pronephric development, we also made heterologous fusion constructs by adding the VP16 activation domain or Engrailed repressor domain to a Tbx2 mutant lacking the C-terminal region that has been shown to confer the repressor activity (Fig. 2K). Injection of *VP16-Tbx2ΔC* mRNA caused the expanded expression of *Pax2* and *WT1* (Fig. 2G,I), whereas, like wild-type *Tbx2*, *EnR-Tbx2ΔC* mRNA

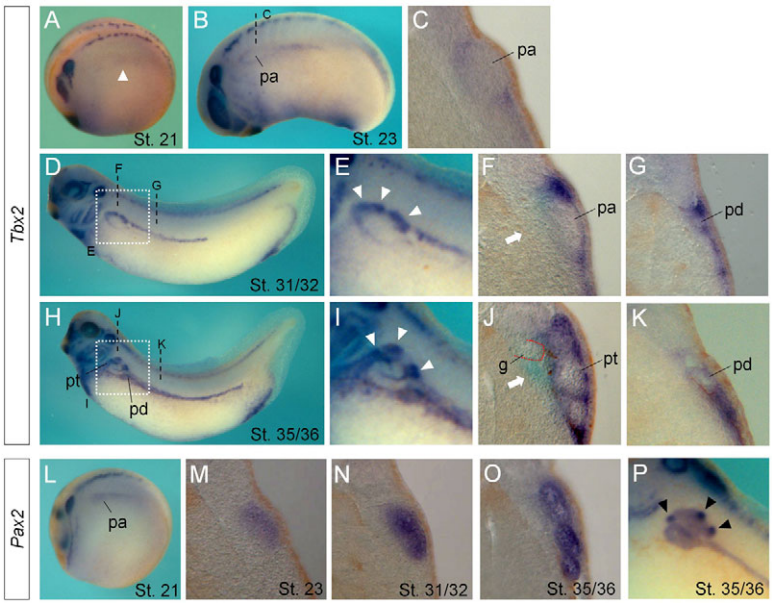

Fig. 1. Pronephric expression of *Xenopus Tbx2* analyzed by in situ hybridization. (**A**,**B**) Expression of *Tbx2* around the pronephric anlage (pa) at stage 21 (A) and 23 (B). Arrowhead indicates weakly expressed *Tbx2* around the pronephric anlage. (**C**) Transverse section of the embryo in B at the level indicated by the dashed line. (**D**) *Tbx2* expression surrounding the developing pronephric tubule and duct at stage 31/32. (**E**) Magnified view of the boxed area in D. Arrowheads indicate the increased expression of *Tbx2* in the dorsal region of the pronephric tubule anlage. (**F**,**G**) Transverse sections of the embryo in D at the levels indicated by the dashed lines. The arrow (F) indicates the absence of expression of *Tbx2* in the medial layer of the intermediate mesoderm (IM). (**H**) *Tbx2* expression enclosing the pronephric tubule (pt) and duct (pd) at stage 35/36. (**I**) Enlarged view of the boxed region in H. Arrowheads point to the expression of *Tbx2* around the tips of nephrostomes. (**J**,**K**) Transverse sections of the embryo in H at the levels indicated by the dashed lines. The pronephric glomus (g) is outlined in red in J. The arrow indicates *Tbx2* expression in the non-glomus mesenchyme in the medial layer of IM. (**L**) *Pax2* expression in the pronephric anlage at stage 21. (**M-O**) *Pax2* expression in transverse sections of the pronephric anlage, tubule and duct at the indicated stages. (**P**) Strong expression of *Pax2* in the tips of nephrostomes (arrowheads).

abrogated the expression of the markers on the injected side of embryos (Fig. 2H,J), indicating that Tbx2 acts as a repressor to control the formation of the pronephric nephron.

Depletion of Tbx2 expands the territory of the pronephric nephron

We further checked the effects of the loss of Tbx2 activity on the development of the pronephros by injecting its antisense morpholino oligonucleotide (MO) or dominant-negative mutant (*Tbx2ΔC*) mRNA. Western blotting demonstrated that Tbx2 MO could specifically inhibit the translation of its mRNA containing the MO target sequence (see Fig. S1 in the supplementary material). To make a stage-specific, inducible dominant-negative mutant, the GR domain was fused to Tbx2ΔC (Tbx2ΔC-GR) (Fig. 2K). At stage 34, *WT1* and *Nephrin* were expressed in the glomus bud domain on the uninjected control side of embryos, whereas their expression expanded to the posterior and ventral regions on the Tbx2 MO- or *Tbx2ΔC-GR*-injected side (Fig. 3A,B; *WT1*, 84%, *n*=45; *Nephrin*, 89%, *n*=45). Specifically, the loss of Tbx2 function caused the expression of *WT1* and *Nephrin*

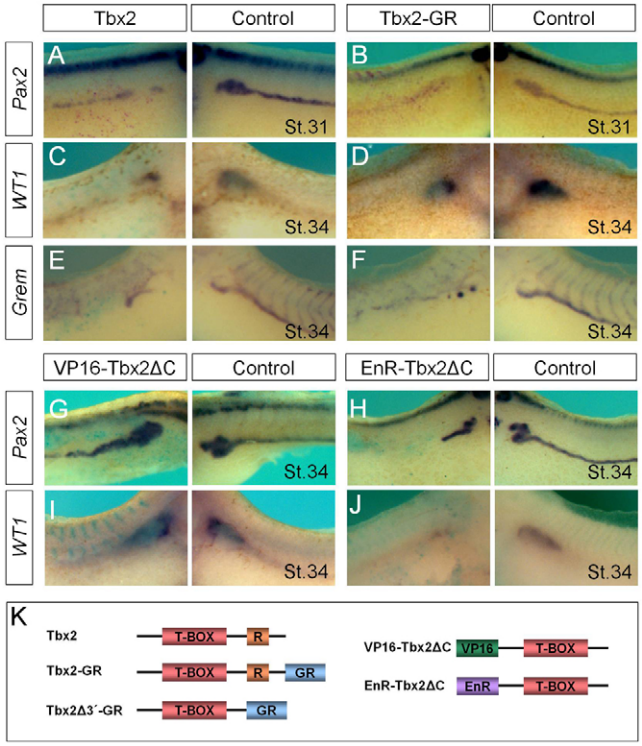

Fig. 2. Ectopic expression of Tbx2 inhibits pronephric nephron formation. (**A-J**) *Xenopus* embryos injected with *Tbx2* (200 pg), *Tbx2-GR* (200 pg), *VP16-Tbx2ΔC* (100 pg) or *EnR-Tbx2ΔC* (100 pg) mRNA were treated (B,D,F) or otherwise (A,C,E,G-J) with dexamethasone (DEX) from stage 22 to 31 or 34 and then subjected to in situ hybridization for the pronephric markers *Pax2*, *WT1* and *Gremlin*. The left and right images in each panel indicate the injected and uninjected control sides of the embryo, respectively. Of note, *VP16-Tbx2ΔC* disorganizes the pronephric tubules (G) and expands the glomus domain (I). (**K**) Tbx2 constructs used in the injection experiments. T-BOX, T-box from Tbx2; R, repressor domain of Tbx2; GR, human glucocorticoid receptor ligand-binding domain; VP16, transactivation domain of VP16; EnR, transrepression domain of Engrailed.

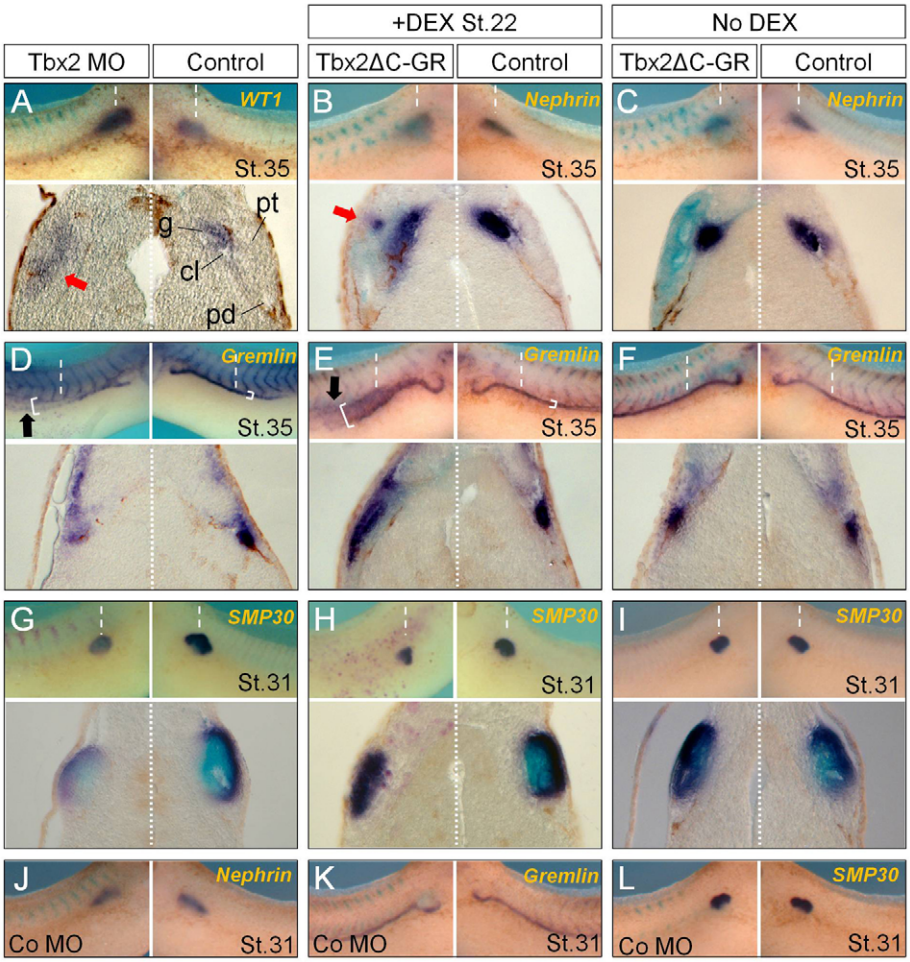

Fig. 3. Loss of Tbx2 function expands the territory of the pronephric duct and glomus but not the tubule. (**A-L**) *Xenopus* embryos injected with Tbx2 MO (10 ng), *Tbx2ΔC-GR* (200 pg) or control MO (Co MO, 10 ng) were subject to in situ hybridization for the glomus-specific markers *WT1* and *Nephrin* and the duct-specific marker *Gremlin* at stage 35 or the tubule-specific marker *SMP30* at stage 31. To activate injected *Tbx2ΔC-GR* mRNA, embryos were treated with DEX from stage 22 to 35 (B,E) or to 31 (H). (A-I) Stained embryos are shown in the upper part of each panel and transverse sections at the levels indicated by the dashed lines are shown below. Left and right parts of each panel show the injected and uninjected control sides, respectively. cl, coelom; g, glomus; pd, pronephric duct; pt, pronephric tubule. Arrows in A,B indicate *WT1* and *Nephrin* expression in the somatic layer of the intermediate mesoderm, respectively. Arrows and bracket in D,E indicate migrating *Gremlin*-expressing cells and the diameter of pronephric duct, respectively. (J-L) Control MO has no effect on the expression of *Nephrin*, *Gremlin* or *SMP30*.

to invade into the somatic layer of the IM, where pronephric tubules are induced, and even into the non-glomus medial layer of the IM (Fig. 3A,B, arrows). In contrast to the control side, the basic components of the pronephric nephron, such as tubule, duct, glomus and coelom, were invisible on the Tbx2-depleted side (Fig. 3A,B).

On the uninjected control side of embryos, *Gremlin*-expressing cells formed a narrow pronephric duct (Fig. 3D,E). Importantly, inhibition of Tbx2 function enlarged the region of the pronephric duct, as revealed by the expanded expression of *Gremlin* (Fig. 3D,E; Tbx2 MO, 71%, *n*=45; *Tbx2ΔC-GR*, 87%, *n*=45). As observed in the tissue sections, the expression of *Gremlin* was detectable in the non-nephric mesenchyme as well as in the pronephric duct epithelium on the Tbx2 MO- or *Tbx2ΔC-GR*-injected side of embryos. Although the territory of the pronephric duct was expanded by the blockade of Tbx2 activity, the pronephric duct precursor cells migrated along the enlarged pronephric duct (Fig. 3D,E, arrows). In addition, whereas *SMP30* was specifically expressed in the branched pronephric proximal tubules on the control side, Tbx2 MO and *Tbx2ΔC-GR* interfered with the branching morphogenesis of pronephric tubules, resulting in misexpression of *SMP30* into the non-branched lump structure (Fig. 3G,H; Tbx2 MO, 75%, *n*=44; *Tbx2ΔC-GR*, 84%, *n*=44). All tested marker genes showed normal expression in embryos without DEX treatment (Fig. 3C,F,I) and in those injected with Control MO (Co MO) (Fig. 3J-L).

To confirm the expansion of the pronephric duct and glomus areas in the embryos devoid of Tbx2 activity as revealed by the staining for *Gremlin*, *WT1* and *Nephrin*, we carried out in situ hybridization against *XC3H-3b* and *Vimentin*, which clearly outline the pronephric nephron (see Fig. S2 in the supplementary material), and sectioned the stained embryos. On the uninjected control side of embryos, both *XC3H-3b* and *Vimentin* were specifically expressed in the non-nephric mesenchyme enclosing each component of the pronephric nephron. Notably, both markers delineated the disorganized and relatively expanded pronephric duct and glomus on the Tbx2 MO- or *Tbx2ΔC-GR*-injected side (see Fig. S2 in the supplementary material).

Since Tbx2 might control the formation of the anterior somites, which are crucial for pronephric patterning (Mauch et al., 2000), we examined the effect of Tbx2 loss of function on the expression of the somite-specific marker *MyoD*. Both Tbx2 MO and *Tbx2ΔC-GR* marginally affected the expression of *MyoD* in the anterior somitic area (see Fig. S3A-C in the supplementary material), suggesting that Tbx2 regulates pronephric morphogenesis independently of somite formation.

Loss of Tbx2 activity leads to enlargement of the pronephric nephron

We next performed immunostaining with pronephric tubule- and duct-specific antibodies to visualize the pronephric components in the embryos lacking or overexpressing Tbx2. As shown in Fig.

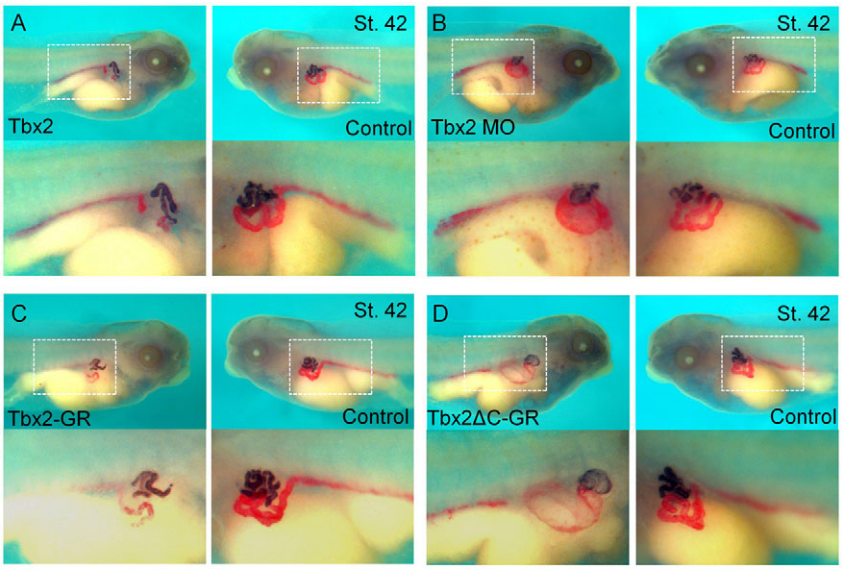

Fig. 4. The pronephric nephron is enlarged by inhibition of Tbx2 activity. (**A-D**) *Xenopus* embryos injected with *Tbx2* mRNA (200 pg), Tbx2 MO (10 ng), *Tbx2-GR* (200 pg) or *Tbx2ΔC-GR* (200 pg) were double stained using pronephric tubule- and duct-specific antibodies (3G8 and 4A6, respectively) at stage 42. To activate injected *Tbx2-GR* and *Tbx2ΔC-GR* mRNA, DEX treatment was from stage 22 to 42 (C,D). Left and right parts of each panel show the injected and uninjected sides of embryos, respectively. Magnified views of the boxed areas are shown beneath.

4A,B, the defective pronephric tubule and duct were observed on the *Tbx2* mRNA-injected side of embryos (86.6%, *n*=75), whereas the pronephric duct on the Tbx2 MO-injected side became enlarged (35.1%, *n*=74) compared with the control side of embryos. Consistently, *Tbx2-GR* also disrupted the formation of the pronephric tubule and duct (65%, *n*=40) and *Tbx2ΔC-GR* caused a dramatically enlarged pronephric duct to develop on the injected side (56.1%, *n*=66) when DEX treatment was from stage 22. The pronephric tubule also appeared to be enlarged by depletion of Tbx2, but not so markedly as the pronephric duct. Even when the *Tbx2ΔC-GR*-injected embryos were treated with DEX from the 8-cell stage onward, 43% of the embryos (*n*=35) had an enlarged pronephric duct (data not shown). Thus, these data strongly suggest that Tbx2 functions to determine the size of the pronephric nephron.

Tbx2 affects the morphogenesis of in vitro induced pronephric nephron

We performed an in vitro kidney induction assay (see Fig. 5A) to further analyze the effects of Tbx2 on pronephric nephron development. As the induced pronephric tissues contain the basic components of the pronephric nephron and express the same nephric marker genes as endogenous nephric tissues (Asashima et al., 2009), this experiment is very effective in elucidating the mechanism of nephrogenesis. Non-induced control *Xenopus* animal caps exhibited the typical lumpy morphology, with no positive staining for *Pax2* or with 3G8 (Fig. 5B,F,J). Notably, induced control animal explants had a bulged morphology (Fig. 5C), with well-developed pronephric tubular structures (Fig. 5G,K); moreover, the *Pax2*-expressing or 3G8-stained cells were clearly observed in these tubular tissues (arrowheads; *Pax2*, 87.5%, *n*=32; 3G8, 81%, *n*=27).

Overexpression of *Tbx2* mRNA caused the induced animal caps to exhibit a typical epidermal shape (Fig. 5D) without any tubular structures, similar to that of non-induced caps (Fig. 5H,L), and the *Pax2* (88%, *n*=27) and 3G8 (77%, *n*=26) stainings were significantly reduced. Interestingly, the induced animal tissues lacking Tbx2 activity were highly bulged (Fig. 5E) but had a rudimentary cell mass at the expense of tubular structures (Fig. 5I,M); nevertheless, *Pax2* and 3G8 staining was still detectable in the condensed inner mass (arrowheads; *Pax2*, 86%, *n*=23; 3G8, 80%, *n*=25). Taken together, this in vitro assay supports the proposal that Tbx2 plays crucial roles in the morphogenesis of the pronephric nephron.

Tbx2 downregulates the expression of *Gremlin* and *Hey1*

We then used RT-PCR analysis to investigate the mechanism by which Tbx2 controls pronephric nephron morphogenesis. Treatment with retinoic acid (RA) and activin induced the ectopic expression of several pronephric markers including *Pax2*, *WT1*, *Gremlin*, *Hey1* and *SMP30* in the ectodermal tissues (Fig. 5N, lane 4). This ectopic induction was strongly inhibited by overexpression of *Tbx2* mRNA (Fig. 5N, lane 5). Importantly, knockdown of Tbx2 increased the transcriptional levels of *Gremlin* and *Hey1* in the RA- and activin-stimulated animal tissues, whereas it had marginally repressive effects on *Pax2*, *WT1* and *SMP30* expression (Fig. 5N, lane 6), indicating that these sets of pronephric markers respond differently to the absence of Tbx2 function.

Since the expression domain of *Gremlin* was reduced and expanded by the gain and loss of Tbx2 function, respectively (Figs 2 and 3), we also examined whether *Hey1* expression could be affected by *Tbx2* in a similar fashion. Overexpression of wild-type *Tbx2* or *Tbx2-GR* mRNA strongly decreased the expression of *Hey1*, which was restricted to the dorso-anterior region of the PA, compared with the control side (Fig. 5O,P; *Tbx2*, 25%, *n*=37; *Tbx2-GR*, 54%, *n*=35). Conversely, the Tbx2 MO and *Tbx2ΔC-GR* resulted in a broadened misexpression of *Hey1* in the dorso-anterior region of the pronephric nephron on the injected side (Fig. 5Q,R; Tbx2 MO, 68%, *n*=35; *Tbx2ΔC-GR*, 71%, *n*=34), suggesting that Tbx2 regulates *Hey1* in the same way as it does *Gremlin*. Moreover, co-injection of *Gremlin* (47%, *n*=45) or *Hey1* (39%, *n*=44) mRNA efficiently restored the defective *Pax2* expression in the Tbx2-overexpressing embryos (Fig. 5S-U). These results indicate that Tbx2 controls pronephric nephrogenesis by repressing the expression of *Gremlin* and *Hey1*.

BMP signaling inhibits the expression of *Hey1* and *Gremlin* by inducing Tbx2

As Tbx2 has been shown to be a target of BMP2/4 in the development of various organs and BMP signaling is crucial for kidney formation (Dudley et al., 1995; Dudley and Robertson,

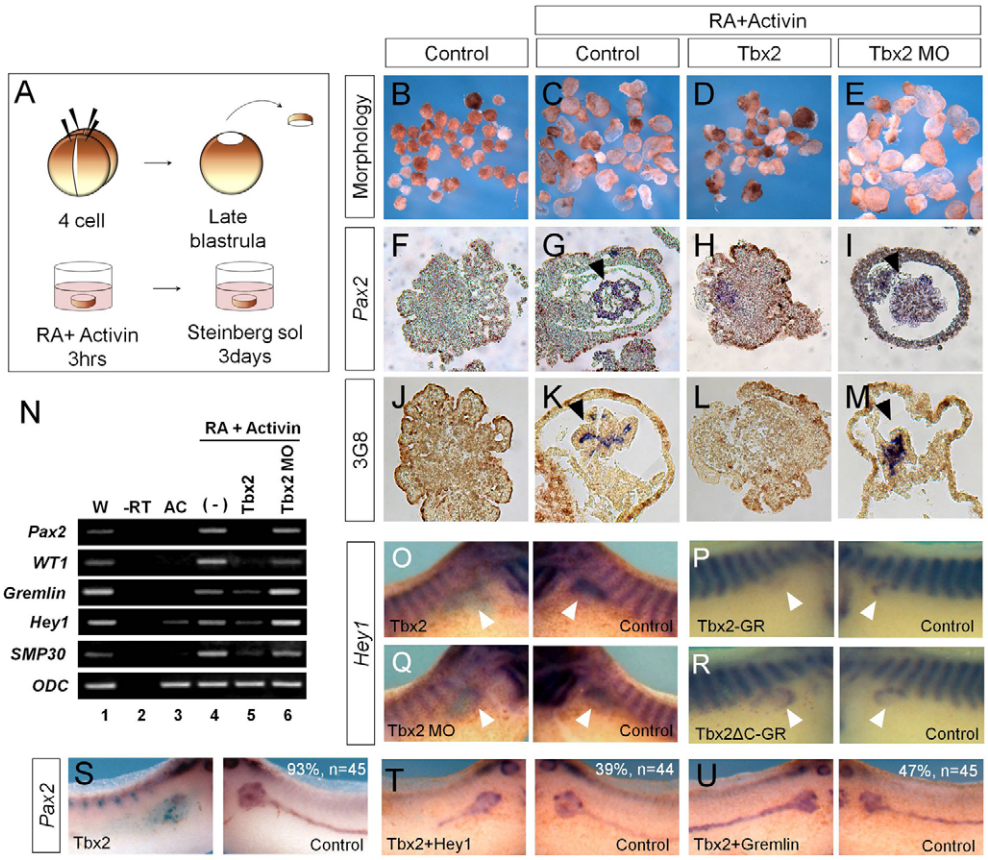

Fig. 5. Tbx2 downregulates the expression of *Gremlin* and *Hey1* to control pronephric morphogenesis. (**A**) Diagram of the in vitro kidney induction assay. RA, retinoic acid. (**B-N**) Four-cell stage *Xenopus* embryos were injected in the animal pole region with *Tbx2* mRNA (100 pg) or Tbx2 MO (10 ng) and then the animal explants were dissected at stage 9.5 and processed for in vitro kidney induction. Subsequently, the animal cap tissues were observed for morphology (B-E), sectioned for in situ hybridization with a *Pax2* antisense probe (F-I) and immunohistochemistry with the tubule-specific antibody 3G8 (J-M) or subjected to RT-PCR analysis (N). W, whole embryo; AC, uninjected animal caps without RA and activin treatment; (–), uninjected animal caps with RA and activin treatment; –RT, negative control without reverse transcriptase; *ODC*, ornithine decarboxylase loading control. Arrowheads (G,K,I,M) indicate *Pax2* expression or 3G8 staining in the induced animal caps. (**O-R**) Embryos injected with *Tbx2* (200 pg), *Tbx2-GR* (200 pg), Tbx2 MO (10 ng) or *Tbx2ΔC-GR* (200 pg) were subject to in situ hybridization with a *Hey1* antisense probe. Arrowheads indicate *Hey1* expression in the pronephros. Embryos in P,R were treated with DEX from stage 22 to 32. (**S-U**) Impaired expression of *Pax2* caused by Tbx2 was restored by co-injection of *Gremlin* or *Hey1*. Embryos injected with *Tbx2* mRNA (200 pg) with or without Hey1 (100 pg) or Gremlin (10 pg) were subject to in situ hybridization for *Pax2* at stage 35. n, total number of embryos analyzed; the percentage of embryos showing the phenotype illustrated is indicated. Left and right parts of each panel show the injected and uninjected control sides of embryos, respectively.

1997; Miyazaki et al., 2000), we examined whether Tbx2 could be regulated by BMP signaling in pronephric nephron morphogenesis. We injected DEX-inducible *Smad1-GR* and *ΔSmad7tevGR/TEV2GR* mRNA for the stage-specific activation and suppression of BMP signaling, respectively (Marom et al., 2005; Wawersik et al., 2005). Treatment of the injected embryos with DEX at stage 22 revealed that *Tbx2* expression was strongly augmented on the *Smad1-GR*-injected side (Fig. 6A; 83.3%, *n*=30) and, conversely, was abrogated on the *ΔSmad7tevGR/TEV2GR*-injected side of embryos (Fig. 6B; 40%, *n*=30). Furthermore, co-injection of *Smad1-GR* and *ΔSmad7tevGR/TEV2GR* constructs restored expression of *Tbx2* to the normal level (Fig. 6E; 57%, *n*=30). In addition, expression of *Pax2* was inhibited (50%, *n*=34) and increased (75%, *n*=33) by injection of *Smad1-GR* and *ΔSmad7tevGR/TEV2GR*, respectively (Fig. 6C,D), reminiscent of the effects of gain and loss of Tbx2 function. *Pax2* expression appeared normal in embryos coexpressing *Smad1-GR* and *ΔSmad7tevGR/TEV2GR* (Fig. 6F; 65%, *n*=34). Similarly, *Hey1* (Fig. 6G; 62.5%, *n*=40) and *Gremlin* (Fig. 6I; 76.2%, *n*=42) expression was increased on the *ΔSmad7tevGR/TEV2GR*-injected side of embryos, as observed in embryos devoid of Tbx2 activity. Notably, co-injection of *Tbx2-GR* counteracted the increased expression of *Hey1* and *Gremlin* caused by *ΔSmad7tevGR/TEV2GR*, restoring it to normal levels (Fig. 6H,J; *Hey1* 67%, *n*=42; *Gremlin*, 75%, *n*=40). Together, these data suggest that BMP signaling modulates the expression of pronephric markers via induction of Tbx2.

Gremlin and Hey1 repress Tbx2 by antagonizing BMP signaling

In agreement with the fact that both Gremlin and Hey1 inhibit BMP signaling (Hsu et al., 1998; Rutenberg et al., 2006), RT-PCR analysis showed that they could attenuate the transcription of *Tbx2*

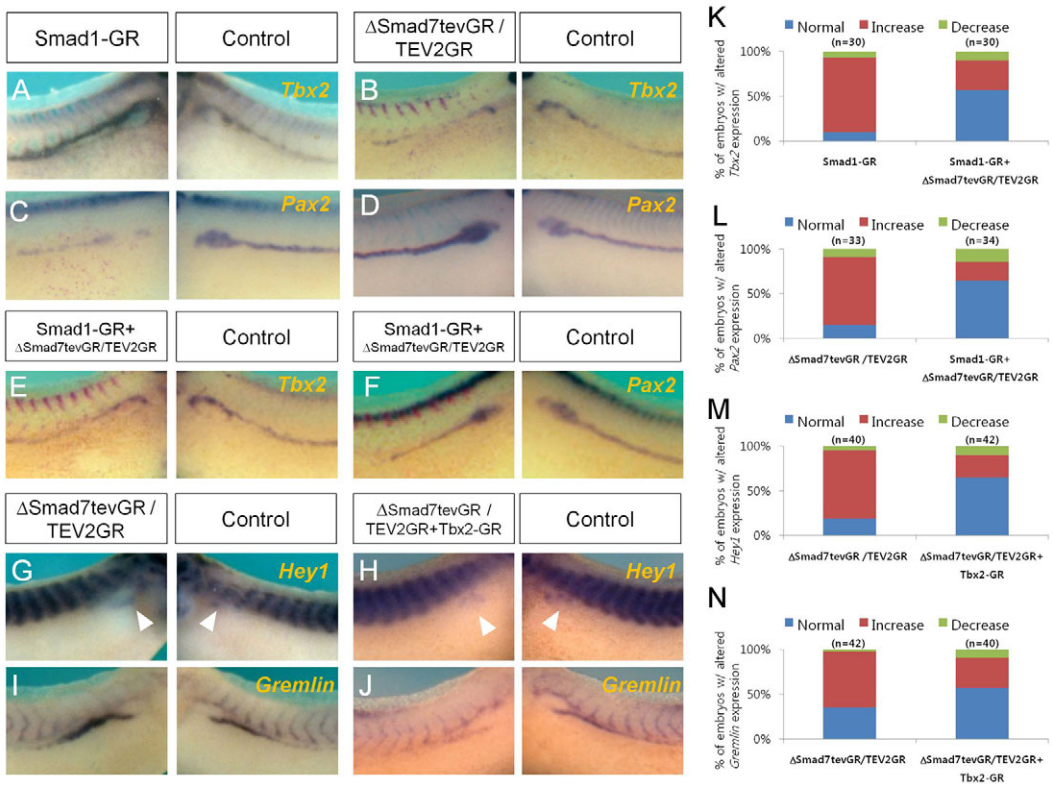

Fig. 6. BMP signaling inhibits the expression of *Hey1* and *Gremlin* via Tbx2. (**A-J**) *Xenopus* embryos injected with the indicated combinations of *Smad1-GR* (250 pg), *ΔSmad7tevGR* (250 pg)/*TEV2GR* (10 pg) and *Tbx2-GR* (200 pg) mRNA were subjected to in situ hybridization for *Tbx2*, *Pax2*, *Hey1* or *Gremlin*. Control shows the uninjected side of the embryo. DEX treatment was from stage 22 to 35. Arrowheads (G,H) indicate *Hey1* expression in the pronephros. (**K-N**) Quantification of rescue experiments shown in A-J. n, total number of embryos analyzed.

and *Vent2*, direct targets of BMP signaling in animal cap cells (Fig. 7A, lanes 4 and 5). Notably, overexpression of Hey1 increased the transcription of *Gremlin* (Fig. 7A, lane 4). In addition to naïve ectodermal cells, *Tbx2* expression in the non-nephric mesenchyme around the pronephros was also significantly diminished by injection of *Hey1* (Fig. 7B; 55%, *n*=40) or *Gremlin* (Fig. 7C; 67.5%, *n*=40) mRNA. Consistent with the RT-PCR data, overexpression of *Hey1* increased the expression of *Gremlin* and vice versa (Fig. 7D,E).

Given that, as shown above, BMP signaling represses the expression of *Gremlin* and *Hey1* via Tbx2, it is possible that the mutual antagonism between BMP signaling and Gremlin or Hey1 might be crucial for proper pronephric development. In line with this hypothesis, overexpression of BMP4 interfered with the ectopic induction of pronephric markers in the RA- and activin-treated animal tissues as analyzed by RT-PCR (Fig. 7F, lane 5). Importantly, this downregulation by BMP4 was reversed by coexpression of Gremlin (Fig. 7F, lane 6). Overexpression of *Gremlin* alone had repressive effects on the induction of some of the pronephric markers, including *Pax8*, *SMP30* and *WT1* (Fig. 7F, lane 8), suggesting that BMP signaling is required to some degree for pronephric nephron development. In addition, co-injection of Tbx2 MO or *Gremlin* rescued the inhibitory effects of BMP4 on the expression of pronephric markers (Fig. 7F, lanes 6 and 7), confirming that the role of BMP signaling during pronephric nephron development is mediated by Tbx2. Of note, the expression of a lateral plate mesoderm marker, *Foxf1*, was strongly increased by BMP4, which was downregulated by Gremlin but not by Tbx2 MO (Fig. 7F, lanes 5-7). This result indicates that as a target of BMP signaling, Tbx2 controls only pronephric mesoderm formation.

DISCUSSION

In this study, we have determined the role of the Tbx2 transcriptional repressor in the formation of the pronephric nephron. Tbx2 is first expressed at the time of pronephric morphogenesis and becomes spatially limited to the non-nephric mesoderm enclosing the pronephric nephron. Given that, unlike *Tbx2*, *Tbx3*, which has functional and structural similarity with *Tbx2,* is detectable only in the ectoderm of the pronephric region (data not shown), the restricted localization of *Tbx2* suggests its unique function in defining the boundary of the pronephric nephron. In line with this assumption, overexpression of Tbx2 abrogates the expression of several pronephric mesoderm-specific markers, thereby interfering with the formation of pronephric nephron components, including the tubules, duct and glomus. Conversely, the loss of Tbx2 function expands the expression domain of these markers, except for those that are tubule specific, which leads to an enlarged pronephric duct and glomus (Fig. 4; data not shown).

In *Xenopus*, the pronephric duct develops independently of the pronephric tubule and glomus, whereas the latter two components affect each other in the anterior region of the IM (Vize et al., 1997). Knockdown of Tbx2 prevents the anterior IM from differentiating into two distinct layers – medial and somatic – as evidenced by the invasion of glomus precursor cells into the somatic layer where the pronephric tubules are induced (Fig. 3). *WT1*, which is specifically expressed in the glomus, represses the expression of the tubule-related genes (Wallingford et al., 1998). Consistently, overexpression of *WT1* expanded the glomus region but strongly impaired the pronephric tubules (see Fig. S4 in the supplementary material). Thus, it is possible that the absence of Tbx2 causes the glomus-specific cells to diffuse into the somatic layer and inhibit the formation of the pronephric tubules. Moreover, *Hey1* and

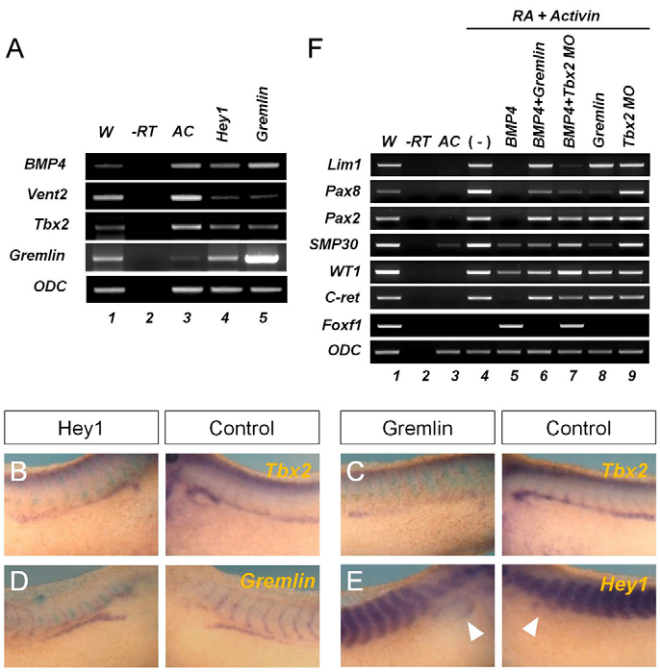

Fig. 7. Gremlin and Hey1 downregulate Tbx2 by antagonizing BMP signaling. (**A**) Animal cap tissues from *Xenopus* embryos injected with *Hey1* (100 pg) or *Gremlin* (10 pg) mRNA were subjected to RT-PCR analysis. (**B-E**) Embryos injected with *Hey1* (100 pg) or *Gremlin* (10 pg) mRNA were subject to in situ hybridization for *Tbx2*, *Gremlin* or *Hey1*. Control shows the uninjected side of the embryo. Arrowheads (E) indicate *Hey1* expression in the pronephros. (**F**) Animal caps from embryos injected with a combination of *Bmp4* (200 pg), *Gremlin* (200 pg) and Tbx2 MO (10 ng) were treated with RA and activin for in vitro kidney induction and then subjected to RT-PCR analysis. W, whole embryo; AC, uninjected animal caps without RA and activin treatment; (–), uninjected animal caps with RA and activin treatment; –RT, negative control without reverse transcriptase; *ODC*, ornithine decarboxylase loading control.

Gremlin, which are major transcriptional targets of Tbx2 in pronephric development, as shown by our results, are expressed in the glomus and duct region. Therefore, the precursor cells that form the glomus and duct are more likely to be affected by Tbx2 depletion than the tubule-specific precursor cells. Together, these results suggest that Tbx2 functions to limit the boundary of the basic components of the pronephric nephron.

BMP signals are crucial in specifying the IM and in regulating kidney development, including nephric duct differentiation and branching morphogenesis (James and Schultheiss, 2005; Miyazaki et al., 2003). We have also confirmed that BMP signaling is essential for the normal specification of the pronephric mesoderm (Figs 6 and 7). It is of interest that the BMP signals regionalize the pronephric mesoderm by inducing Tbx2 in the tissue surrounding the pronephric nephron. Thus, inhibition of BMP signaling expands the expression of pronephric markers similarly to the loss of Tbx2 function. Notably, knockdown of Tbx2 upregulates the expression of *Gremlin* and *Hey1* only among cells expressing several markers of pronephric mesoderm. Gremlin is a secreted cysteine-knot protein and functions as an antagonist of BMP ligands (Hsu et al., 1998). Hey1, which is a downstream mediator of Notch signaling, directly inhibits the transcription of *Bmp2* in the chamber region of the heart for regional specification (Rutenberg et al., 2006). Our analysis also showed that the transcription of *Bmp4* was decreased by injection of Hey1 in ectodermal cells (Fig. 7A). Hey1 also has a role in glomerular development and pronephros patterning (Taelman et al., 2006). Although Hey1 mediates Notch signaling, it does not rescue the pronephric defects caused by inhibition of Notch signaling, suggesting that it cannot fully compensate for the loss of Notch signals in the control of pronephric development.

We found that overexpression of Gremlin or Hey1 reduces *Tbx2* expression and expands the expression of pronephric duct-specific markers, reminiscent of the effects of the inhibition of BMP signaling (Fig. 7). Tbx2, which is induced by BMP signaling, is expressed in the non-nephric mesoderm around the nephron, whereas *Gremlin* and *Hey1* are restricted to the nephric mesoderm. Given their mutually exclusive expression patterns, the antagonism between BMP/Tbx2 and Gremlin or Hey1 could function to demarcate the boundary of the pronephric nephron by establishing the correct level of BMP signaling or Tbx2 activity. Tbx2 has been implicated in regional specification and boundary formation for various organs, including the hypothalamus, limb and heart (Manning et al., 2006; Sasagawa et al., 2002; Suzuki et al., 2004; Yamada et al., 2000). Interestingly, Tbx2 is also induced by BMP signals and inhibits *Hey1* or *Gremlin* expression in the formation of these organs (Rutenberg et al., 2006; Suzuki et al., 2004). In addition, BMP signaling and Hey1- or Gremlin-mediated inhibition of BMP signals are important for tubulogenesis in the lung, mammary gland and thymus (Cebra-Thomas et al., 2003; Collins et al., 2004; Rowley et al., 2004; Shi et al., 2001; Tomita et al., 1999; Yamada et al., 2000; Zavadil et al., 2004). *Tbx2* is specifically expressed in the mesenchyme, but not in the epithelium, of the branching tubule in these tubular organs (Chapman et al., 1996), although its roles remain unknown. Taken together, it is tempting to speculate that the negative regulatory loop between BMP/Tbx2 and Gremlin or Hey1 might be a general mechanism for boundary formation and tubulogenesis in diverse forms of organogenesis.

The size of organs appears to be determined by a variety of factors. In *Drosophila*, the sizes of two flight appendages – the wing and haltere – are regulated by the distribution of the long-range morphogen Decapentaplegic (Dpp), which is controlled by Hox genes (Crickmore and Mann, 2006). In addition, the sizes of pancreas and liver are limited by the number of embryonic progenitor cells (Stanger et al., 2007) and by circulating tissue-specific factors (Stanger, 2008a), respectively. However, the size determinants of the branched organs, such as the kidney, lung and trachea, remain unknown. We have shown that the gain and loss of Tbx2 function control the size of the embryonic nephron. This regulation appears to be achieved by alteration of the rate of cell proliferation, as assayed by phospho-histone H3 staining (Fig. S5A in the supplementary material). Tbx2 functions to modulate the level of BMP activity and this could indirectly affect the number of cells in the pronephric region. Alternatively, Tbx2 could regulate cell proliferation directly by repressing genes such as *NMyc1*. In support of this, Tbx2 has been shown to reduce cell proliferation by directly suppressing NMyc1 in the non-chamber region of the developing heart (Cai et al., 2005) and NMyc1 expression has been detected in the pronephric nephron of *Xenopus laevis* (Vize et al., 1997). We have also observed that *NMyc1* expression is strongly increased on the Tbx2 MO-injected side of the embryo (Fig. S5B in the supplementary material). Possibly, the increased expression of NMyc1 could result in the expanded size of the pronephric

nephron. Therefore, it appears that Tbx2 controls, directly or indirectly, the fate and proliferation of the cells in the pronephric tissue via the transcriptional inhibition of key genes.

Furthermore, depletion of Tbx2 appears to disrupt the radial arrangement of cells in the pronephric mesoderm (see Fig. S5C in the supplementary material) and the migration of the pronephric duct precursor cells along the narrow path located between the somite and lateral plate mesoderm (see Fig. S2A in the supplementary material). These phenotypes suggest the possible involvement of Tbx2 in these morphogenetic processes during pronephros formation. Wnt signaling is essential for tubulogenesis of the *Xenopus* pronephros and murine metanephros (Saulnier et al., 2002; Stark et al., 1994). In addition, Wnt/β-catenin signaling is required for *Xenopus* pronephric tubule, duct and glomus development (Lyons et al., 2009). Notably, several lines of evidence show that Tbx2 function is connected with Wnt signaling during embryogenesis (Rowley et al., 2004). In zebrafish, *tbx2b* is regulated by the Wnt signaling molecule Fz7 and mediates cell movement (Fong et al., 2005). Given that *Xenopus Fz7* is also expressed in the pronephros, it is possible that Tbx2 regulates, downstream of Wnt signaling, the pronephric cell movement and rearrangement.

The current study suggests a functional role of Tbx2 in defining the boundary of the pronephric nephron. Tbx2 is induced by BMP signals in the non-nephric mesoderm and inhibits the transcription of the nephric mesoderm-specific markers *Gremlin* and *Hey1*. These nephric mesodermal molecules antagonize BMP signaling, thereby restricting the activity of the BMP signals and Tbx2. Further studies are warranted to investigate whether this type of regulatory loop exemplified by BMP/Tbx2 and Gremlin/Hey1 functions during tubulogenesis and boundary formation in other branched organs. In addition, it will be necessary to examine whether Tbx2 is involved in the morphogenetic processes in nephrogenesis and how this role could contribute to the determination of nephron size. An enlarged nephron is one of the major characteristics of oligomeganephronia (OMN), the most common form of human congenital renal hypoplasia (Suzuki et al., 2005). Some genetic factors, including *PAX2* and *HNF1B*, have been identified in human OMN (Sagen et al., 2003; Salomon et al., 2001), and enlarged glomeruli and tubular structures have been associated with *HNF1B* mutation. It might prove clinically significant to investigate whether Tbx2 is also a factor in some congenital kidney diseases.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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