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# Cv2, functioning as a pro-BMP factor via twisted gastrulation, is required for early development of nephron precursors

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ABSTRACT

The fine-tuning of BMP signals is critical for many aspects of complex organogenesis. In this report, we show that the augmentation of BMP signaling by a BMP-binding secreted factor, Crossveinless2 (Cv2), is essential for the early embryonic development of mammalian nephrons. In the *Cv2*-null mouse, the number of cap condensates (clusters of nephron progenitors, which normally express Cv2) was decreased, and the condensate cells exhibited a reduced level of aggregation. In these $Cv2^{-/-}$ condensates, the level of phosphorylated Smad1 (pSmad1) was substantially lowered. The loss of a *Bmp7* allele in the $Cv2^{-/-}$ mouse enhanced the cap condensate defects and further decreased the level of pSmad1 in this tissue. These observations indicated that Cv2 has a pro-BMP function in early nephrogenesis. Interestingly, the renal defects of the $Cv2^{-/-}$ mutant were totally suppressed by a null mutation of *Twisted gastrulation* (*Tsg*), which encodes another BMP-binding factor, showing that Cv2 exerts its pro-BMP nephrogenic function Tsg-dependently. By using an embryonic kidney cell line, we presented experimental evidence showing that Cv2 enhances pro-BMP activity of Tsg. These findings revealed the molecular hierarchy between extracellular modifiers that orchestrate local BMP signal peaks in the organogenetic microenvironment.



## Introduction

In terrestrial amniotes, the kidney is an indispensable and complex organ that maintains fluid homeostasis and blood pressure. Its anlage is a tissue called the metanephros. In the mouse, metanephric development starts about embryonic day (E) 10.5 with the demarcation of the metanephric blastema in the caudal part of the intermediate mesoderm, followed by reciprocal inductions between the blastema and the Wolffian duct (Vainio and Lin, 2002). In response to metanephric blastema-derived signals, the ureteric bud forms from the Wolffian duct, invades the metanephric blastema, and successively branches to form the collecting ducts. Conversely, ureteric bud-derived signals induce the formation of metanephric blastema-derived condensates (cap condensates) around the tips of collecting ducts; the cells forming the condensates will ultimately give rise to the nephrons, which carry out the functions of the adult kidney (Kobayashi et al., 2008). Although the signaling networks that regulate duct branching have been extensively studied (Shah et al., 2004), relatively little is known about the molecular mechanism of how cap condensates are formed and maintained (Kobayashi et al., 2008; Oxburgh et al., 2004).

The Bone Morphogenetic Protein (BMP) family is a class of secreted signaling proteins that belong to the transforming growth factor-beta (TGFβ) superfamily; they have diverse effects on the control of embryogenesis, including kidney development (Cain et al., 2008; Godin et al., 1998; Hogan, 1996; Simic and Vukicevic, 2005). An intriguing feature of BMP signaling is the presence of various extracellular BMP inhibitors (i.e., anti-BMP factors), such as Chordin, Noggin, Follistatin, Cerberus, and Gremlin (Glinka et al., 1997; Hemmati-Brivanlou et al., 1994; Hsu et al., 1998; Lamb et al., 1993; Sasai et al., 1995, 1994; Smith and Harland, 1992). Moreover, recent studies show that there are some proteins that bind to BMPs extracellularly and augment their signaling (i.e., pro-BMP factors). One protein with pro-BMP functions is Crossveinless2 (Cv2; also called Bmper), which enhances BMP signaling during wing cross-vein formation in *Drosophila*, as well as in neural crest emigration in the chick embryo, dorsal-ventral patterning of the zebrafish gastrula, and some cultured cell lines (Coles et al., 2004; Conley et al., 2000; Kamimura et al., 2004; Kelley et al., 2009; Moser et al., 2007; Rentzsch et al., 2006; Serpe et al., 2008).

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Interestingly, other reports have shown Cv2 to have an anti-BMP role in various in vitro and in vivo contexts (Ambrosio et al., 2008; Binnerts et al., 2004; Coles et al., 2004; Harada et al., 2008; Kelley et al., 2009; Moser et al., 2003; Rentzsch et al., 2006; Zhang et al., 2007, 2008). For instance, Cv2 exerts both pro- and anti-BMP activities when injected into the zebrafish embryo (Rentzsch et al., 2006). In the *Xenopus* gastrula, Cv2 acts predominantly as an anti-BMP factor (Ambrosio et al., 2008). While endocytic internalization is proposed to contribute to Cv2's anti-BMP activity, this machinery appears to be restricted to a limited range of cell types (Kelley et al., 2009). Biochemical and crystal structure analyses suggest that vertebrate Cv2 may interfere with BMP ligand-receptor binding via its Chordin-type cysteine-rich domain (CR1; Zhang et al., 2007; Zhang et al., 2008), although the in vivo relevance of such molecular interactions remains elusive. More recently, Cv2 was shown to bind other proteins, such as Chordin (Ambrosio et al., 2008), suggesting that Cv2's function is complex, and its role as a pro- or anti-BMP factor may be context-dependent. Therefore, careful investigation is required to identify the in vivo role of Cv2 in different developmental contexts.

Previously, by showing a genetic enhancement between *Bmp4* and *Cv2*, we demonstrated that Cv2 functions as a pro-BMP factor in vertebral and eye development (Ikeya et al., 2006). In the same study, we found kidney defects (hypoplasia) in *Cv2*-null mouse embryos. Our previous report showed that Cv2 acts in the same direction with Kcp, which functions as a pro-BMP factor in a different context (Ikeya et al., 2006; Lin et al., 2005). However, our previous study could not tell whether Cv2 in the developing kidney was required as a pro- or anti-Bmp factor, because of the lack of genetic evidences showing functional interaction between Cv2 and Bmp ligands.

Here, we report that Cv2 plays an essential role as a pro-BMP factor in mouse kidney development. We found that Cv2 promotes the BMP-dependent formation of the cap condensates, and we present genetic evidence that the pro-BMP function of Cv2 is dependent on the presence of Tsg, another BMP modulator. These results demonstrate that an extracellular system for modulating local BMP signals via Cv2 and Tsg plays a key role in the early steps of mouse nephron development.

## Materials and methods

### *Mutant mice and crosses*

Mice carrying mutations in *Cv2*, *Bmp7*, *Tsg*, and *Smad1* were described previously (Hayashi et al., 2002; Ikeya et al., 2006; Luo et al., 1995; Nosaka et al., 2003). We crossed $Cv2^{+/-}$ mice with $Bmp7^{+/-}$, $Tsg^{-/-}$ or $Smad1^{+/-}$ mice to obtain compound heterozygotes. No obvious defects were observed in the compound heterozygotes, and we used them for further intercrosses. Genotypes were confirmed by PCR (Ikeya et al., 2006, 2008). Animals were housed in environmentally controlled rooms in accordance with RIKEN guidelines for animal experiments.

### *LacZ staining, histology, immunohistochemistry, and statistics*

LacZ staining, histology, and immunohistochemistry were performed as described previously (Ikeya et al., 2006). Primary antibodies and dilutions were as follows: anti-alpha-catenin, 1:500 (Sigma, rabbit polyclonal); anti-BF2, 1:500 (Abcam, goat polyclonal); anti-cadherin-11, 1:500 (R&D, goat polyclonal); anti-Cv2, 1:1000 (R&D, goat polyclonal); anti-E-cadherin, 1:500 (Takara, ECCD2); anti-β-galactosidase, 1:5000 (Cappel, rabbit polyclonal) or 1:2000 (AbD Serotec, goat polyclonal); anti-laminin, 1:500 (Chemicon, AL-4); anti-NCAM, 1:1000 (Chemicon, rabbit polyclonal); anti-Pax2, 1:200 (Zymed, rabbit polyclonal); anti-phospho-Smad1/5/8, 1:30 (Cell Signaling, rabbit polyclonal); and anti-WT1, 1:50 (Santa Cruz, rabbit polyclonal). For staining with the anti-WT1 antibody, citrate buffer, pH 6, was used for antigen retrieval (Zymed) in a 2100 Retriever (PickCell Laboratories). The anti-Cv2 polyclonal antibody recognizes both N- and C-halves of cleaved Cv2 (data not shown).

The signal intensity of the phospho-Smad1/5/8 staining was compared as follows, using the ImageJ software (National Institutes of Health, Bethesda, MD). We stained sections with phospho-Smad1/5/8-specific antibody and DAPI and acquired images by scanning the sections with a confocal microscope (LSM510 (Zeiss)). The images were trimmed into smaller ones showing either cap condensates or collecting ducts, and we divided the total signal intensity of phospho-Smad1/5/8 in the trimmed regions by DAPI-positive area. We defined this value as the "average signal intensity" of phospho-Smad1/5/8 and compared it across images. No differences were observed among the average signal intensities of the collecting ducts, regardless of the genotype. We regarded average signal intensities from cap condensates that were less than two thirds of the collecting duct average intensity as "reduced."

The numbers of nephrons and nephron progenitors at E18.5 were counted as described previously (Ikeya et al., 2006). We scored eight to 20 embryos of each genotype to obtain these numbers. The number of LacZ-positive cap condensates at E13.5, 14.5, and 15.5 was obtained from six to 30 kidneys from each genotype.

Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software).

### *Cell culture, transfection, siRNA, and luciferase assay*

HEK293T cells were maintained in DMEM/10% FCS (HyClone). To examine the effect of Cv2 on BMP signaling, reverse transfections were performed in a 24-well cell culture plate (BD Falcon) using FuGene6 (Roche, Basel, Switzerland) with a total of 210 ng DNA [ 100 ng of BRE-luc (Korchynskyi and ten Dijke, 2002), 10 ng of phRL-null (Promega), and the indicated dose of CIG-mCv2 and CIG-LacZ (Megason and McMahon, 2002)] per well, in DMEM/1% FCS. Annealed and purified siRNA duplexes were obtained from Ambion (Austin, TX) and were added at 75 ng per well 4 h prior to the cDNA transfection with X-treme Gene (Roche, Basel, Switzerland). After 16 h of treatment, the cells were lysed and assayed for luciferase activity using the dual luciferase reporter assay system (Promega, Madison, WI), according to the manufacturer's instructions.

## Results

### *Enhancement of hypoplastic phenotypes in the $Cv2^{-/-}$ kidney by $Bmp7^{+/-}$ mutation*

We previously demonstrated that *Cv2*-null mice display a reduced kidney size and lower nephron number than wild-type mice (Ikeya et al., 2006). To examine the role of Cv2 during nephrogenesis, we first analyzed *Cv2*'s expression patterns during kidney development in *nLacZ*-knock-in mice ($Cv2^{+/nLacZ}$). At E11.5, the metanephric blastema expressed $Cv2^{nLacZ}$, whereas the ureteric buds were negative for it (Figs. 1A–C). At E12.0, the maturating stromal cells located in the central portion of the kidney became $Cv2^{nLacZ}$-negative (asterisk in Fig. 1D). At E13.5 and E14.5, the $Cv2^{nLacZ}$ expression was mainly restricted to the cap condensates (Figs. 1E–H, and data not shown) and to portions of the forming nephrons (pretubular aggregates, comma-shaped body, and S-shaped body) and Bowman's capsules (Supplementary Figs. S1A–D). Immunohistochemical analyses confirmed that cap condensate cells positive for Pax2 strongly expressed $Cv2^{nLacZ}$, but the peripheral stroma, which was positive for BF2, and collecting ducts did not (Supplementary Figs. S2A–C). From E14 until birth, the $Cv2^{nLacZ}$ expression was restricted to the cap condensates and its derivatives (data not shown).

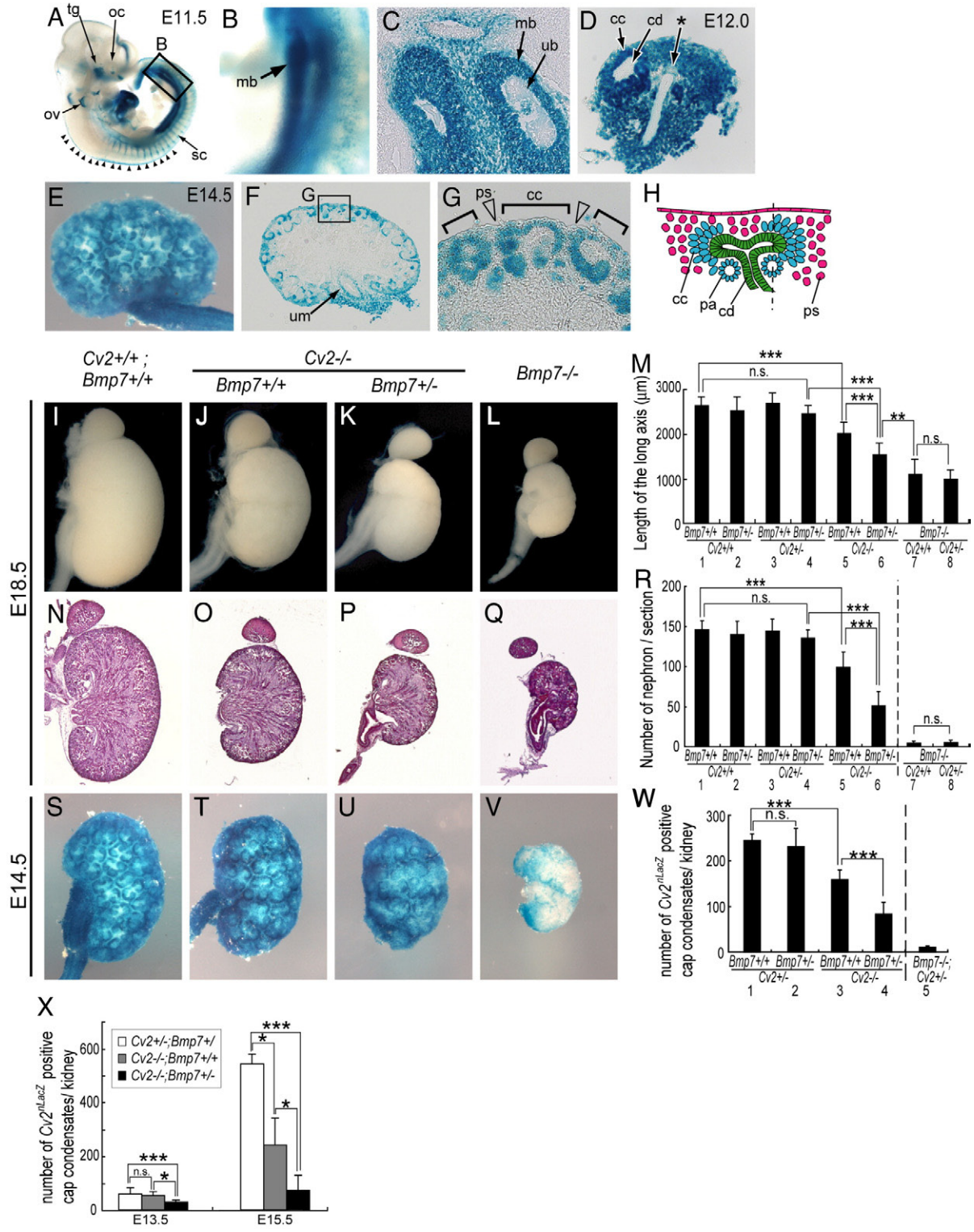


**Fig. 1.** Cooperative roles of *Cv2* and *Bmp7* in kidney development. (A–G) Expression of *Cv2* analyzed with *nLacZ* knock-in mice. (A–C) At E11.5, LacZ staining was observed in the trigeminal ganglion (tg), optic cup (oc), otic vesicle (ov), sclerotome (sc), roof plate of the neural tube (triangles), and metanephric blastema (mb), but not in the ureteric bud (ub). (D) At E12.0, the central portion of the metanephric mesenchyme became $Cv2^{nLacZ}$ negative (asterisk). cd, collecting duct; cc, cap condensates. (E–G) At E14.5, $Cv2^{nLacZ}$ was preferentially expressed in the cap condensates (bracket in G) and the ureteric mesenchyme (um). Triangles in (G) indicate that the peripheral stroma (ps) was $Cv2^{nLacZ}$-negative. (H) Schematic representation of the cortical region of the embryonic kidney. pa, pretubular aggregate. (I–L) External appearances of the control, $Cv2^{-/-}$, $Bmp7^{+/-}$;$Cv2^{-/-}$, and $Bmp7^{-/-}$ kidneys at E18.5. (M) Length of the long axis. (N–Q) Longitudinal sections stained with hematoxylin and eosin at E18.5. (R) The number of nephrons in the maximal longitudinal sections. (S–V) External views at E14.5 stained with $Cv2^{nLacZ}$. (W, X) The number of $Cv2^{nLacZ}$-positive cap condensates at E14.5 (W, Tukey test), and E13.5 and E15.5 (X, Bonferroni test). Error bars show S.D.; n.s., no significant difference; ***$P<0.001$; **$P<0.01$; *$P<0.05$. In (R) and (W), statistical analyses of the $Bmp7^{-/-}$ samples were performed separately because of significant differences among the S.D.s.

Although these expression patterns, as well as the *Cv2*-null phenotypes, suggest a crucial role for Cv2 in nephrogenesis, the molecular mechanism of Cv2's action, and particularly whether it functioned as a pro-BMP or anti-BMP factor in this developmental context, was unclear. Among the *Bmp* genes, *Bmp7* is strongly expressed in the cap condensate, as well as in the ureteric bud, collecting duct, and forming nephron (Supplementary Fig. S3A; Dudley and Robertson, 1997; Godin et al., 1998), and its mutation causes progressive renal hypoplasia (Dudley et al., 1995; Luo et al., 1995). In addition, Cv2 binds BMP7 with a high affinity (Zhang et al., 2007). These findings suggested that Cv2 might interact with BMP7 in kidney development. We therefore tested their functional interaction by crossing *Cv2* mutants with *Bmp7* mutants (Figs. 1I–X).

At E18.5, the loss of one allele of *Bmp7* in the $Cv2^{+/+}$ or $Cv2^{+/-}$ background had no obvious effects on kidney development (Figs. 1M, R, lanes 1–4). Similarly, the loss of one *Cv2* allele in the $Bmp7^{-/-}$ background had little effect on kidney size or nephron number (Figs. 1M, R, lanes 7, 8). In contrast, the deletion of a single *Bmp7* allele in the $Cv2^{-/-}$ background caused further reductions in the kidney size and nephron number at E18.5 than seen in $Cv2^{-/-}$ mice at the same age (Figs. 1J, K, O, P; Figs. 1M, R, lanes 5, 6).

A similar genetically enhanced reduction in the number of cap condensates (marked by the expression of $Cv2^{nLacZ}$) was evident during the early stages of kidney development, even at E14.5 and E15.5 (Figs. 1S–X). In addition, the enhanced decrease in the components of the forming nephrons (comma- and S-shaped bodies) was obvious in the $Bmp7^{+/-}$;$Cv2^{-/-}$ kidney, particularly at E15.5 (Fig. 1X and Supplementary Fig. S4).

These observations support the idea that Cv2 plays a pro-BMP role in the early phases of nephron formation.

*Cv2 is essential for high levels of BMP signaling in cap condensates*

These findings prompted us to study the expression and functions of Cv2 proteins in the early embryonic kidney. To this end, we performed immunohistochemical analysis of Cv2 protein in the developing kidney (Fig. 2). At E12.5 and E14.5, the Cv2 protein had accumulated in two regions: the pericellular region of the $Cv2^{nLacZ}$-expressing cap condensates (punctate signals; arrows in Figs. 2G–I) and the basement membrane of the collecting ducts (continuous signals; arrowheads in Figs. 2B, E). No immunostaining was observed in the $Cv2^{-/-}$ kidney, demonstrating the specificity of the antibody (Fig. 2F). Both the pericellular and the basement membrane signals co-localized with fibronectin (Figs. 2G–I) and laminin (data not shown), suggesting that Cv2 protein is densely accumulated in the extracellular matrix. This finding is in accordance with the previous work demonstrating Cv2's co-localization with extracellular matrix (Rentzsch et al., 2006; Serpe et al., 2008).

To identify the site of action of Cv2 in renal development, we next analyzed the levels of intracellular BMP signaling in wild-type and mutant mice by detecting a downstream component of BMP signaling, phosphorylated Smad1/5/8 (pSmad1), which is frequently used to assay BMP activity. At E14.5, high pSmad1 signals were observed in both the cap condensates (cc) and the tips of the collecting ducts (cd) in wild-type and $Bmp7^{+/-}$ embryos (Fig. 3A and Supplementary Fig. S5). In contrast, in $Cv2^{-/-}$, $Bmp7^{+/-}$;$Cv2^{-/-}$, and $Bmp7^{-/-}$ embryos, pSmad1 staining was reduced in the cap condensates, whereas no substantial change was observed in the collecting ducts (Figs. 3B–D). Fig. 3I shows the percentages of cap condensates with reduced pSmad1 signals (in this analysis, signal levels comparable to those in the collecting duct cells were considered to be high). As shown

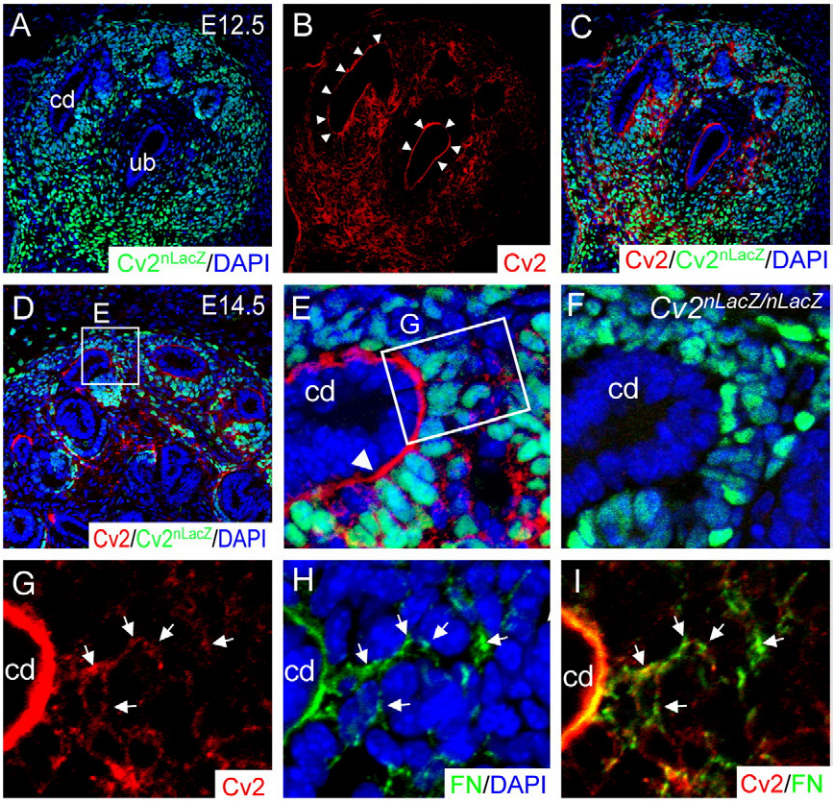


**Fig. 2.** Distribution of the Cv2 protein. Cv2 protein was accumulated in both the pericellular region of the cap condensate and the basement membrane of the collecting duct. (A–C) Immunohistochemistry with anti-Cv2 antibody at E12.5. Arrowheads, accumulation of Cv2 on the surface of the collecting duct and ureteric bud. (D–I) At E14.5, anti-Cv2 staining was observed in the pericellular region of the $Cv2^{nLacZ}$-positive cells (arrows in G–I) and the basement membrane of the collecting duct (arrowhead in E). (F) No signal was detected in the $Cv2^{-/-}$ mutants. Punctate pericellular signals co-localized with fibronectin (FN; arrows in G–I).

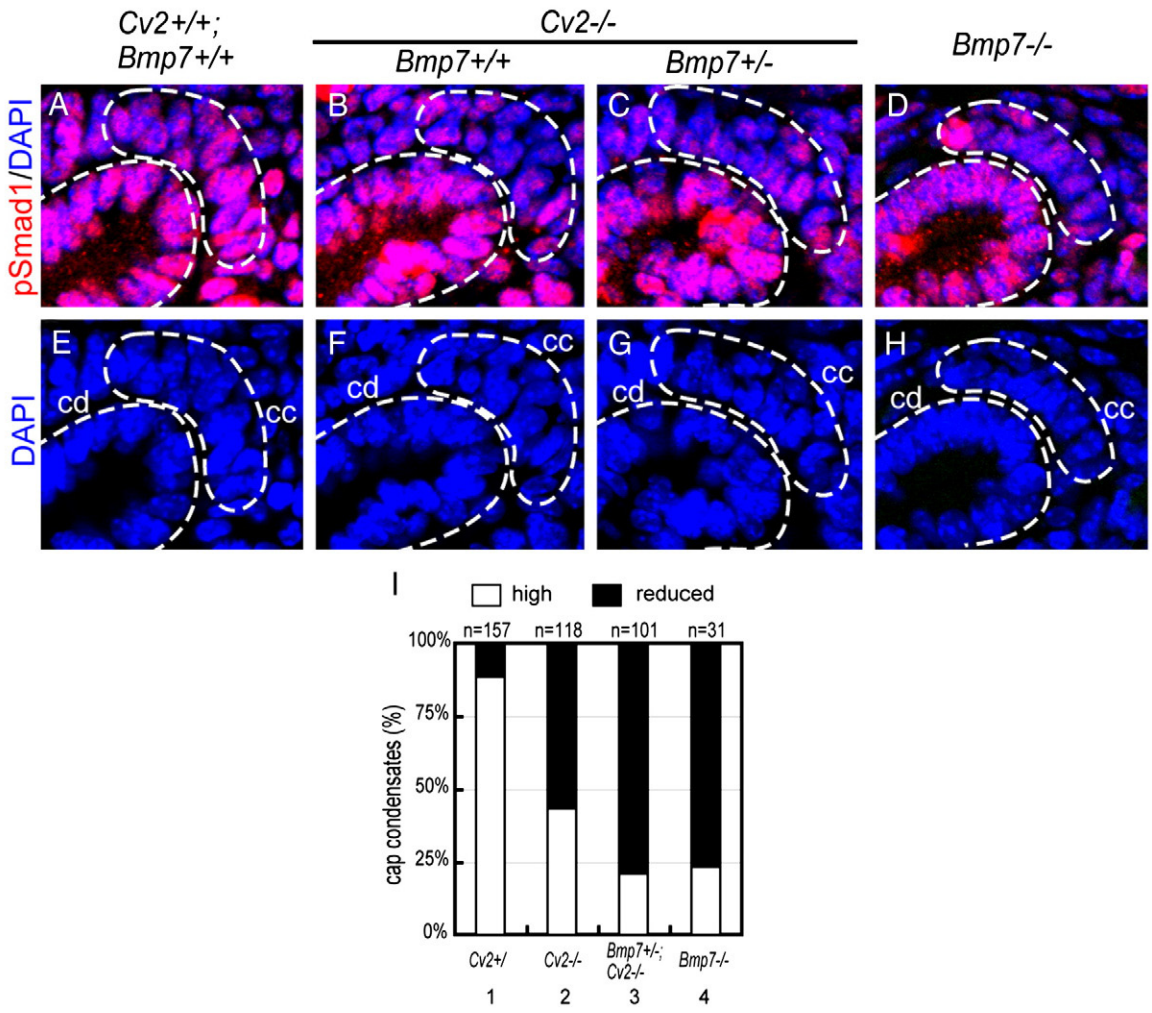


**Fig. 3.** A pro-BMP role of *Cv2* in the cap condensates but not in the collecting ducts. Staining intensity of pSmad1 was decreased in cap condensates of $Cv2^{-/-}$, $Bmp7^{+/-};Cv2^{-/-}$, and $Bmp7^{-/-}$ kidneys, but it was unchanged in the collecting ducts from all genotypes. Blue, DAPI; red, phospho-Smad1/5/8 (pSmad1)-specific antibody. (I) Percentages of cap condensates showing reduced pSmad1 signal intensity. Under the $Cv2^{-/-}$ background, the $Bmp7^{+/+}$ and $Bmp7^{+/-}$ groups exhibited a significant differences ($P$ value = 0.0007) by Chi-square test.

(Fig. 3I, lanes 2 and 3), the additional deletion of one *Bmp7* allele enhanced the reduction of pSmad1 in the $Cv2^{-/-}$ background, suggesting that Cv2 acts in the same direction as BMP7 (i.e., as a pro-BMP factor).

These observations indicate that Cv2 is essential for enhancing BMP signals in the cap condensate during kidney organogenesis, but that the high BMP signals in the collecting ducts are independent of Cv2, suggesting a tissue-specific mode of Cv2's action.

### *Incomplete cellular aggregation in the $Cv2^{-/-}$ cap condensates*

During nephrogenesis, cap condensates are formed as compact cell aggregates at the tips of the collecting ducts (cc in Fig. 4A). As shown above, BMP signaling was attenuated in the cap condensates of $Cv2^{-/-}$ and $Bmp7^{+/-};Cv2^{-/-}$ mutants. In addition, thin section analysis revealed impaired cell–cell attachment in the $Cv2^{-/-}$ and $Bmp7^{+/-};Cv2^{-/-}$ cap condensates (see loosely packed aggregates in Figs. 4B, C; E14.5), although the cells looked healthy and did not exhibit signs of apoptosis such as picnosis. These observations indicate that the loss of Cv2's pro-BMP function not only reduced the cap condensate number but also caused abnormal cellular aggregation.

Next, we further examined the cellular aggregation by immunostaining. While adhesion molecules such as NCAM and cadherin11 (also alpha-catenin) were distributed uniformly in the cell–cell interface regions in the cap condensate of control embryos (Figs. 4E, I, M), these protein appeared discontinuous or punctate in the $Cv2^{-/-}$ and $Bmp7^{+/-};Cv2^{-/-}$ mutants (arrowheads in Figs. 4F, G, J, K, N, O). These changes did not seem to be caused by a loss of specific cell types, since the cell type-specific markers (Pax2 and p75 for the cap condensate and BF2 for the peripheral stroma) were expressed normally in these mice (Figs. 4Q–S, and data not shown). Rather, these results indicate that a local augmentation of BMP signaling by Cv2 is crucial for the proper formation of cap condensates, including the cap–cell aggregation, during kidney development.

To focus in on which developmental step was most dependent on Cv2, we investigated the marker expression and aggregation of the cap cells in the $Bmp7^{-/-}$ kidney. We found, as reported previously, that the number of $Pax2^{+}$ cells in the $Bmp7^{-/-}$ cap condensates was severely reduced (Fig. 4T; Dudley et al., 1995; Luo et al., 1995). The adhesion molecules were found in a discontinuous pattern (Figs. 4H, L, P), resembling those in the *Cv2* mutants. In addition, the morphology of $Bmp7^{-/-}$ cap condensates were substantially impaired (even more drastically than $Cv2^{-/-}$ and $Bmp7^{+/-};Cv2^{-/-}$ condensates), consisting of cells with a generally round shape (Fig. 4D). These results imply that the complete loss of BMP7 signaling affected the cap condensate in terms of both the cellular presence (Pax2 expression; as reported previously (Dudley et al., 1995; Dudley and Robertson, 1997; Luo et al., 1995)) and the formation of the aggregates. In contrast, the

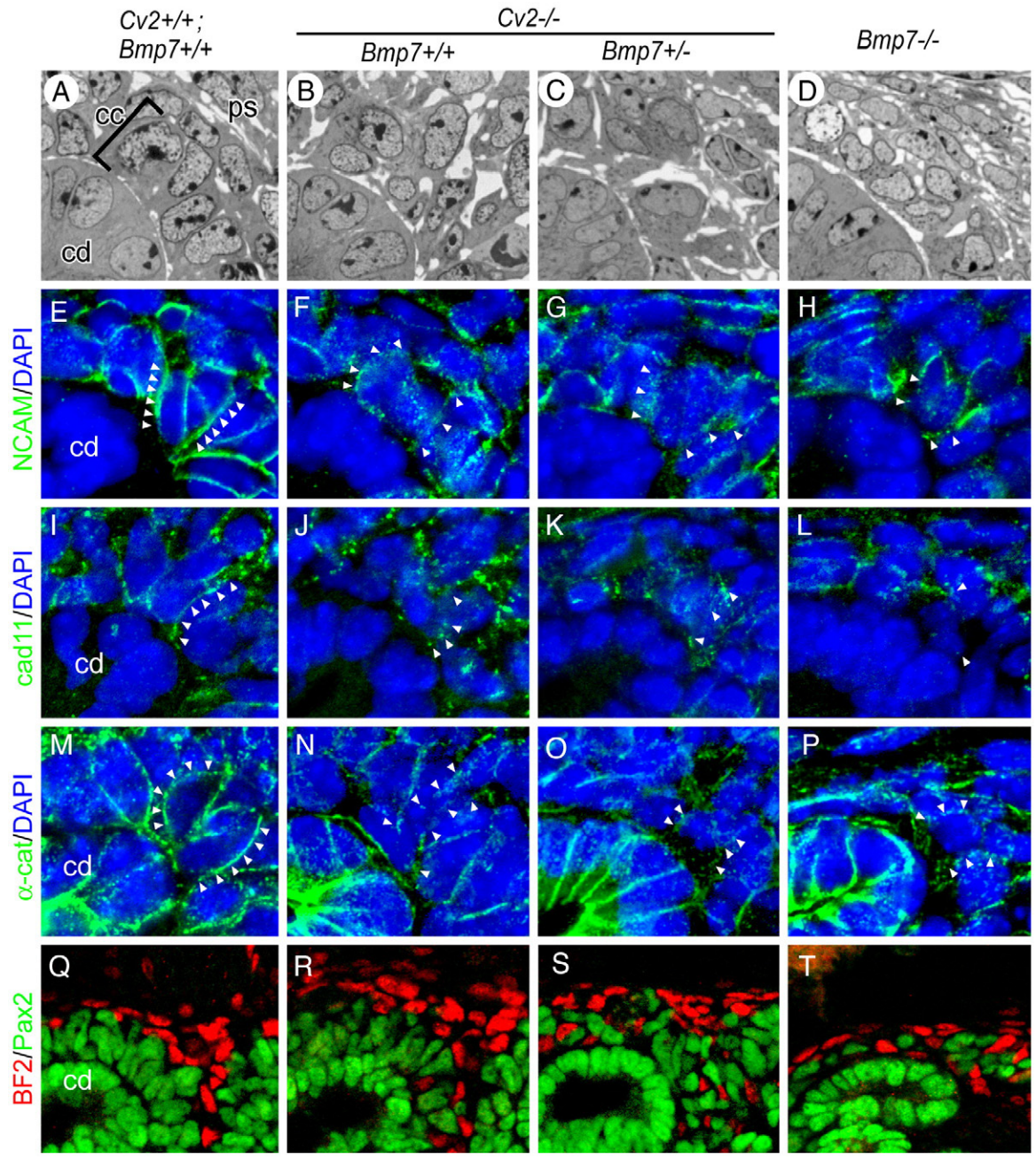


**Fig. 4.** Cv2 required for the cellular aggregation of the cap condensates. (A–D) Plastic thin sections stained with toluidine blue. In *Cv2*$^{-/-}$ and *Bmp7*$^{+/-}$;*Cv2*$^{-/-}$ kidneys, cap condensate cells adhered loosely to one another. In the *Bmp7*$^{-/-}$ kidney, the cells on the tip of the collecting duct became round. (E–P) Disturbed distribution of adhesion proteins in the cap condensates of the mutants. Immunohistochemistry with (E–H) anti-NCAM, (I–L) anti-cadherin11, and (M–P) anti-alpha-catenin. (Q–T) Immunohistochemistry with anti-Pax2 (green) and anti-BF2 (red). The Pax2-positive cap condensates and collecting ducts and the BF2-positive peripheral stroma developed normally in the *Cv2*$^{-/-}$ and *Bmp7*$^{+/-}$;*Cv2*$^{-/-}$ mutants. In the *Bmp7*$^{-/-}$ kidney, the number of Pax2-positive cells was markedly reduced.

attenuation of BMP signaling caused by the loss of Cv2 preferentially impaired the formation of the cellular aggregates.

### *Tsg mutation is epistatic to the Cv2 mutation in the kidney-defect phenotype*

Taken together, our genetic and histochemical analyses demonstrated that Cv2 is an essential pro-BMP factor for the development of cap condensates in the early embryonic kidney. An obvious remaining question is how Cv2 promotes BMP signaling in these cells.

Very recently, two independent studies (from De Robertis' and our groups) reported a genetic interaction between *Cv2* and *Tsg* in skeletal development (Ikeya et al., 2008; Zakin et al., 2008). The major skeletal defects of the *Cv2*-null mutant mice (in the thoracic and lumbar vertebrae) are suppressed in *Tsg*$^{-/-}$;*Cv2*$^{-/-}$ embryos, which show moderate skeletal phenotypes (Nosaka et al., 2003; Petryk et al., 2004; Zakin and De Robertis, 2004). This suggests that the *Tsg* mutation is epistatic to the *Cv2* mutation in skeletal development. However, the precise molecular and cellular mechanism of this gene interaction in embryonic development was still unknown.

With this in mind, we examined possible interactions between *Cv2* and *Tsg* in kidney development (*Tsg* expression is detected diffusely in the developing kidney (Supplementary Fig. S3B; Nosaka et al., 2003)). In nephrogenesis, as opposed to skeletal development, interactions with the *Tsg* mutation can be analyzed rather simply, because the *Tsg* mutation itself does not cause significant embryonic kidney

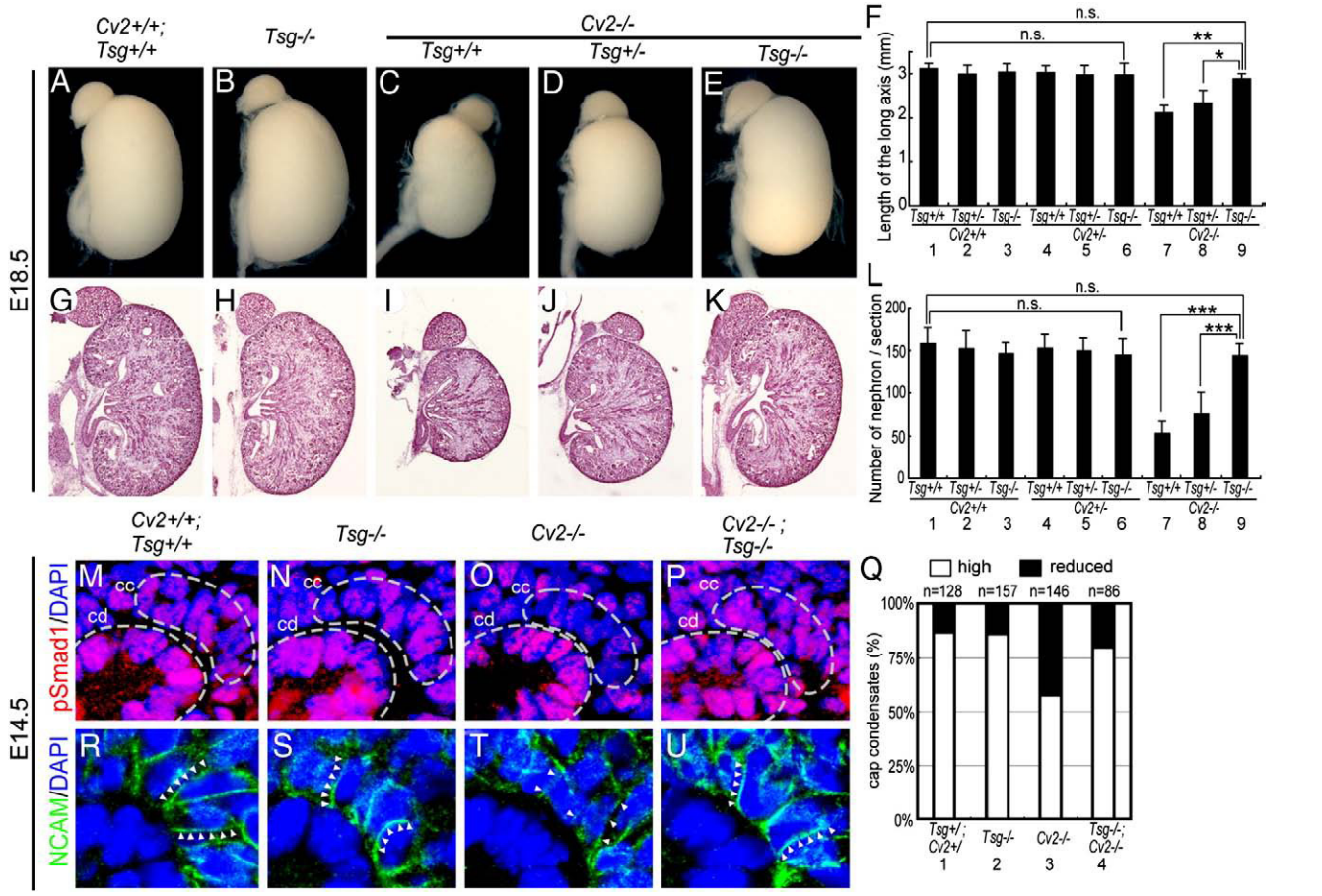


**Fig. 5.** Evidence for a *Tsg*-dependent pro-BMP function of *Cv2* in vivo. Renal phenotypes of $Cv2^{-/-}$ rescued by the deletion of both alleles of *Tsg* at E18.5 and E14.5. (A–E) External views at E18.5. (F) Length of the long axis. (G–K) Histological sections at E18.5. (L) The number of nephrons in the maximal longitudinal sections. (M–P) Immunohistochemistry with anti-pSmad1 at E14.5. (Q) Percentages of cap condensates exhibiting reduced pSmad1 signal intensity. (R–U) Immunohistochemistry with anti-NCAM at E14.5. Error bars show S.D.; n.s., no significant difference; $***P<0.001$; $**P<0.01$; $*P<0.05$ (Bonferroni test).

phenotypes (Nosaka et al., 2003). In the $Cv2^{+/+}$ and $Cv2^{+/-}$ backgrounds, the loss of both *Tsg* alleles did not significantly affect kidney size or nephron number at E18.5 (Figs. 5A, B, G, H; Figs. 5F, L, lanes 1–6). In contrast, when combined with the $Cv2^{-/-}$ mutation, the elimination of *Tsg* completely suppressed the renal defects otherwise present in *Cv2*-null mutants (Figs. 5C–E, I–K; Figs. 5F, L, lanes 1, 7–9). The *Tsg* deletion also restored the levels of BMP signaling (reduced pSmad1 levels) in the cap condensates of *Cv2*-null mutants at E14.5 (Figs. 5M–Q) and rescued the impaired cell–cell adhesion in the condensates (Figs. 5R–U).

These in vivo findings suggest a unidirectional dependence in which the pro-BMP function of Tsg requires Cv2, while Cv2 function is not dependent on Tsg activity.

### *Tsg-dependent mechanism of Cv2's pro-BMP function in cultured embryonic kidney cells*

The suppression of the $Cv2^{-/-}$ kidney phenotypes by the *Tsg* mutation implied that, as a whole, Cv2 and Tsg act in opposite directions. That is, they appeared to play pro-BMP and anti-BMP roles, respectively, during nephrogenesis. Interestingly, previous studies suggested that Tsg can act as an anti-BMP or pro-BMP factor, depending on the context (Chang et al., 2001; Harland, 2001; Larrain et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001). For instance, genetic interaction studies of mouse *Tsg*-null mutations with *Bmp4* and *Bmp7* mutations show *Tsg* acting as a pro-BMP factor in head development and posterior mesodermal patterning (Zakin and De Robertis, 2004; Zakin et al., 2005). In *Xenopus* embryos, the Chordin–Tsg complex binds to BMPs and inhibits their signaling more efficiently than Chordin, an anti-BMP factor, does alone (anti-BMP activity of Tsg; Oelgeschlager et al., 2000; Scott et al., 2001). In the presence of the Chordin-degrading enzyme Xolloid, on the other hand, Tsg dislodges BMP from cleaved Chordin fragments, resulting in enhanced BMP signaling (a pro-BMP activity; Oelgeschlager et al., 2000).

Given the context-dependent bidirectional functions of Tsg and our findings in this study, the following hypothetical models could explain the functional interaction between Cv2 and Tsg in nephrogenesis. (1) The pro-BMP factor Cv2 and the anti-BMP factor Tsg act independently on BMP signals as simple antagonists (Fig. 6A, Model a). (2) Cv2 acts as a pro-BMP factor by interfering with the anti-BMP function of Tsg (Fig. 6A, Model b). (3) Tsg has simultaneous dual functions as both a pro-BMP and an anti-BMP factor, while Cv2 acts as a co-factor to strengthen the pro-BMP aspect of Tsg's functions (Fig. 6A, Model c).

Given that the fine-tuning of BMP signals is vital for kidney organogenesis (Cain et al., 2008), the phenotypical discrepancy between the normal kidney development of $Tsg^{-/-}$ embryos and the marked hypoplasia of *Cv2*-null embryos is rather difficult to explain with hypothetical Models a and b, which predict hyperactive BMP levels in $Tsg^{-/-}$ kidneys. In contrast, the BMP activity level under the $Tsg^{-/-}$ condition would be less affected in the hypothetical Model c, in which the simultaneous loss of both the anti- and pro-BMP functions could reduce the extent of change in signaling strength (Supplementary Fig. S6B). Consistent with this idea, we have not seen substantial changes in pSmad1 levels in $Tsg^{-/-}$ embryonic kidneys compared with control kidneys (Fig. 5N and our preliminary

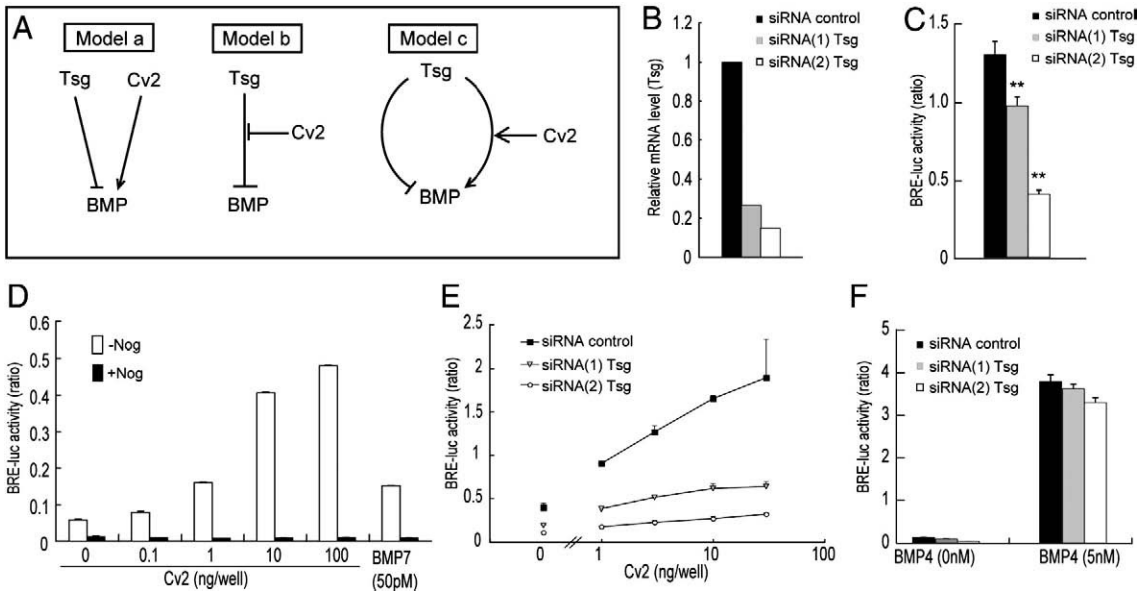


**Fig. 6.** Cv2 enhances pro-BMP activity of Tsg in HEK293T cell. (A) Models of the interaction between Tsg and Cv2. (B) Knockdown efficiency of Tsg mRNA. Expression levels were determined by quantitative real-time PCR analysis. (C) siRNA targeting of *Tsg* attenuates BMP signaling. Error bars show S.D.; $^{**}P<0.01$ (Dunnet test). (D) Dose-dependent activation of BMP signaling by *Cv2* in HEK293T cells. (E) siRNA targeting of *Tsg* reduces dose-dependent activation of BMP signaling by *Cv2*. (F) *Tsg* knockdown cells respond normally to treatment with high levels BMP4.

observations). Thus, Model c is consistent with the in vivo phenotype, at least in this respect.

To test the predictive ability of this model, one essential question is whether Cv2 can in fact function as a co-factor for the pro-BMP activity of Tsg in kidney development. This question is particularly relevant in light of a *Xenopus* study that suggests that Cv2 enhances the anti-BMP activity of Tsg in a different developmental context (dorsal-ventral patterning during gastrulation) (Ambrosio et al., 2008). Therefore, we next examined whether Cv2 promotes BMP signaling under the condition in which Tsg predominantly exerts a pro-BMP activity over an anti-BMP function.

In a series of preliminary experiments, we found that a human embryonic kidney-derived cell line, HEK293T, expresses *Tsg* as well as *Cv2* and *BMPs* (Supplementary Fig. S7), and that endogenous Tsg acted predominantly as a pro-BMP factor, since, when Tsg was knocked down by siRNAs (see Fig. 6B for the knockdown efficiency), the BMP signaling reporter (*BRE-luc*) activity was reduced accordingly (Fig. 6C). In this cell line, the expression of exogenous *Cv2* (introduced by plasmid transfection) strongly augmented the BMP signal in a dose-dependent manner, showing a pro-BMP activity (Fig. 6D, open columns). This augmentation appeared to depend on extracellular BMP signaling, since the addition of Noggin to the culture medium completely suppressed it (Fig. 6D, closed columns). Importantly, there was little augmentation of BMP signaling by *Cv2* in the *Tsg*-depleted HEK293T cells (Fig. 6E). This absence of the *Cv2*-induced increase in the *Tsg*-depleted cells was not owing to a general loss of the cellular BMP signaling pathway, because the *BRE-luc* activity was strongly stimulated by high concentrations of BMP4 also in these cells (Fig. 6F).

These data show that Cv2 functions as a pro-BMP factor in the presence of Tsg, which has a pro-BMP role in this embryonic kidney cell line, supporting the idea that Cv2 can enhance the pro-BMP activity of Tsg at least under certain situations (Fig. 6A, Model c).

## Discussion

In this report, we demonstrated that Cv2 plays an essential pro-BMP role in early nephrogenesis. The cap condensate is the embryonic kidney tissue that normally expresses Cv2, and its development was substantially affected by the *Cv2*-null mutation, even during very early histogenesis. The loss of Cv2 directly attenuated the BMP signaling in this tissue, as assessed by its reduced pSmad1 levels (Fig. 3; in contrast, pSmad2 levels were largely unaffected; Supplementary Fig. S8). In contrast, the *Cv2* mutation did not substantially affect the high BMP signaling levels in the neighboring collecting duct cells, which do not normally express Cv2. Taken together, these observations suggest that Cv2 is a local (or short-range) enhancer of BMP signaling that mainly acts in a tissue-autonomous fashion. In other words, the tissue augments its own BMP response by expressing Cv2.

At least three mechanistic explanations for the context-dependent pro-BMP function of Cv2 have been advocated so far: (1) a cleaved Cv2 protein, rather than a full-length one, exerts a pro-BMP activity (shown in a zebrafish study; Rentzsch et al., 2006). (2) Cv2 is a biphasic BMP modulator acting in a dose-dependent manner that, only at a low dose, facilitates the binding of BMPs to their type I receptor (shown in a *Drosophila* study; Serpe et al., 2008). (3) Cv2 increases the local concentration of diffusible Chordin/Tsg/BMP protein complexes (e.g., on the ventral side in the case of the *Xenopus* embryo), which release active BMPs to their cell surface receptors upon the cleavage of Chordin by tolloid proteinases (proposed in a *Xenopus* study; Ambrosio et al., 2008). In this case, the entrapment of BMP into the complexes is an anti-BMP process, while the release of BMP from the accumulated Chordin/Tsg/BMP complexes (i.e., reservoir complexes for BMP) serves as a pro-BMP step.

Of the three proposals (which are not mutually exclusive), the last one (Mechanism 3) fits our in vivo and in vitro data particularly well. First, our immunostaining results showed that Cv2 proteins are associated with the pericellular matrix (Fig. 2), which could make Cv2 less diffusible. Since Cv2 can physically interact with Chordin, Tsg and BMP (Ambrosio et al., 2008), the Cv2-bound pericellular matrix supposedly contributes to the local accumulation of Chordin/Tsg/BMP complexes. Second, the *Cv2*-null mutation specifically affected the cap condensates, which are normally surrounded by auto/paracrine Cv2. Third, Cv2 functions as a pro-BMP factor in the presence of Tsg, which is evidence that Cv2 might function in a complex, as proposed for Mechanism 3.

In addition, Mechanism 3 appears to be compatible with Model c (Fig. 6A), in which pro- and anti-BMP functions of Tsg can co-exist. In this interpretation, which combines Model c and Mechanism 3, Cv2 preferentially enhances the pro-BMP activity of Tsg; this activity is expected to depend heavily on the Cv2-mediated local high concentration of reservoir complexes that potentially release BMP. In contrast, the inactivation of BMPs by their entrapment in the Chordin–Tsg complex (anti-BMP) is Cv2-independent presumably because the entrapment process itself does not require the specific localization of the complexes. By using an embryonic kidney cell line, in which the pro-BMP function of Tsg is predominantly observed (Fig. 6E), we presented the practical feasibility of Model c (Fig. 6A), at least with respect to the cooperative pro-BMP function of Cv2 and Tsg. Consistent with Model c and also Mechanism 3, Cv2 exerts pro-BMP function in the presence of Tsg in this cell culture system. In the future investigations, other models must also be ruled out, including one in which Cv2 acts as an inhibitor of Tsg's anti-BMP activity, rather than as a co-factor of its pro-BMP activity (a sort of fusion of Models b and c).

This report mainly focused on the pro-BMP role of Cv2. Genetic analyses of *Bmp7;Cv2* mutants provide firm evidences that Cv2 acts predominantly as a pro-BMP factor in early nephrogenesis. In addition, a genetic enhancement in the nephron number was observed between *Cv2* and *Smad1* (Supplementary Fig. S9), indicating that Cv2 works cooperatively not only with the extracellular BMP ligand (BMP7) but also with the major transducer of BMP receptor signaling. Although these data do not argue the presence of a minor anti-BMP function of Cv2, it is considered the total Cv2 activity, at least on balance, is deviated toward the pro-BMP direction in the context of kidney development.

This report also focused on its genetic interactions with Tsg. Whereas it is beyond the scope of this genetics-based study, identifying the detailed molecular mechanisms underlying the pro-BMP function at the protein level is an important topic for future comprehensive study (Umulis et al., 2009). For instance, it will be intriguing to show the different kinetics of BMP release from complexes of Cv2 with Tsg versus Cv2 with Chordin, or the concentration or presentation of the ligand-bearing complexes to the receptor. It is also interesting to learn whether the role of mammalian Tsg in kidney development in fact depends on Chordin, especially given that Chordin and Chordin-related genes are expressed in the embryonic kidney (Supplementary Fig. S10). Because good antibodies for immunohistochemical studies of Tsg, Chordin and the tolloids are not available (our preliminary observations), we cannot currently examine whether Tsg–Chordin complexes are concentrated or specifically degraded near the cap condensate. Visualizing the dynamic localization, diffusion and processing of the complex proteins is a challenging but critical future task.

Our preliminary study showed that the decrease of nephron numbers in $Cv2^{-/-}$ kidneys (E14.5) was also moderately enhanced by adding a $Bmp4^{+/-}$ mutation similar to that observed with $Bmp7^{+/-}$ mutation (Supplementary Fig. S11). On the other hand, during late nephrogenesis, the $Bmp4^{+/-}$ mutation strongly enhanced the hydroureter phenotypes in the $Cv2^{-/-}$ kidneys (Supplementary Fig. S12). In this case, the formation of the ureter mesenchyme, which expresses *Bmp4* but not *Bmp7*, was strongly inhibited, suggesting that BMP4 cooperates with Cv2 independently of BMP7, at least in this context. Thus, whether BMP7 acts as a homodimer or a heterodimer with BMP4 in the Cv2 interaction is a remaining question for further investigation.

## Conclusion

From our in vivo and in vitro observations, we conclude that Cv2, in concert with Tsg, shapes the signaling landscape in the complex organogenetic microenvironment of the kidney, creating locally restricted peaks in the BMP signal.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.013.

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