Bone morphogenetic protein antagonists and kidney

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Introduction

Bone morphogenetic proteins (BMPs) are phylogenetically conserved signaling molecules that belong to the transforming growth factor (TGF)-β superfamily [1–4]. Although these proteins were first identified by their capacity to promote endochondral bone formation [5–7], they are involved in the cascades of body patterning including nephrogenesis. Furthermore, BMPs play important roles after birth in pathophysiology of several diseases including osteoporosis [8], arthritis [5], pulmonary hypertension [9, 10], and kidney diseases [11–13]. Several BMPs are expressed in the kidney, and the expression level and pattern of each BMP varies dynamically during embryogenesis and kidney disease progression. BMP-7 is the most abundant BMP during kidney development [14], whereas the level of BMP-4, BMP-6 and BMP-7 are comparable in adult healthy kidneys (S. Yamada, unpublished data). BMP-2 is hardly detectable in developing and adult kidneys.

BMP-7 in kidney disease and development

BMP-7, also known as osteogenic protein-1 (OP-1), is a 35-kDa homodimeric protein, and kidney is the major site of BMP-7 synthesis during embryogenesis and in postnatal development [14–17]. Its genetic deletion in mice leads to severe impairment of kidney development resulting in perinatal death [18, 19]. Kidney development is essentially normal until embryonic day (E) 14.5; however, the metanephric mesenchymal cells fail to differentiate subsequently, resulting in a low number of nephrons in newborn kidneys. The mutant kidneys also suffered massive apoptosis in the uninduced mesenchymal cells, demonstrating that BMP-7 is essential for their continued survival, proliferation and differentiation. Borovecki et al. [20] demonstrated that iodinated BMP-7 (^{125}I-BMP-7) injected through the tail vein of pregnant mice passed across the placenta and localized in developing fetal organ including

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kidneys until E14, indicating the possibility that maternal circulating BMP-7 might rescue the lack of embryonic BMP-7 in early development.

Bmp-7 null mice also have an eye defect and minor skeletal patterning defects [18, 19]. Although Bmp-7 is expressed at diverse sites in the developing mouse embryos, the tissue defects in *bmp-7* null embryos are confined to certain organs. Dudley et al. [21] demonstrated the overlapping expression domain of BMPs and the possibility that BMP family members can functionally substitute for BMP-7 at sites where they colocalized. Oxburgh et al. [22] further supported the idea by demonstrating that the *bmp-4* knock-in allele in the *bmp-7* locus rescued the kidney development, and suggesting that BMP family members can function interchangeably.

Expression of BMP-7 in adult kidney is confined to distal convoluted tubules, collecting ducts and podocytes of glomeruli (Fig. 1) [23], and the expression decreases in several kidney disease models [24–28]. Recently, several reports indicate that the administration of pharmacological doses of BMP-7 inhibits and repairs chronic renal injury in animal models [25–27, 29–31]. The administration of BMP-7 is reported to reverse TGF-β1-induced epithelial-to-mesenchymal transition (EMT) and induce mesenchymal-to-epithelial transition (MET) *in vitro* [32, 33], inhibit the induction of inflammatory cytokine expression in the kidney [23], attenuate inflammatory cell infiltration [30], and reduce apoptosis of tubular epithelial cells in renal disease models [34]. Collectively, BMP-7 plays critical roles in repairing processes of the renal tubular damage in kidney diseases.

However, the physiological role and precise regulatory mechanism of endogenous BMP-7 remain elusive. Although many groups reported the possible actions of BMP-7 on proximal tubule epithelial cells (PTEC) in adult kidney injury [23], it is poorly understood which cells are the main source of BMP-7 in the circumstances, and how endogenous BMP-7 can be delivered to PTEC. BMP-7 might be delivered from adjacent distal nephron segments *via* the intervening interstitium, or alternatively, might be delivered from the glomerulus or *via* the circulation. Bosukonda et al. [35] reported that injected ^{125}I-BMP-7 in rats is found within glomeruli, proximal convoluted tubules, and medullary collecting tubules. *In situ* hybridization using a BMPR-II riboprobe demonstrated similar localization with ^{125}I-BMP-7 (Fig. 1A), and immunostaining of BMPR-II localized the receptor to glomeruli and proximal tubules. Further study is needed to determine whether BMP receptors are expressed in the apical or basolateral membrane of PTEC, which implies the route of endogenous BMP-7 delivery.

BMP-4 in urinary tract development

In mouse embryos, Bmp-4 is expressed in mesenchymal cells surrounding the Wolffian duct and ureter stalk [21, 36, 37]. *Bmp-4* null embryos die between E6.5 and

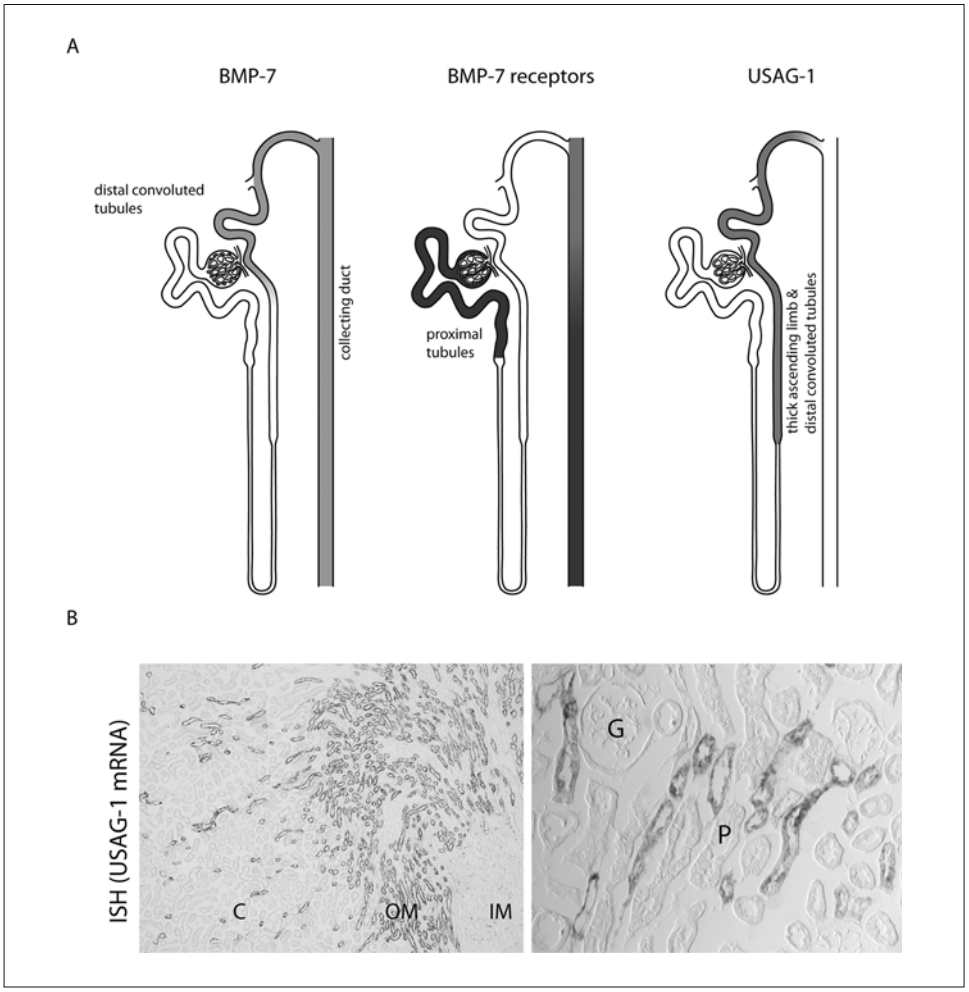

Figure 1
Expression of bone morphogenetic protein (BMP)-7, its receptor, and uterine sensitization-associated gene (USAG)-1. (A) Schematic illustration demonstrating the nephron segments in which BMP-7, its receptors and USAG-1 are expressed. (B) Localization of USAG-1 mRNA in the adult kidney.

10.0, indicating the essential role of BMP-4 in early embryonic development [38]. *Bmp-4* heterozygous null mutant mice displays abnormalities that mimic human congenital anomalies of the kidney and urinary tract (CAKUT), including hypo/dysplastic kidneys, hydroureter, ectopic ureterovesical junction, and double collecting system [36]. Further *in vivo* and *in vitro* studies clarified that BMP-4 inhibits ectopic

budding from Wolffian duct and stimulates the elongation of the branching ureter within the metanephros [37].

As mentioned above, the knockin allele of *bmp-4* in the *bmp-7* locus efficiently rescues kidney development in *bmp-7* null embryos [22], indicating that BMP-4 and BMP-7, sharing only minimal sequence similarity, can function interchangeably to activate essential pathways in kidney development. The results indicate the distinct phenotypes in *bmp-4* null embryos and *bmp-7* null embryos simply reflect differences in expression domains of these two molecules.

Extracellular modification of BMP activity

The local activity of endogenous BMP is precisely regulated at multiple steps: intracellulary, at the membrane site, and extracellulary (Fig. 2). In this review, we focus on the extracellular modification of BMP signaling.

At the membrane, the transmembrane protein BAMBI (BMP and activin membrane-bound inhibitor) functions as a pseudoreceptor to interfere with BMP, activn, and TGF-β signaling in *Xenopus* [39, 40]. BAMBI and its mammalian homologue Nma are structurally related to type I serine/threonine kinase receptors in the extracellular domain, but lack the intracellular serine/threonine kinase domain. BAMBI/Nma stably associate with type II receptors, thus preventing the formation of active receptor complex.

Recently, repulsive guidance molecule (RGMA) [41], DRAGON (RGMB) [42, 43], and hemojuvelin [44] are reported to act as BMP-activating co-receptors. These are glycosyl phosphatidyl inositol (GPI)-anchored proteins, which form a complex with BMP type I receptors and enhance receptor binding to BMP-2 and BMP-4, potentiating their biological effects.

In the extracellular space, BMP signaling is precisely regulated by certain classes of molecules termed as BMP antagonists [45, 46]. BMP antagonists function through direct association with BMPs, thus prohibiting BMPs from binding their cognate receptors. The interplay between BMP and their antagonists fine-tunes the level of available BMPs, and governs developmental and cellular processes as diverse as establishment of the embryonic dorsal–ventral axis [47], induction of neural tissue [48], formation of joints in the skeletal system [5] and neurogenesis in the adult brain [49].

In addition to the modulation by BMP antagonists, high affinity binding of BMP to extracellular matrix modifies the local activity of BMP. Vukicevic et al. [50] previously showed that BMP-7 binds to basement membrane components including type IV collagen. In addition, Gregory et al. [51] recently demonstrated that the prodomain of BMP-7 targets BMP-7 complex to the extracellular matrix. In most tissues, BMP mRNA expression and BMP protein are found colocalized. Restricted diffusion of BMP proteins is considered to increase its local concentration.

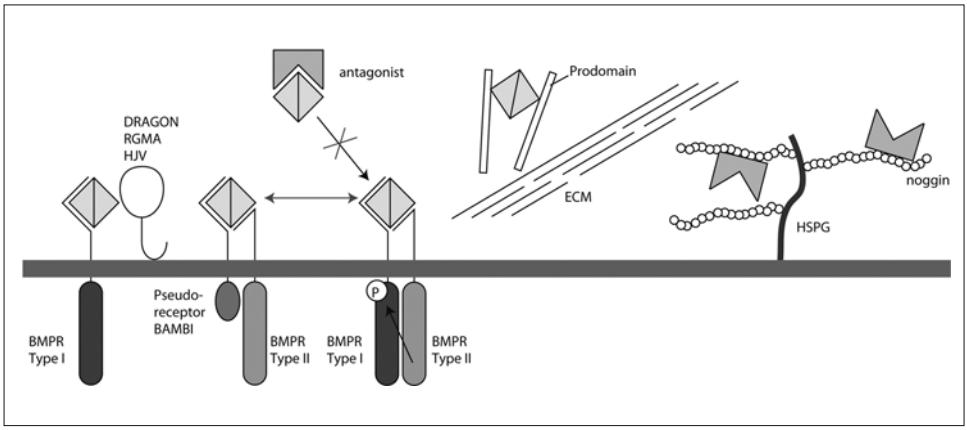

Figure 2
Extracellular modulation of BMP signaling. Modified from [45]. ECM; extracellular matrix.

Heparin sulfate proteoglycans (HSPGs) are also reported to shape the BMP gradient at the cell surface. Jiao et al. [52] recently reported that HSPGs mediate BMP-2 internalization and modulate BMP-2 osteogenic activity, while other groups reported that BMP antagonists such as chordin and noggin are retained at cell surface and regulated diffusion by binding to HSPGs [53].

BMP antagonists in the kidney

Classification and expression of BMP antagonist in the kidney

BMP antagonists have a secretory signal peptide and cysteine arrangement consistent with the formation of the cystine knot structure and represent a subfamily of cystine knot superfamily, which comprises of TGF-β, growth differentiation factors (GDFs), gonadotropins, and platelet-derived growth factors, and BMPs [54]. Recently, Avsian-Kretchmer et al. [55] classified BMP antagonists into three subfamilies based on the size of the cystine knot: the DAN family (eight-membered ring), twisted gastrulation (Tsg) (nine-membered ring) and chordin and noggin (10-membered ring). They further divided the DAN family into four subgroups based on a conserved arrangement of additional cysteine residues outside of the cystine knots: (1) PRDC and gremlin, (2) coco and Cer1 homologue of *Xenopus* Cerberus, (3) Dan, and (4) USAG-1/wise/ectodin and sclerostin. This subdivision is almost consistent with the phylogenic tree based on the overall amino acid sequence similarity shown in Figure 3.

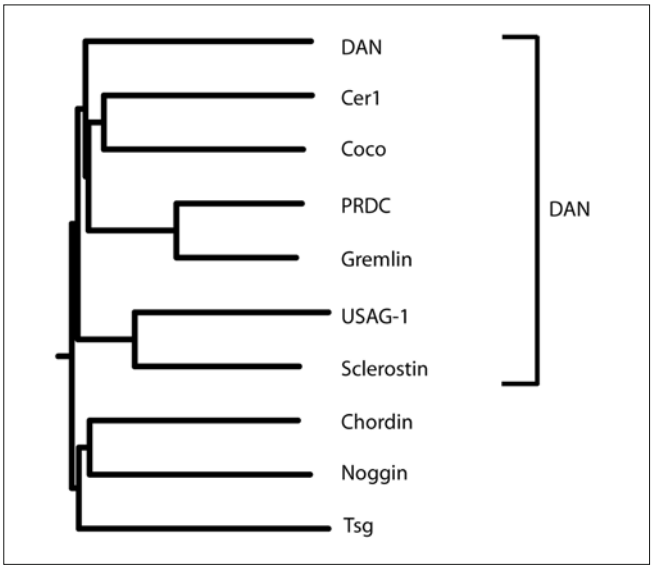

Figure 3
Phylogenetic tree of human BMP antagonists based on the overall amino acid sequence similarity of representative members from each subfamily. The GenomeNet server at http://www.genome.jp/ was used for phylogenetic tree construction. Modified from [66].

To compare the expression level of BMP antagonists in the kidney, our group utilized modified real-time PCR and demonstrated that USAG-1 is by far the most abundant BMP antagonist in adult kidney (Fig. 4A), as well as in embryogenesis (Fig. 4B). In the following section, we review the papers describing the possible role of BMP antagonists in the kidney.

USAG-1: the most abundant BMP antagonist in the kidney

Discovery and characterization of USAG-1 as a BMP antagonist

Through a genome-wide search for kidney-specific transcripts, our group found a novel gene, which encodes a secretory protein with a signal peptide and cysteine-rich domain [56]. The rat orthologue of the gene was previously reported as a gene of unknown function that was preferentially expressed in sensitized endometrium of rat uterus, termed uterine sensitization-associated gene-1 (*USAG-1*) [57]. Amino acid sequences encoded in rat and mouse cDNAs are 97% and 98% identical to the human sequence respectively, indicating high degrees of sequence conservation.

Domain search predicted this protein to be a member of the cystine-knot superfamily, and homology search revealed that USAG-1 has significant amino acid

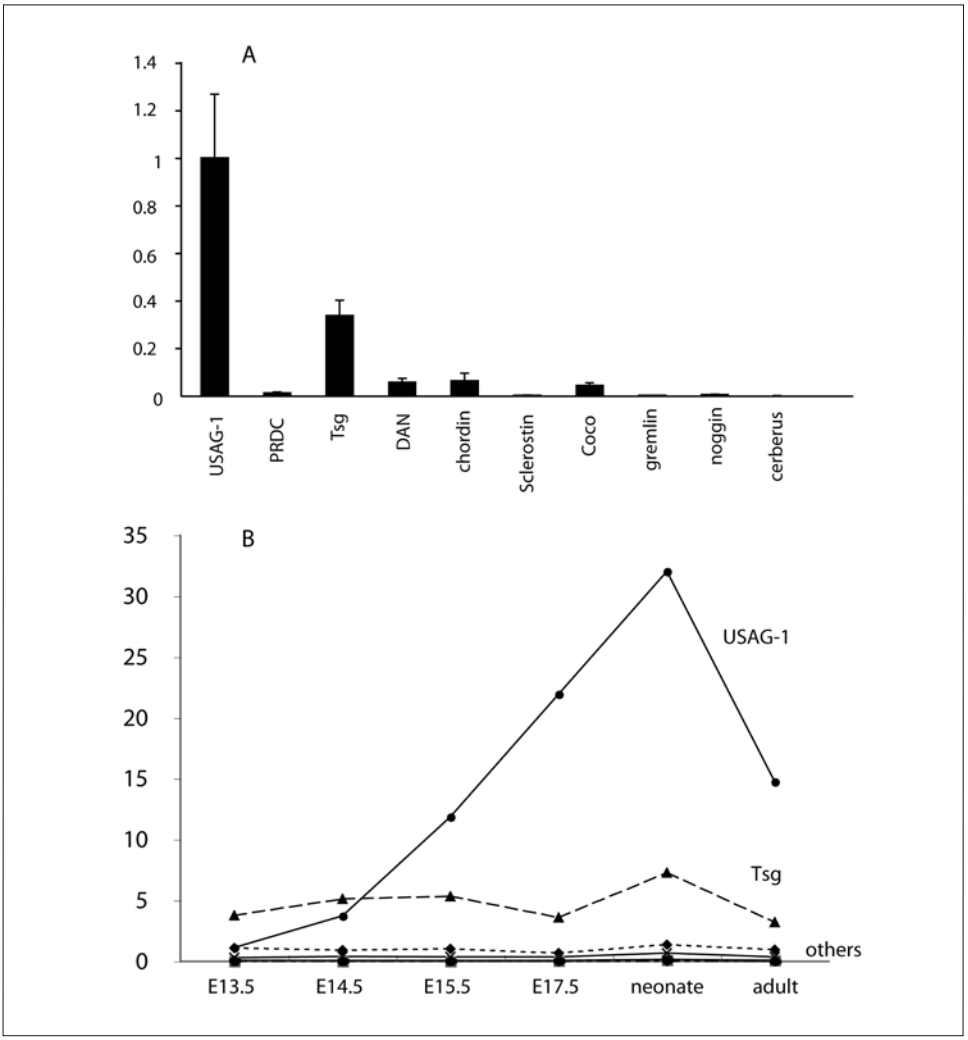

Figure 4
Expression of BMP antagonists in adult healthy kidney (A) and developing kidney (B). Tsg; twisted gastrulation.

identities (38%) to sclerostin, the product of the *SOST* gene (Fig. 4). Mutations of *SOST* are found in patients with sclerosteosis, a syndrome of sclerosing skeletal dysplasia. Sclerostin was expressed in bones and cartilages, and subsequently shown to be a new member of BMP antagonist [58–61], as well as a modulator of Wnt signaling [62–64].

USAG-1 protein is a 28–30-kDa secretory protein [56] and is heavily glycosylated (A. Yoshioka, unpublished data). USAG-1 behaves as a monomer, although a number of BMP antagonists form disulfide-bridged dimers. This is consistent with the fact that USAG-1 protein does not have the extra cysteine residues present in noggin and DAN, which are necessary to make inter-molecular disulfide bridges. Recombinant USAG-1 protein physically interacts with BMP-2, -4, -6, and -7, leading to the inhibition of alkaline phosphatase activities (ALP) induced by each BMP in C2C12 cells and MC3T3-E1 cells dose-dependently [56, 65], while sclerostin only inhibits BMP-6 and BMP-7 activities [58]. The activity of USAG-1 as a BMP modulator was also confirmed *in vivo* using *Xenopus* embryogenesis [56]. Injection of synthetic RNA encoding BMP antagonists to the ventral portion of *Xenopus* embryos inhibits the ventralizing signal of endogenous BMP, and induces dorsalizing phenotypes of the embryos including secondary axis formation and hyperdorsalization. The injection of as little as 100 pg USAG-1 mRNA was sufficient to cause secondary axis formation, and injection of increasing doses of mRNA up to 1000 pg led to a corresponding increase in the frequency of dorsalization phenotypes, while embryos developed normally when irrelevant mRNA was injected.

USAG-1 as a central regulator of renoprotective action of BMP-7

In adult tissues, the expression of USAG-1 was by far the most abundant in the kidney and is restricted to the thick ascending limb, and distal convoluted tubules (Fig. 1) [56, 66–68]. Thus, the cellular distribution of USAG-1 overlaps with that of BMP-7 in the distal convoluted tubules. Together with the fact that PTECs are the site of injury in many types of kidney diseases, and that the PTECs express the receptors for BMP-7, we hypothesized a working model of the regulation of the reno-protective action of BMP-7: in renal injury, PTECs are mainly damaged and undergo apoptosis or EMT to fibroblast-like mesenchymal cells. BMP-7 secreted from distal tubules binds to the receptors on the cell surface of PTECs, and inhibits apoptosis and EMT. USAG-1 is also secreted from distal tubules, binds to BMP-7, and inhibits the reno-protective actions of BMP-7 by reducing the amount of available BMP-7.

To evaluate this working model, our group generated *usag-1* null mice, and induced acute and chronic renal disease models in which the renal tubules were mainly damaged [68]. *usag-1* null mice exhibited prolonged survival and preserved renal function in acute and chronic renal injuries. Renal BMP signaling, assessed by phosphorylation of Smad proteins, is significantly enhanced in *usag-1* null mice during renal injury, indicating that the preservation of renal function is attributed to enhancement of endogenous BMP signaling. Furthermore, the administration of neutralizing antibody against BMP-7 abolished reno-protection in *usag-1* null mice, indicating that USAG-1 plays a critical role in the modulation of reno-protective action of BMP, and that inhibition of USAG-1 will be a promising means of devel-

opment of novel treatment for kidney diseases. In addition, we demonstrated that the expression of USAG-1 in the kidney biopsy could be a diagnostic tool to predict renal prognosis [69].

USAG-1 as a context-dependent activator and inhibitor of Wnt signaling

Itasaki et al. [70] reported that *wise*, a *Xenopus* orthologue of USAG-1, functions as a context-dependent activator and inhibitor of Wnt signaling in *Xenopus* embryogenesis. They also demonstrated the physical interaction between wise/USAG-1 and Wnt co-receptor LRP6, and that Wise/USAG-1 can compete with Wnt8 for binding to LRP6. Recently, they demonstrated that the cellular localization of Wise/USAG-1 has distinct effects on the Wnt pathway readout [71]. While secreted Wise/USAG-1 either synergizes or inhibits the Wnt signals depending on the partner ligand, enndoplasmic reticulum (ER)-retained Wise/USAG-1 consistently blocks the Wnt pathway. ER-retained Wise/USAG-1 reduces LRP6 on the cell surface, making cells less susceptible to the Wnt signal. Further studies are needed to clarify the biological function of USAG-1 *in vivo*; however, it might be possible that these two proteins possess dual activities, and play as a molecular link between Wnt and BMP signaling pathway.

Gremlin: Essential for kidney development and a possible role in fibrosis

Gremlin was identified from a *Xenopus* ovarian library for activities inducing secondary axis [72]. Gremlin is a 28-kDa protein, and binds to BMP-2/4 and inhibits their binding to the receptors. *Gremlin* null mice are neonatally lethal because of the lack of kidneys and septation defects in lung [73]. In early limb buds, mesenchymal gremlin is required to establish a functional apical ectodermal ridge and the epithelial-mesenchymal feedback signaling that propagates the sonic hedgehog morphogen [74]. In the *gremlin* null embryos, metanephric development is disrupted at the stage of intiating ureteric bud outgrowth and genetic lowering of BMP-4 levels in *gremlin* null embryos completely restores ureteric bud outgrowth and branching morphogenesis, indicating that initiation of metanephric kidney development requires the reduction of BMP-4 activity by the antagonist gremlin in the mesenchyme, which in turn enables ureteric bud outgrowth and establishment of autoregulatory GDNF/WNT11 feedback signaling [75].

Gremlin is also known as DRM (down-regulated by v-*mos*) because it was identified as a gene that down-regulated in *mos*-transformed cells [76, 77]. Another name for gremlin is IHG-2 (induced in high glucose 2) because its expression in cultured kidney mesangial cells is induced by high ambient glucose, mechanical strain, and TGF-β [78]. The expression of gremlin is not detected in adult healthy kidney, but is increased in streptozotocin-induced diabetic nephropathy model [24], as well

as in human diabetic nephropathy. Although some expression of gremlin is observed in occasional glomeruli, gremlin expression was prominent in areas of tubulointerstitial fibrosis, where it colocalized with TGF-β expression [79]. The authors of the study also demonstrated that gremlin expression correlated well with the tubulointerstitial fibrosis, and the result is consistent with previous reports demonstrating the up-regulation of gremlin in fibrosis of other organs [80, 81].

Recently, Sun et al. [82] reported a novel intracellular regulatory mechanism by which Gremlin interacts with BMP-4 precursor, prevents secretion of mature BMP-4, and therefore inhibits BMP-4 activity more efficiently. Furthermore, they defined a 30-amino acid peptide sequence within the Gremlin DAN domain that is essential for BMP-4 interaction. This result implies that the level of BMP-4 mRNA expression does not truly reflect BMP-4 activity when Gremlin and BMP-4 are co-expressed within the same cell. Similar regulatory mechanisms may be utilized by other DAN family proteins.

Noggin: Effective tool to inhibit BMP signaling

Noggin is a 32-kDa glycoprotein secreted by Spemann organizer of *Xenopus* embryos, and is found to rescue dorsal development in the ultraviolet-induced ventralized embryos [83]. Noggin antagonizes the action of BMPs, induces neural tissues and dorsalizes ventral mesoderm [84]. Noggin binds to BMP-2 and BMP-4 with high affinity and to BMP-7 with low affinity, and prevent BMPs from binding to its receptors. Groppe et al. [85] reported the crystal structure of Noggin bound to BMP-7, which shows that Noggin inhibits BMP signaling by blocking the molecular interfaces of the binding epitopes for both type I and type II receptors. The BMP-7-binding affinity of site-specific variants of Noggin is correlated with alterations in bone formation and apoptosis in chick limb development, showing that Noggin functions by sequestering its ligand in an inactive complex. The scaffold of Noggin contains a cystine knot topology similar to that of BMPs; thus, ligand and antagonist seem to have evolved from a common ancestral gene.

In mice, Noggin is expressed in the node, notochord, dorsal somite, condensing cartilage, and immature chondrocytes, and null mutation of Noggin results in serious developmental abnormalities including failure of neural tube formation, and dismorphogenesis of the axial skeleton and joint lesions [86–88].

In the healthy kidney, Noggin is not expressed, but its high binding affinity to BMP is utilized as a tool to inhibit BMP signaling in certain cell type. Recently, it was reported that overexpression of noggin in podocytes leads to the development of mesangial expansion, indicating the importance of endogenous BMP signaling in the maintenance of glomerular structure [89]. Because the expression of noggin is almost undetectable in healthy and diseased kidney, other negative regulator of endogenous BMP might play a role in glomerular mesangial expansion.

Crim1: A membrane-bound antagonist and a role in glomerular development

Crim1 is a transmembrane protein possessing cysteine-rich repeat (CRR), and plays a role in the tethering of growth factors at the cell surface [90]. Crim1 binds to BMP-4 and -7 *via* the CRR-containing portion, and functions as a BMP antagonist in three different ways: Crim1 binding with BMP-4 and -7 occurs when these proteins are co-expressed within the Golgi compartment of the cell and leads to (i) a reduction in the production and processing of pre-protein to mature BMP, (ii) tethering of pre-BMP to the cell surface, and (iii) an effective reduction in the secretion of mature BMP. Hence, Crim1 modulates BMP activity by affecting its processing and delivery to the cell surface.

Crim1 is expressed in a spatially and temporally restricted manner during organogenesis of the limbs, kidney, lens, pinna, erupting teeth, and testis [91–93]. During metanephric development, Crim1 is expressed in the ureteric tree, the early condensing mesenchyme and distal comma-shaped bodies. As the nephron elongates, Crim1 becomes expressed in the proximal end of the S-shaped bodies [91]. In later stages of development, Crim1 is also detected in podocyte and mesangial cells in glomeruli [94].

A gene-trap mouse line with an insertion of β-Geo cassette into intron 1 of the *Crim1* gene (*Crim1*$^{KST264/KST264}$) is a Crim1 hypomorph, and displayed perinatal lethality with defects in multiple organ systems [95]. In the kidney, *Crim1*$^{KST264/KST264}$ mice displayed abnormal glomerular development, including enlarged capillary loops, podocyte effacement, and mesangiolysis [94]. When outbred, homozygotes that reached birth displayed marked albuminuria. The podocytic co-expression of Crim1 with vascular endothelial growth factor-A (VEGF-A) suggested a role for Crim1 in the regulation of VEGF-A action. Crim1 and VEGF-A were shown to interact directly, providing evidence that CRR-containing proteins can bind to non-TGF-β superfamily ligands.

In addition, a homologue of Crim1 in *Caenorhabditis elegans*, crm-1, is reported to facilitate BMP signaling to control body size in *C.s elegans* [96].

Kielin/chordin-like protein: BMP agonist with a role in kidney injury

Lin et al. [97] recently identified a cDNA clone from an embryonic kidney library that contained multiple CRRs. The entire coding lesion was similar to the *Xenopus* kielin protein, and thus was named kielin/chordin-like protein (KCP). KCP is a secretory protein with 18 CRRs, and increases the binding of BMP-7 to its receptor and enhances downstream signaling pathways. The expression of KCP was detected in developing nephrons, but not in adult healthy kidneys. *kcp* null mice developed normally. When introduced in a kidney injury model, *kcp* null mice showed reduced levels of phosphorylated Smad1, and were susceptible to developing renal interstitial fibrosis, and more sensitive to tubular injury.

In contrast to the enhancing effect on BMPs, KCP inhibits both activin A- and TGF-β1-mediated signaling through the Smad2/3 pathway. KCP binds directly to TGF-β1 and blocks the interactions with its receptors. Consistent with this inhibitory effect, primary renal epithelial cells from *KCP* null cells are hypersensitive to TGF-β1 [98].

Crossveinless 2:Another BMP agonist related to kielin

Crossveinless 2 (Cv2) is also closely related to kielin, and was first identified in a fly mutant study as a gene required for the formation of cross-veins in the fly wings [99]. Genetic studies in flies showed that the formation of these veins required high Bmp signaling activity, and that Cv2 was essential for enhancing the local Bmp signal near the receiving cells. By contrast, the *in vivo* role of the vertebrate counterpart of Cv2 remains to be elucidated, as some reports indicate that Cv2 is an anti-BMP factor [100], while others describe its pro-BMP activity [101]. Analysis of *cv2* null mice terminated the argument, and demonstrated that Cv2 is a pro-BMP factor in mouse embryogenesis [102].

In *cv2* null mouse, gastrulation occurs normally, but a number of defects are found in Cv2-expressing tissues such as the skeleton. The defects of the vertebral column and eyes in the *cv2* null mouse are substantially enhanced by deleting one copy of the *bmp-4* gene, suggesting a pro-Bmp role of Cv2 in the development of these organs. In addition, *cv2* null mice exhibit kidney hypoplasia, and the phenotype is synergistically enhanced by the additional deletion of *kcp*, that encodes a pro-Bmp protein structurally related to Cv2 (see previous section).

Conclusions

In conclusion, BMPs and their modulators play important roles in kidney injury as well as in kidney development. Because negative and positive modulators of BMP signaling regulate and define the boundaries of BMP activity, further understanding of these modulators would give valuable information about their pathophysiological functions and provide a rationale for a therapeutic approach against these proteins.

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