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Author(s): Tanaka, Mari; Asada, Misako; Higashi, Atsuko Y.; Nakamura, Jin; Oguchi, Akiko; Tomita, Mayumi; Yamada, Sachiko; Asada, Nariaki; Takase, Masayuki; Okuda, Tomohiko; Kawachi, Hiroshi; Economides, Aris N.; Robertson, Elizabeth; Takahashi, Satoru; Sakurai, Takeshi; Goldschmeding, Roel; Muso, Eri; Fukatsu, Atsushi; Kita, Toru; Yanagita, Motoko

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Loss of the BMP antagonist USAG-1 ameliorates disease in a mouse model of the progressive hereditary kidney disease Alport syndrome

Mari Tanaka,1 Misako Asada,2 Atsuko Y. Higashi,1 Jin Nakamura,2 Akiko Oguchi,2 Mayumi Tomita,3 Sachiho Yamada,1 Nariaki Asada,2 Masayuki Takase,2 Tomohiko Okuda,4 Hiroshi Kawachi,5 Aris N. Economides,6 Elizabeth Robertson,7 Satoru Takahashi,8 Takeshi Sakurai,9 Roel Goldschmeding,10 Eri Muso,11 Atsushi Fukatsu,3 Toru Kita,1 and Motoko Yanagita1,2,3

The glomerular basement membrane (GBM) is a key component of the filtering unit in the kidney. Mutations involving any of the collagen IV genes (COL4A3, COL4A4, and COL4A5) affect GBM assembly and cause Alport syndrome, a progressive hereditary kidney disease with no definitive therapy. Previously, we have demonstrated that the bone morphogenetic protein (BMP) antagonist uterine sensitization–associated gene-1 (USAG-1) negatively regulates the renoprotective action of BMP-7 in a mouse model of tubular injury during acute renal failure. Here, we investigated the role of USAG-1 in renal function in Col4a3−/− mice, which model Alport syndrome. Ablation of Usag1 in Col4a3−/− mice led to substantial attenuation of disease progression, normalization of GBM ultrastructure, preservation of renal function, and extension of life span. Immunohistochemical analysis revealed that USAG-1 and BMP-7 colocalized in the macula densa in the distal tubules, lying in direct contact with glomerular mesangial cells. Furthermore, in cultured mesangial cells, BMP-7 attenuated and USAG-1 enhanced the expression of MMP-12, a protease that may contribute to GBM degradation. These data suggest that the pathogenic role of USAG-1 in Col4a3−/− mice might involve crosstalk between kidney tubules and the glomerulus and that inhibition of USAG-1 may be a promising therapeutic approach for the treatment of Alport syndrome.

Introduction

The renal glomerular basement membrane (GBM) contributes importantly to maintenance of the structural integrity of the glomerular capillaries (1, 2). Type IV collagen is the major component of the GBM, and its mutations have been linked to the genetic disorder Alport syndrome, a progressive hereditary kidney disease associated with sensorineural deafness (3). With a genetic frequency of about 1 in 5000 people, it counts among the more prevalent of known genetic disorders (4). The disease is caused by mutations in any one of the genes encoding the α3, α4, and α5 chains of type IV collagen (COL4A3, COL4A4, and COL4A5) (5–7), and a mutation affecting 1 of these chains forming the α3/α4/α5(IV) collagen network can alter or abolish the GBM expression not only of the corresponding chain but also of the other 2 chains (8). The GBM in Alport syndrome instead retains the fetal α1/α1/α2(IV) collagen network (9), which confers an increased susceptibility to proteolytic enzyme, leading to progressive destruction of the GBM with subsequent hematuria and proteinuria, glomerulosclerosis and ultimately end-stage renal disease. The current therapy is limited to dialysis and transplantation, with a higher risk of anti-GBM disease in the transplanted organs due to immune reaction against the type IV collagen chains.

Bone morphogenetic protein-7 (BMP-7) is a promising candidate to treat Alport syndrome. BMP-7 belongs to the TGF-β superfamily (10), and the kidney is the major site of BMP-7 expression during both embryogenesis and postnatal development (11). Pharmacological doses of BMP-7 can repair damaged renal tubules and preserve renal function in several models of renal diseases, including the Col4A3 knockout model of Alport syndrome (12–20). However, the exact role of endogenous BMP-7 and its mechanism of action remain unclear. In addition, the administration of recombinant BMP-7, whose target cells are widely expressed throughout the body, might also produce some undesired extrarenal effect.

The local activity of endogenous BMPs is controlled by certain classes of binding molecules that act as positive or negative regulators of BMP signaling activity (10, 21–24). BMP antagonists function through direct association with BMP, thus inhibiting the binding of BMP to its receptors and defining the boundaries of BMP activity.

The product of uterine sensitization–associated gene-1 (USAG-1) acts as a kidney-specific BMP antagonist, and USAG-1 binds to and inhibits the biological activity of BMP-7 (22, 25). USAG-1 is...
Usag1+/−Col4a3−/− mice showed less glomerular and tubular injury. (A) Representative histological findings in Usag1+/−Col4a3−/− mice (WT/KO) and Usag1+/−Col4a3−/− mice (KO/KO) at 6 weeks and 10 weeks of age. Scale bars: 100 μm. (B and C) Quantitative assessment of the number of glomeruli, percentages of sclerotic and hemorrhagic glomeruli, and tubulointerstitial fibrosis score in Usag1+/−Col4a3−/− mice (WT/KO) and Usag1+/−Col4a3−/− mice (KO/KO) at 6 weeks (B, n = 5) and 10 weeks of age (C, n = 10). Bars indicate the mean ± SD. **P < 0.01; ***P < 0.05. (D) Electron microphotographs in Usag1+/−Col4a3−/− mice (WT/KO) and Usag1−/−Col4a3−/− mice (KO/KO) at 4 weeks and 10 weeks of age. Arrows indicate the splitting of GBM. Scale bars: 5 μm. (E) Immunostaining for α1(IV) and α3(IV) collagen in the glomeruli of WT littermates (WT/WT), Usag1+/−Col4a3−/− mice (WT/KO), and Usag1−/−Col4a3−/− mice (KO/KO) at 6 weeks of age. Podocin was used as a podocyte marker. Note the positive staining for α1(IV) collagen along with the GBM of Usag1+/−Col4a3−/− mice (WT/KO) and Usag1−/−Col4a3−/− mice (KO/KO), while the staining is restricted to mesangial areas in the glomeruli of WT littermates.
expressed in distal tubules and colocalizes with BMP-7 in distal convoluted tubules and connecting tubules (26). Furthermore, Usag1−/− mice are resistant to tubular injury such as acute renal failure and interstitial fibrosis, and Usag1 is the central negative regulator of BMP function in the adult kidney (27). Because in adults the expression of USAG-1 is confined to the kidneys, target organ-specific therapies than the administration of BMP-7 (23). For this, it will be important to first elucidate the role of USAG-1 in the pathology of progressive glomerular injury.

Here we show that genetic ablation of USAG-1 significantly attenuated the disease progression and preserved renal function in Col4a3−/− mice, a model for human Alport syndrome. The observations in this study suggest that USAG-1 might contribute to the pathogenesis of renal deterioration by a mechanism we believe to be novel that involves crosstalk between the macula densa of the distal tubules and the mesangium of the belonging glomerulus. In addition, we demonstrate that in the kidney of Col4a3−/− mice, TGF-β signaling includes phosphorylation of Smad1/5/8, transcription factors classically considered to be the downstream effectors of BMP signaling.

Results

Loss of USAG-1 slows progression of glomerular injury in Alport mice.

Col4a3−/− mice, a mouse model of human Alport syndrome, develop progressive glomerulonephritis associated with tubulointerstitial fibrosis leading to renal failure. Kidneys from Col4a3−/− mice showed irregular thickening and splitting of the GBM at 4 weeks of age by electron microscopy. At 5 weeks of age, proteinuria is initiated, and at 6 weeks of age, minor glomerular lesion is occasionally observed by light microscopy. At 10 weeks of age, severe glomerular lesions associated with tubulointerstitial fibrosis are observed, and renal function deteriorates.

To test the role of USAG-1 in the progression of end-stage renal disease originating from glomerular injury, mice deficient in both Col4a3 gene and Usag1 gene were generated (Usag1−/−Col4a3−/− mice). A histological examination of the kidneys from Usag1−/−Col4a3−/− mice revealed segmental sclerosis and intraglomerular hemorrhage at 6 weeks of age, while these changes were almost completely absent in Usag1−/−Col4a3−/− mice (Figure 1, A and B). At 10 weeks of age, Usag1−/−Col4a3−/− mice demonstrated glomerulosclerosis associated with inflammatory cell infiltration, interstitial fibrosis, tubular atrophy, and cast formation, while these changes significantly decreased in Usag1−/−Col4a3−/− mice (Figure 1, A and C).

An ultrastructural analysis of GBM using transmission electron microscopy at 4 weeks of age showed that Usag1−/−Col4a3−/− mice had extensive splitting of the GBM, while Usag1+/−Col4a3−/− mice showed almost normal GBM structure (Figure 1D). Usag1−/−Col4a3−/− mice at 10 weeks of age also exhibited a significant preservation of GBM structure in comparison with age-matched Usag1+/−Col4a3−/− mice (Figure 1D).

The immunostaining of α1(IV) or α3(IV) collagen was performed to compare the glomerular localization of α1(IV) collagen in both genotypes (Figure 1E). The expression of α1(IV) collagen was detected in the kidney of both Usag1+/−Col4a3−/− and Usag1−/−Col4a3−/− mice, while the expression was confined to mesangial area in the WT mice. The expression of α3(IV) collagen was absent in the GBM of both Usag1+/−Col4a3−/− and Usag1−/−Col4a3−/− mice, while the expression was detected along the GBM in the WT mice. Therefore, regardless of the presence or absence of USAG-1, no alteration was observed in the glomerular localization of α(IV) collagen.

Usag1−/−Col4a3−/− mice showed less albuminuria, preserved renal function, and longer life span. An analysis of urinary albumin excretion at 6 weeks of age is shown in Figure 2A, demonstrating significantly less albuminuria in Usag1−/−Col4a3−/− mice than in Usag1+/−Col4a3−/− mice. The systolic blood pressure of Usag1−/−Col4a3−/− mice at 5 weeks of age was slightly lower than that of Usag1+/−Col4a3−/− mice (Supplemental Figure 2; supplemental material available online with this article; doi:10.1172/JCI39569DS1). Renal function of Usag1−/−Col4a3−/− mice at 10 weeks of age, as assessed by serum creatinine and blood urea nitrogen, was also significantly preserved in comparison with that of Usag1+/−Col4a3−/− mice (Figure 2B), consistent with the results of renal histology and urinary albumin excretion. Furthermore, upon aging beyond 13 weeks, Usag1−/−Col4a3−/− mice showed less mortality than Usag1+/−Col4a3−/− mice (Supplemental Figure 1).

Inflammatory cytokine expression was significantly reduced in Usag1−/−Col4a3−/− mice. As previously reported, the mRNA of inflammatory cytokines such as TNF-α, IL-1β, monocyte chemoattractant protein-1 (MCP-1), and TGF-β was upregulated in the kidneys of Usag1+/−Col4a3−/− mice at 10 weeks of age (28, 29). In Usag1−/−Col4a3−/− mice, however, increases in inflammatory cytokines were signifi-
results indicate that enhanced phosphorylation of Smad1/5/8 in the kidneys of Usag1–/–Col4a3–/– mice with the expression of BMP-7 (Figure 4D). Taken together, these models suggested that TGF-β signaling and related phospho-Smad1/5/8 expression 

Enhanced Smad1/5/8 phosphorylation in Usag1–/–Col4a3–/– mice, but not in Usag1+/–Col4a3+/– mice, was possibly activated by TGF-β signaling. Next, the activation of Smad signaling was examined. The traditional view of the TGF-β superfamily signaling pathways assumes 2 distinct branches: a TGF-β branch that signals through Smad2/3 and a BMP branch that signals through Smad1/5/8 (30).

We observed increased phosphorylation of Smad2, the TGF-β signal transducer, in the kidneys of Usag1–/–Col4a3–/– mice as compared with WT mice as well as Usag1+/–Col4a3+/– mice (Figure 3B), consistent with high expression of TGF-β in Usag1–/–Col4a3–/– mice (Figure 3A). The phosphorylation of Smad1/5/8, the classical BMP signal transducer, was expected to be reduced in the kidneys of Usag1+/–Col4a3+/– mice due to generally low expression of BMP-7 in kidney disease models (12, 17, 26, 31). However, the phosphorylation of Smad1/5/8 was unexpectedly increased in the kidneys of Usag1–/–Col4a3–/– mice in comparison with WT mice as well as Usag1+/–Col4a3+/– mice.

Recently, several groups demonstrated that TGF-β activates Smad1/5 in addition to Smad2/3 in endothelial cells through novel receptor complexes (32–34). Thus, we hypothesized that the increased phosphorylation of Smad1/5/8 in the kidneys of Usag1–/–Col4a3–/– mice might also have resulted from high expression of TGF-β. To test this hypothesis, we administered TGF-β to various types of cells including MDCK cells, primary mesangial cells, NRK cells (rat tubule epithelial cells), NIH3T3 cells, and HeLa cells, and demonstrated that TGF-β can activate the phosphorylation of Smad1/5/8 in addition to Smad2/3 in all these cell types (Figure 4B). The phosphorylation of Smad1/5/8 was induced by TGF-β at concentrations as low as 1 ng/ml (Figure 4C). Furthermore, the phosphorylation of Smad1/5/8 in the kidneys of Usag1–/–Col4a3–/– mice correlated well with renal TGF-β as well as with serum creatinine levels, but not with the expression of BMP-7 (Figure 4D). Taken together, these results indicate that enhanced phosphorylation of Smad1/5/8 in Col4a3–/– mice might be due to TGF-β signaling and attenuated phosphorylation of Smad1/5/8 in Usag1–/–Col4a3–/– mice might reflect reduced expression of TGF-β and disease severity.

Usag1–/–Col4a3–/– mice showed less expression and activity of MMPs in the kidneys. Previous reports have demonstrated the important roles of MMPs in increasing susceptibility of defective Alport GBM to proteolytic degradation (9). The expression of MMP mRNA reported to be involved in this model was analyzed in the kidneys of 10-week-old mice, and this demonstrated strong upregulation of MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12 in the kidneys of Usag1–/–Col4a3–/– mice, while the expression of these MMPs was significantly less increased in the kidneys of Usag1+/–Col4a3+/– mice (Figure 5A). The proteolytic activity of these MMPs in the kidney extracts was also demonstrated by casin and gelatin zymography. Casein zymography showed a significant reduction in MMP-7 and MMP-12 activities (Figure 5B), and gelatin zymography demonstrated significant reduction of MMP-2 activity in the kidneys of 10-week-old Usag1–/–Col4a3–/– mice (Figure 5C). Also, the bands seen at 57 and 45 kDa in gelatin zymography, possibly representing MMP-3 activity (35), were significantly less in the kidneys of Usag1–/–Col4a3–/– mice. Recently, it was demonstrated that expression of MMP-12 was markedly upregulated in the glomeruli of Usag1–/–Col4a3–/– mice, and inhibition of MMP-12 preserved the integrity of GBM (29). Immunostaining for MMP-12 demonstrated a significant upregulation in the glomeruli and interstitium of Usag1–/–Col4a3–/– mice, while the induction was attenuated in Usag1+/–Col4a3+/– mice (Figure 5D). While MMP-12 is expressed by macrophages as well as glomerular cells such as podocytes (29, 36, 37), immunostaining of CD11b, a marker of monocytes and tissue macrophages, failed to demonstrate any significant infiltration of macrophages or monocytes in the glomeruli of 10-week-old Usag1–/–Col4a3–/– mice (data not shown), suggesting that the glomerular MMP-12 in Alport mice was not due to the macrophages that infiltrated the glomeruli.
USAG-1 was not expressed in the Alport glomeruli. USAG-1 is expressed predominantly in the distal tubules, more specifically, in the thick ascending limb, distal convoluted tubules, and connecting tubules in adult kidneys, and not expressed in glomeruli (26). In situ hybridization was used to determine the expression of USAG-1 in Usag1+/Col4a3−/− mice and demonstrated that USAG-1 expression was not detectable in the glomeruli of Usag1+/Col4a3−/− mice either (Figure 6A) and was confined to tubules.

USAG-1 colocalizes with BMP-7 in the macula densa. Deficiency of USAG-1 significantly attenuated glomerular pathology in the Col4a3−/− mouse model of Alport syndrome in spite of the absence of USAG-1 expression in glomeruli. Further experiments focused on the part of the distal tubule that came in contact with its own glomerulus, the macula densa (Figure 6B). To investigate potential mechanisms that are responsible for the beneficial effect of USAG-1 deficiency in Alport syndrome, the effect of BMP-7 and USAG-1 in cultured mesangial cells was examined. The expression of MMP-12 in cultured mesangial cells was upregulated by the administration of IL-1β and TGF-β, but not by the administration of MCP-1 (Figure 6D), in spite of the fact that MCP-1 is reported to stimulate MMP-12 expression in podocytes (29). The administration of BMP-7 suppressed TGF-β–induced MMP-12 upregulation in mesangial cells, and simultaneous administration of USAG-1 antagonized the suppressive effect of BMP-7 (Figure 6E). These results indicate that USAG-1 might enhance MMP-12 expression in the glomeruli by suppressing the inhibitory effect of BMP-7 and exacerbate glomerular disease progression in Alport syndrome.

Discussion

This study demonstrates that USAG-1 accelerates glomerular pathogenesis in a mouse model of human Alport syndrome, possibly through the crosstalk between the kidney tubules and its own glomerulus. Usag1+/Col4a3−/− mice demonstrated attenuated glomerular disease progression and preserved renal function in comparison with Usag1−/−Col4a3−/− mice and significantly decreased...
expression and activity of MMPs, which play key roles in disease progression of Alport syndrome. Furthermore, USAG-1 and BMP-7 colocalized in the macula densa, a part of the distal tubules in contact with its own glomerulus, and BMP-7 reduced MMP-12 expression in mesangial cells, which was antagonized by USAG-1.

**USAG-1 exacerbates glomerular injuries as well as tubular interstitial fibrosis.** Tubular damage and interstitial fibrosis are the final common pathways leading to end-stage renal disease (ESRD) (38, 39) irrespective of the nature of the initial renal injury, and the degree of tubular damage parallels the impairment of renal function (39). Severe tubulointerstitial fibrosis is observed following glomerular injury in Col4a3−/− mice, and this exacerbates renal function. Because Usag1−/− mice were resistant to tubulointerstitial fibrosis (27), this resistance might contribute at least in part to the preservation of renal function in Usag1−/−Col4a3−/− mice. In addition, Usag1−/−Col4a3−/− mice showed preserved GBM with less albuminuria in the early stage when tubular injury has yet to appear. Therefore, Usag1−/−Col4a3−/− mice were resistant to both glomerular and tubular injuries.

**USAG-1 increases the expression of MMP in Col4a3−/− mice.** The molecular mechanisms by which the altered GBM composition in Alport syndrome causes renal pathogenesis remain unclear. It is proposed that abnormal persistence of α1/α1/α2(IV) collagen network in the adult GBM is associated with increased susceptibility to proteolysis by proteases in Alport syndrome (3, 9) and pharmacological ablation of MMP activities, especially MMP-12, leads to a significant attenuation in Alport disease progression (29, 40). The expression and activities of MMPs were significantly upregulated in the kidneys of Usag1−/−Col4a3−/− mice, which is consistent with previous reports, and they were suppressed in the kidneys of Usag1−/−Col4a3−/− mice. The suppression of MMP activities probably contributed, at least in part, to slow glomerular pathogenesis in Usag1−/−Col4a3−/− mice. In addition, the administration of BMP-7 decreased the expression of MMP-12 in cultured mesangial cells, and USAG-1 antagonized the action of BMP-7. Therefore, USAG-1 might increase the expression of MMP in glomeruli and accelerate GBM destruction in Col4a3−/− mice.

**Other possible roles of USAG-1 and BMP-7 in glomerular pathogenesis in Col4a3−/− mice.** In addition to the inhibitory effect on MMP expression, BMP-7 possibly inhibits the progression of glomerular pathogenesis in Col4a3−/− mice at multiple steps. BMP-7 reduces the damage in podocytes (20, 41, 42) and mesangial cells (43–45) and attenuates the expression of inflammatory cytokines (46) and
apoptosis in several types of cells (12, 41). Antagonizing the beneficial effects of BMP-7 by USAG-1 might enhance these injuries and accelerate glomerular pathogenesis in Alport syndrome.

USAG-1 secreted from distal tubules reaches the glomerulus and accelerates glomerular injury. Although the mechanism by which USAG-1 secreted from distal tubules reaches the glomerulus and exacerbates glomerular pathogenesis is not entirely clear, a crosstalk may exist between the distal tubule and the glomerulus in the same nephron.

The distal tubule of a nephron makes contact with the vascular pole of its glomerulus from which the distal tubule originated. At this point, there is a plaque of very specialized and differentiated cells in the distal tubule known as the macula densa (Figure 6B). The macula densa detects changes in the distal tubular fluid composition and transmits signals to the adjacent extraglomerular mesangial cells and afferent arterioles (47–53). Extraglomerular mesangial cells are anatomically in continuity with the glomerular mesangial cells and transmit the signal from the macula densa to the glomeru-
The signals from the macula densa to the mesangial cells involve small diffusible substances (ATP or adenosine) released from the macula densa (47, 54). Multiphoton imaging demonstrates the water flow across the macula densa into the mesangial cell field at varying osmolarities in the luminal fluid (47). The histological characteristics of the macula densa also support the idea of substance transportation from the macula densa to mesangial cells: the nuclei of macula densa cells are apically located, while most of cell organelles tend to be located basally and laterally, thus indicating that the substances from the macula densa are possibly secreted basolaterally (Figure 7A). In addition, the basement membrane of the macula densa is fused with the basement membrane of the extraglomerular mesangial cells, indicating the lack of a barrier to interfere with the substance transportation from the macula densa to the glomeruluss (Figure 7A). Furthermore, Hugo et al. demonstrated that extraglomerular mesangial cells function as reserve cells for glomerular mesangial cells (55). Extraglomerular mesangial cells stimulated by substances secreted from the macula densa migrate into glomerulus after glomerular injury and repopulate as mesangial cells.

As shown in Figure 6C, both USAG-1 and BMP-7 are expressed in the macula densa, and USAG-1 secreted from the basolateral membrane of macula densa could inhibit the action of BMP-7 on the adjacent mesangial cells. In the experiments using cultured mesangial cells, BMP-7 significantly attenuated TGF-β–induced MMP-12 upregulation, and the inhibitory effect of BMP-7 was abolished by the addition of USAG-1. Moreover, BMP-7 reduced TGF-β–induced cytotoxicity in mesangial cells (data not shown). Therefore, USAG-1 might exacerbate glomerular pathogenesis in Alport syndrome through accelerating upregulation of GBM-degrading enzyme and cytotoxicity by inhibiting the renoprotective effects of BMP-7 (Figure 7B).

An alternative possibility that may explain the effect of USAG-1 on glomerular injury is an interaction between circulating USAG-1 and BMP-7 in plasma. BMP-7 is present in plasma at a concentration range of 100–300 pg/ml (12). Due to the lack of an effective ELISA system, the plasma level of USAG-1 remains to be determined. If an appropriate amount of USAG-1 is present in the circulation, it could therefore bind to BMP-7 and thus inhibit its activity. To test the effect of circulating USAG-1 in the progression of Col4a3–/– mice, we performed systemic gene transfer of USAG-1 expression vector to Usag1–/–Col4a3–/– mice and demonstrated that the difference in albuminuria between the gene transfer group and the control group was not statistically significant (Supplemental Figure 3).

**Figure 7** Hypothetical model for involvement of USAG-1 secreted from distal tubules in the pathogenesis of glomerular damage in Alport syndrome. (A) The macula densa (red), a part of the distal tubules, lies beside the extraglomerular mesangial cells (green), and the nuclei of the macula densa cells are apically located, suggesting the possibility that substances (blue) might be secreted from the basolateral membrane of the macula densa. Note that the basement membrane of the macula densa cells is continuous with the basement membrane of the extraglomerular mesangial cells. (B) In Usag1–/–Col4a3–/– mice (WT/KO), the mesangial cells are activated (purple) and secrete MMPs (scissors), which degrade GBM. Following GBM destruction, podocytes residing on the GBM are damaged (brown) and albuminuria is observed. BMP-7 (blue) secreted from the macula densa is captured by USAG-1, which is also secreted from the macula densa. As a consequence, BMP-7 cannot bind to its receptors and exert its renoprotective action. In Usag1–/–Col4a3–/– mice (KO/KO), BMP-7 secreted from the macula densa can bind to its receptors on mesangial cells and podocytes, and protect fragile alport GBM from degradation.

**Methods**

**Mice.** The Usag1–/– mice used in this study have been described previously (27), and Col4a3–/– mice were purchased from Jackson Laboratory (JAX mice strain 129-Col4a3tm1Dec/+) (62). Usag1–/–Col4a3–/– mice were generated by breeding Usag1–/– and Col4a3–/– mice. Col4a3–/– littermates (Usag1–/–Col4a3–/– mice) and WT littermates (Usag1–/–Col4a3–/– mice) served as controls. All animal studies were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, and performed in accordance with the guidelines of Kyoto University.
Age-matched mice were used for all studies. The ages of mice used in each experiment are described below.

Assessment of albuminuria. The mice were placed in metabolic cages, and urine was collected over a 24-hour period. During the urine collection, mice were allowed free access to food and water. Urinary albumin concentration was measured using the Albuwell M assay kit (Exocell).

Renal histopathology and electron microscopy. The kidneys were fixed in Carnoy’s solution and embedded in paraffin. Sections (2-μm thick) were stained with PAS for routine histological examination, and the degree of morphological change was determined for ten 10-week-old mice and five 6-week-old mice per group by experienced pathologists who were blinded to the genotypes. The following parameters were evaluated: percentage of hemorhagic glomeruli and sclerotic glomeruli; and tubular atrophy/interstitial fibrosis score. Tubular atrophy/interstitial fibrosis was graded as follows: grade 0, 0%–24%; grade 1, 25%–49%; grade 2, 50%–74%; grade 3, ≥75%.

Frozen sections of the kidneys were immunostained as previously described (63). The primary antibodies were against podocin (64), α1 (H11), and α3 (H51) chains of type IV collagen (a gift from Y. Sado; ref. 65), MCP-1 (R&D Systems), MMP-12 (Santa Cruz Biotechnology Inc.), nNOS (Cayman Chemical and Abcam), and LacZ (Cappel Laboratory). For double staining with β-gal, immunostaining was performed before β-gal staining to avoid the possibility that the deposition of X-gal might interfere with the antibody binding to the antigen. For electron microscopy, portions of the cortex were fixed in 2% glutaraldehyde and post-fixed in 1% osmic acid. After embedding, ultrathin sections were stained with uranyl acetate and lead citrate.

β-Gal staining and in situ hybridization. β-Gal staining and in situ hybridization were performed as described previously (26, 66). The probe for in situ hybridization of USAG-1 mRNA contained the 1.0-kb open reading frame with GC content of 52.6%. Hybridization was detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase and Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (Roche Diagnostics).

Immunoblots. Whole-kidney tissue was homogenized in RIPA buffer and subjected to immunoblotting as previously described (67). The primary antibodies were anti-phospho-Smad1/5/8 (Cell Signaling Technology), phospho-Smad2 (Upstate Biotechnology), and GAPDH (Fitzgerald Industries).

Quantification of mRNA by real-time RT-PCR. Real-time RT-PCR was performed as described previously (27). Specific primers were designed using Primer Express software (Applied Biosystems). Serially diluted cDNA was used to generate the standard curve for each primer, and the PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 95°C for 15 seconds, and 60°C for 1 minute for 40 cycles.

Cell cultures. Mouse mesangial cells were established from glomeruli isolated from a 4-week-old normal mouse (C57BL/6J) and characterized as described previously (68). Cells of passage numbers 18 to 21 were cultured in DMEM/F12 containing 20% fetal calf serum.

Assessment of MMP mRNA expression in mesangial cells. Mesangial cells were seeded at a concentration of 5 × 10^5/mL. After 24 hours, the culture medium was replaced with DMEM containing 0.5% bovine serum albumin. The cells were incubated for 72 hours with 10 ng/mL MCP-1 (R&D Systems), 250 pg/mL IL-1β (R&D Systems), or 3 ng/mL TGF-β (R&D Systems) in the presence or absence of 20 ng/mL BMP-7 (R&D Systems) and then were analyzed for MMP mRNA expression by real-time RT-PCR. All experiments were performed in quadruplicate.

Production of recombinant USAG-1/Flag protein. A recombinant C-terminally Flag-tagged USAG-1 protein (USAG-1–Flag) was produced using the Baculovirus Expression System (Invitrogen) and purified from culture medium by affinity absorption on anti-FLAG M2 affinity beads (Sigma-Aldrich). Protein concentrations were estimated by Coomassie staining.

Zymography. Renal proteins were extracted as previously described (69). Samples standardized for protein concentration of 60 μg/lane were electrophoretically separated in 10% SDS-polyacrylamide gels that contained 1 mg/mL gelatin or casein. After separation, gels were placed in 2.5% Triton X-100 in PBS, washed, and incubated in developing buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl2, and 0.02% Brij-35) overnight at 37°C. The gels were stained with 0.5% Coomassie blue R250 and then destained with 10% acetic acid, 40% methanol solution until the gelatinolytic bands were clearly seen.

Systemic gene transfer. Usag1−/−Col4a3−/− mice were injected with 300 μg of pcDNA3.1mUSAG-1 (cDNA for mouse USAG-1 cloned into the pcDNA3.1 expression vector) into the tibialis anterior muscle at 6 weeks as described (70) and were analyzed at 8 weeks for urinary albumin and renal histology.

Statistics. Data are presented as the mean ± SD. Statistical significance was assessed by Student’s t test for 2 group comparisons and by ANOVA, followed by Fisher’s protected least significant difference post-hoc test for multiple group comparisons. Significance was defined as a value of P < 0.05.

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Address correspondence to: Motoko Yanagita, Career-Path Promotion Unit for Young Life Scientists, Kyoto University Graduate School of Medicine, Yoshida-konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. Phone: 81.75.753.9310; Fax: 81.75.753.9311; E-mail: motoy@kuhp.kyoto-u.ac.jp.

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