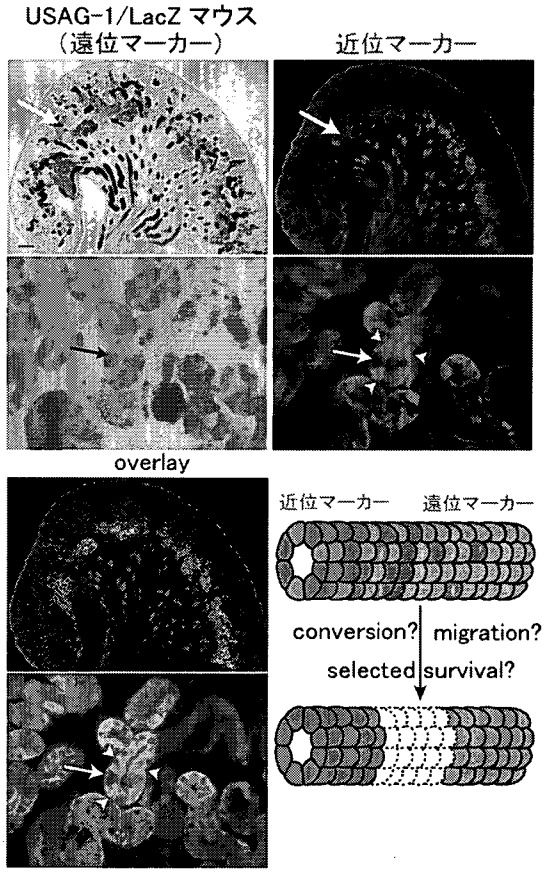
間葉系細胞集団がどのようにしてセグメントごとに異なる分化を遂げてネフロンをかたちづくるのか、そのセグメント特異的な分化のメカニズムはほとんど明らかにされておらず、再生医療を臨床応用する上での壁になっている。今回の結果は、未分化な尿細管の段階からそれぞれの細胞に cell fate が決まっていることを示唆するものである。

**図7 近位、遠位分化におけるパッチワーク現象**
**USAG-1/LacZ マウス**



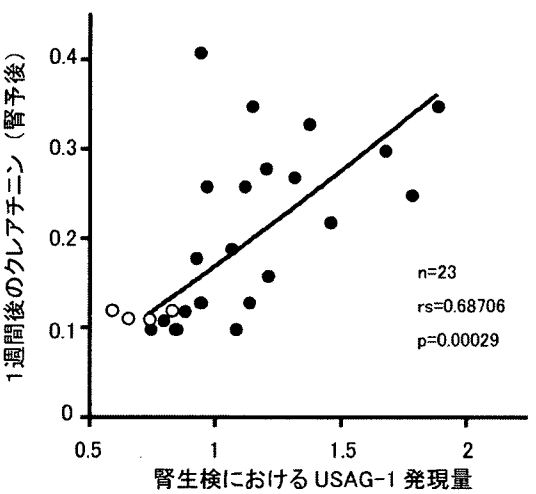

③ 腎 USAG-1 発現と予後に関する解析

申請者は①に述べたように、USAG-1 強陽性の再生尿細管が多いほど葉酸腎症が重度であることに気づき、腎 USAG-1 発現と将来の腎予後の相関を検討した。その結果、腎生検サンプルにおける USAG-1 発現量が多いほど、将来の腎機能が悪いことが明らかになった（図8）。

腎疾患患者の最大の関心事はいつ透析になるか（腎予後）であるが、現時点では腎障害がかなり進展するまで腎予後を正確に予知することは難しい。さらに医療者にとっても、将来腎機能が低下する群を正しく把握して早期から治療を行なうことが出来れば医療経済上極めて意義が大きい。

さらに USAG-1 の発現は腎臓に限局するので、腎臓における USAG-1 発現量は血中 USAG-1 濃度に反映されることが期待される。その場合には、血液検査で腎予後判定が可能となり、検診時のスクリーニングとしての有用性が極めて高い。USAG-1 は発現が腎臓特異的であること、発現量も多いこと、変化の幅が大きいことなど、よいバイオマーカーとしての要件を備えている。

**図8 腎 USAG-1 発現量は将来の腎機能と相関する**




④ 初代尿細管培養細胞を用いた USAG-1 発現に関する検討

腎障害時には尿細管が脱分化して Epithelial-to-mesenchymal transition (EMT) を起こし線維芽細胞様に変化すると言われている。申請者は研究計画の項で述べたように、新生仔の腎臓から初代尿細管培養細胞を樹立する方法を確立し、その細胞を用いて尿細管細胞を分化、脱分化させた際の USAG-1 の発

現変化を検討した。市販されている尿細管細胞株にEMTを惹起すると30−50%の細胞しか反応しないが、申請者が確立した細胞は100%が反応した。
その結果、USAG-1 は脱分化(EMT)時には発現が低下し、再分化時には発現が増加することが明らかとなった。これはUSAG-1 が尿細管障害時に発現低下し、再生尿細管で強発現するという上記の結果と一致している。
さらに申請者はUSAG-1/LacZ ノックインマウスからも同様の手法を用いて初代尿細管培養細胞を樹立し、その LacZ 活性をプレートリーダーで定量する方法を確立した。さらに BMP-7/LacZ ノックインマウス(Prof. Robertson から入手)からも同様に初代尿細管培養細胞を樹立した。
これらの細胞株を用いて chemical compound library のスクリーニングを行なうことで、USAG-1 発現を抑制し、BMP-7 発現を促進するような化合物を同定することが可能になった

⑤ 腎細胞癌モデルとUSAG-1 発現に関する検討

申請者は研究計画の項で紹介したラット腎発がんモデルにおける USAG-1 の発現を検討した。
その結果、USAG-1 は腎尿細管細胞が癌化し、その分化度が低くなるにつれ、発現低下することが明らかになった。
申請者らはヒト腎細胞癌においても、USAG-1 発現が低いことが悪性度のマーカーになりうると考えている。

4 結論、考察

USAG-1はよい腎予後マーカーとしての可能性がある。さらにUSAG-1発現解析から尿細管セグメント分化メカニズムの手がかりをえた。

5 健康危険情報
該当事項なし。

6 研究発表は主任研究者と重複。

# IV. 研究成果の刊行に関する一覧表

**レビュー誌への発表の主なもの（和文）**

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2柳田素子　Annual Review腎臓２００６
BMP-7と腎臓特異的BMP拮抗分子USAG-1

3柳田素子　医学のあゆみ
新規BMP antagonistであるUSAG-1(Uterine sensitization-associated gene-1)は腎不全治療薬のターゲットである

4柳田素子　医学のあゆみ
腎病変の発症進展に果たすGas6の役割

5柳田素子　化学と生物
USAG-1、BMP、そして腎不全治療薬の開発

6 柳田素子　「腎と透析」　これだけは知っておきたい分子腎臓学２００７
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Biochemical and Biophysical Research Communications 316 (2004) 490–500
BBRC
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# USAG-1: a bone morphogenetic protein antagonist abundantly expressed in the kidney

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**Abstract**

Bone morphogenetic proteins (BMPs) play critical roles in cellular proliferation, differentiation, and programmed cell death in multiple tissues. An increasing body of recent evidence has suggested that classes of molecules collectively termed BMP antagonists play important roles for the local regulation of BMP actions by binding BMPs and neutralizing their activities. Uterine sensitization-associated gene-1 (USAG-1) was previously reported as a gene of unknown function, preferentially expressed in sensitized endometrium of the rat uterus. Here, we show that USAG-1 is abundantly expressed in the kidney and functions as a BMP antagonist. Recombinant USAG-1 binds directly to BMPs and antagonizes the BMP-mediated induction of alkaline phosphatase in C2C12 cells. USAG-1 also induces formation of secondary axis and/or hyperdorsalization when its mRNA is injected to *Xenopus* embryos. In the early stage of mouse embryogenesis, USAG-1 is expressed in the first and second branchial arches and in metanephros, while in later stages the expression is confined to renal tubules and ameloblasts of teeth. Postnatally, the expression is further restricted to distal tubules of kidney, in a pattern similar to the localization of BMP-7, which has been shown to be important in the development of kidney and preservation of adult renal functions under pathological stresses. Collectively, we suggest that USAG-1 is a BMP antagonist that interacts with BMP-7 in the developing and adult kidney.


*Keywords:* Sclerostin; Ectodin; Wise; *Xenopus*; Mouse

Bone morphogenetic proteins (BMPs) are phylogenetically conserved signaling molecules that belong to the transforming growth factor (TGF)-β superfamily [1]. Although these proteins were first identified by their capacity to promote endochondral bone formation, they are now shown to be responsible for many signaling processes in proliferation, differentiation, and programmed cell death [2]. Furthermore, recent studies show that BMPs play important roles in neuronal differentiation, ventral mesoderm formation in *Xenopus* embryos, and pathophysiology of primary pulmonary hypertension in human [3–5].

The local concentration of active BMP is controlled not only by the precise regulation of its spatial and temporal expression [6], but also by certain classes of molecules that have been recently recognized as BMP antagonists [1]. These BMP antagonists function through direct association with BMPs, thus prohibiting

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BMPs from binding their cognate receptors [7–9]. One class of BMP antagonists possesses a secretory signal peptide and cysteine-rich domain, forming a subfamily of the cystine-knot superfamily of secreted factors [7].

BMP-7, the most abundant BMP in the fetal and adult kidney, is required for normal kidney development in mammals; *BMP7* null mice die shortly postpartum due to renal dysplasia [10,11]. Recent studies have demonstrated that the expression of BMP-7 is decreased in acute and chronic renal diseases. Moreover, systemic administration of recombinant BMP-7 leads to repair of severely damaged renal tubular epithelial cells, in association with reversal of chronic renal injuries [12–15]. The mechanism of this action involves the reversal of epithelial-to-mesenchymal transition of tubular epithelial cells with the induction of expression of genes such as E-cadherin, a key epithelial cell adhesion molecule [15]. These actions of BMP-7 are reminiscent of the effects that this morphogen exerts during development. Collectively, BMP-7 plays critical roles in normal development of the kidney, and postnatally in repairing processes of the renal tubular damage in kidney diseases.

The indispensable roles of BMP-7 in the kidney led us to postulate that BMP antagonist(s) may modulate the renal activities of BMP-7. So far the only known BMP antagonist expressed in the kidney is gremlin [16]. Intriguingly, deletion of the *gremlin* gene in mice resulted in defects of renal morphogenesis [17]. However, although gremlin can antagonize BMP-2 and BMP-4 [18,19], the effect of gremlin on BMP-7 has not been demonstrated. These observations suggest the existence of additional BMP antagonist(s) that interfere with the actions of BMP-7 in the kidney.

Through a genome-wide search for kidney-specific human transcripts, we found a novel gene, which encodes a secretory protein with a signal peptide and cysteine-rich domain. The rat ortholog of the gene was previously reported as a gene of unknown function that was preferentially expressed in sensitized endometrium, termed uterine sensitization-associated gene-1 (USAG-1) [19]. However, its biological role and expression patterns in embryonic and adult tissues have not been described. A search of the GenBank dbEST database on the amino acid sequence of USAG-1 detected only one similar EST entry, which encodes sclerostin, a recently identified BMP antagonist expressed in bones [20,21]. This led us to speculate that USAG-1 might be a BMP antagonist expressed in the kidney. To evaluate this hypothesis, we examined whether recombinant USAG-1 antagonizes the activities of BMPs in vitro and in vivo.

## Materials and methods

*Plasmid construction.* Human USAG-1 cDNA clone (DKFZP564D206) was purchased from Invitrogen (Carlsbad, CA) and subcloned into pCS2 vector for in vitro transcription (pCS2-hUSAG1). Human USAG-1 tagged with Flag and 6× His at its C-terminus were each cloned into pEF4 expression vector (Invitrogen) (pEF4-hUSAG1-Flag and pEF4-hUSAG1-His, respectively). We also constructed a plasmid, which includes the prolactin signal sequence followed by Flag tag [22] that was joined to USAG-1 residue 24, and a Myc tag in the C-terminus (pEF4-Flag-hUSAG1-Myc).

*Cell culture and transfection.* The C2C12 mouse myoblast cell line was obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) and 100 U/ml penicillin. COS-7 cells were cultured in DMEM supplemented with 10% FBS and plasmids were transfected using the LipofectAMINE reagent (Invitrogen) following manufacturer's instructions. Cells were cultured and maintained at 37 °C in a humidified atmosphere of 95% air/5% $CO_2$.

*Western blotting.* COS7 cells transfected with the designated plasmids were washed with phosphate-buffered saline (PBS) and solubilized in a buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation, applied to 15% SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose filter (Protran) (Schleicher & Schuell, Keene, NH). Membranes were subjected to immunoblotting with the designated antibodies, followed by visualization using the Enhanced Chemiluminescence detection system (Pharmacia, Piscataway, NJ). Antibodies used were: anti-Flag M1 and M2 monoclonal antibodies (Sigma, St. Louis, MO), anti-Myc monoclonal antibody and anti-His polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-BMP-2, -4, and -7 monoclonal antibodies (R&D systems, Minneapolis, MN). Rabbit polyclonal anti-USAG-1 antibody was generated by immunizing rabbits with a synthetic peptide corresponding to the N-terminal sequence of secreted USAG-1 (FKNDATEILYSHC), which was conjugated with keyhole limpet hemocyanin, using the Imject Maleimide Activate mcKLH kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

*Co-immunoprecipitation.* Lysates of COS7 cells transfected with a pEF4 mock vector or pEF4-hUSAG1-Flag (see above) were pre-incubated with BMPs for 1 h at room temperature in 1 ml binding buffer containing 137 mM NaCl, 8.1 mM $Na_2HPO_4$, 2.68 mM KCl, 1.47 mM $KH_2PO_4$, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 0.2% NP40, and 1 mg/ml BSA. After 1 h, 30 μl of anti-Flag M2 affinity gel (Sigma) was added to each reaction and incubated overnight. Gel was washed three times with 1 ml binding buffer containing 0.2% Tween 20 and subjected to immunoblotting with anti-BMP antibodies.

*Preparation of culture supernatant.* COS7 cells transfected with pEF4-hUSAG1-His (see above) were cultured in serum-free DMEM for 72 h, and the conditioned medium (100 ml) was concentrated to 100 μl using Centriprep YM10, Centricon YM10, and Microcon YM10 sequentially (Millipore, Bedford, MA).

*Assays for alkaline phosphatase activity.* BMP-2, -4, -7, and noggin-Fc fusion protein were purchased from R&D systems. C2C12 cells were plated at a density of 500 cells/well in a 96-well plate. After 24 h, C2C12 cells were stimulated with the designated BMP and indicated amounts of concentrated medium for 48 h. Cells were washed and extracted with a lysis buffer as described previously [23,24], and alkaline phosphatase activity was determined using *p*-nitrophenylphosphate (Sigma) as substrate.

*Xenopus embryo manipulation and microinjection.* Embryo manipulations and microinjections were performed as described previously [3]. Capped synthetic mRNA of human USAG-1 was generated by in vitro transcription of linearized templates by using the Megascript kit (Ambion, Austin, TX). mRNA was injected into the animal pole or into the marginal zone of a ventral blastomere at the four-cell stage.

*Reverse transcription-PCR analysis.* Total RNA was isolated from pooled (at least 15) animal caps and subjected to reverse

transcription (RT)-PCR analysis as described previously [25] by using the following conditions: 94 °C for 5 min, followed by designated numbers of cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. Primers for neural cell adhesion molecule (NCAM), muscle actin, and histone H4 have been described previously [25]. PCR products were visualized by electrophoresis on agarose gels followed by ethidium bromide staining.

*Northern blots.* Northern blot membranes were purchased from BD Biosciences (Palo Alto, CA). Membranes were hybridized with a full-length human and mouse USAG-1 cDNA probe random-prime labeled with [$^{32}$P]dCTP (10 mCi/ml; Amersham, Buckinghamshire, UK).

*In situ hybridization.* In situ hybridization was performed as previously described [26]. The sense or anti-sense USAG-1 riboprobes were prepared using mouse USAG-1 cDNA (621 bp) as a template.

*Luciferase assays.* 293T cells were transfected using LipofectAMINE 2000 (Invitrogen) with a total of 2 μg of various concentrations of plasmids as described [27]: 0.2 μg of pTOP-tk-luciferase reporter plasmid, 0.05 μg of internal control pRL-tk *Renilla* reporter plasmid (Promega, Madison, WI), the designated amount of Wnt1 expression vector (pCS2-mWnt1), pCS2-hUSAG1, DKK1 expression vector (pcDNA-hDKK1-Flag), and control vector (pCS2 expression vector) as stuffer. Luciferase activities were measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

## Results

### *Primary structure of USAG-1*

During a search of kidney-specific human transcripts in EST databases, we found the cDNA DKFZP564 D206. An inter-specific search for EST sequences homologous to this cDNA revealed that it is a human ortholog of the rat gene named uterine sensitization-associated gene-1 (USAG-1), which was previously reported as a transcript preferentially expressed in sensitized endometrium [19] (Fig. 1A). A mouse ortholog was also found from databases as RIKEN cDNA named 0610006G05Rik. Amino acid sequences encoded in the rat and mouse cDNAs are 97% and 98% identical to the human sequence, respectively, indicating high degrees of sequence conservation. A web-based domain prediction software (Simple Modular Architecture Research Tool: SMART) and signal peptide prediction software (SignalP) predicted this protein to be a member of the cystine-knot superfamily with an N-terminal signal peptide

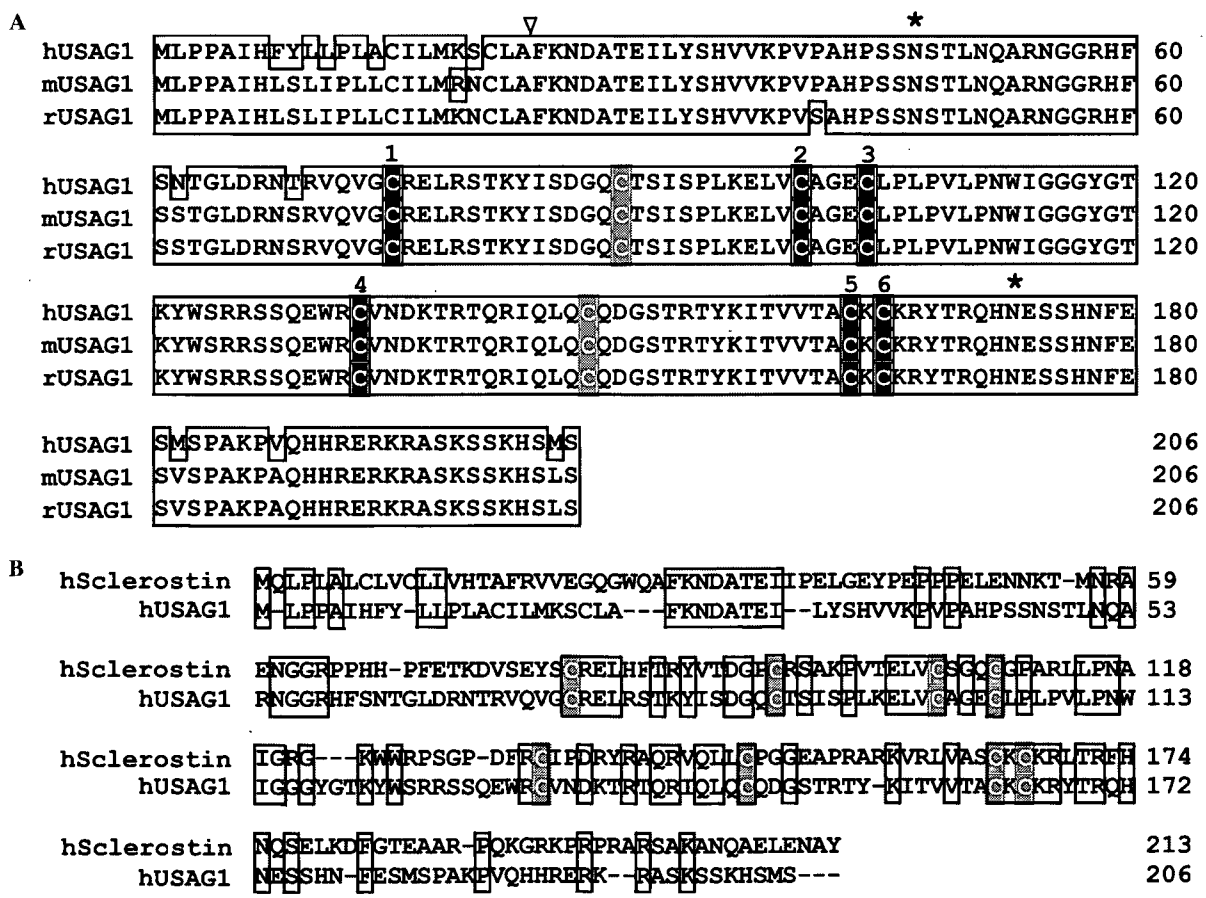


Fig. 1. Sequence alignment of USAG-1. (A) Alignment of encoded amino acid sequences of human, mouse, and rat USAG-1. Six cysteine residues (labeled 1–6) that constitute the predicted cystine-knot fold are shown in black boxes. The extra cysteine residues, conserved among species but not among other BMP antagonists, are shown in gray boxes. Residues shared by more than two species are enclosed. Putative cleavage site of signal sequence is shown by an arrowhead and potential sites for N-linked glycosylation by asterisks. (B) Alignment of human USAG-1 and human sclerostin, the *Sost* gene product. Common amino acid residues are enclosed. Note that all eight cysteine residues (shown in gray boxes) are conserved in both proteins.

of 23 amino acids. Further, homology searches revealed that USAG-1 has significant amino acid identities (38%) to sclerostin, the product of the *Sost* gene (Fig. 1B). Mutations of *Sost* are found in patients with sclerosteosis, a syndrome of sclerosing skeletal dysplasia [28]. Sclerostin is subsequently shown to be a new member of BMP antagonist expressed in bones and cartilages [20,21]. We therefore postulated that USAG-1 might be a BMP antagonist expressed in the kidney.

*Secreted form of human USAG-1*

To examine whether USAG-1 is a secreted protein, the culture medium of COS7 cells transfected with a mock vector or the pEF4-hUSAG1-His expression plasmid (see Materials and methods) was subjected to immunoblotting (Fig. 2A). Two major bands with apparent molecular masses of 28–30 kDa were detected only in the culture medium of COS7 cells transfected with pEF4-hUSAG1-His, showing that the His-tagged USAG-1 was indeed secreted. The observed molecular masses on SDS–PAGE were higher than the calculated mass of recombinant USAG-1 (20.1 kDa). The USAG-1 sequence contains possible N-glycosylation sites at conserved Asn47 and Asn173 (Fig. 1A), and the shift of molecular mass may be due to glycosylation at these sites. The two major bands of USAG-1 might also be due to heterogeneity of glycosylation.

Since a number of BMP antagonists, such as noggin and DAN, are known to form disulfide-bridged dimers [29], we next examined whether USAG-1 also forms a dimer. Because tags at the C-terminus might interfere with the formation of dimers, we transfected COS7 cells with native form of human USAG-1 (pCS2-hUSAG1; see Materials and methods) and the lysates were subjected to immunoblotting with anti-USAG-1 antibody raised against an N-terminal sequence of USAG-1 after the signal peptide. USAG-1 contained in the lysates of COS7 cells transfected with pCS2-hUSAG1 exhibited essentially the same apparent molecular mass under non-reducing and reducing conditions (Fig. 2B). Similar results were obtained with concentrated culture medium of COS7 cells transfected with pCS2-hUSAG1, indicating that human USAG-1 was secreted as a monomer (data not shown). This is consistent with the fact that USAG-1 does not have the extra cysteine residues present in noggin and DAN, which are necessary to make inter-molecular disulfide bridges.

To examine whether the N- or C-terminus of USAG-1 protein is proteolytically processed, several constructs

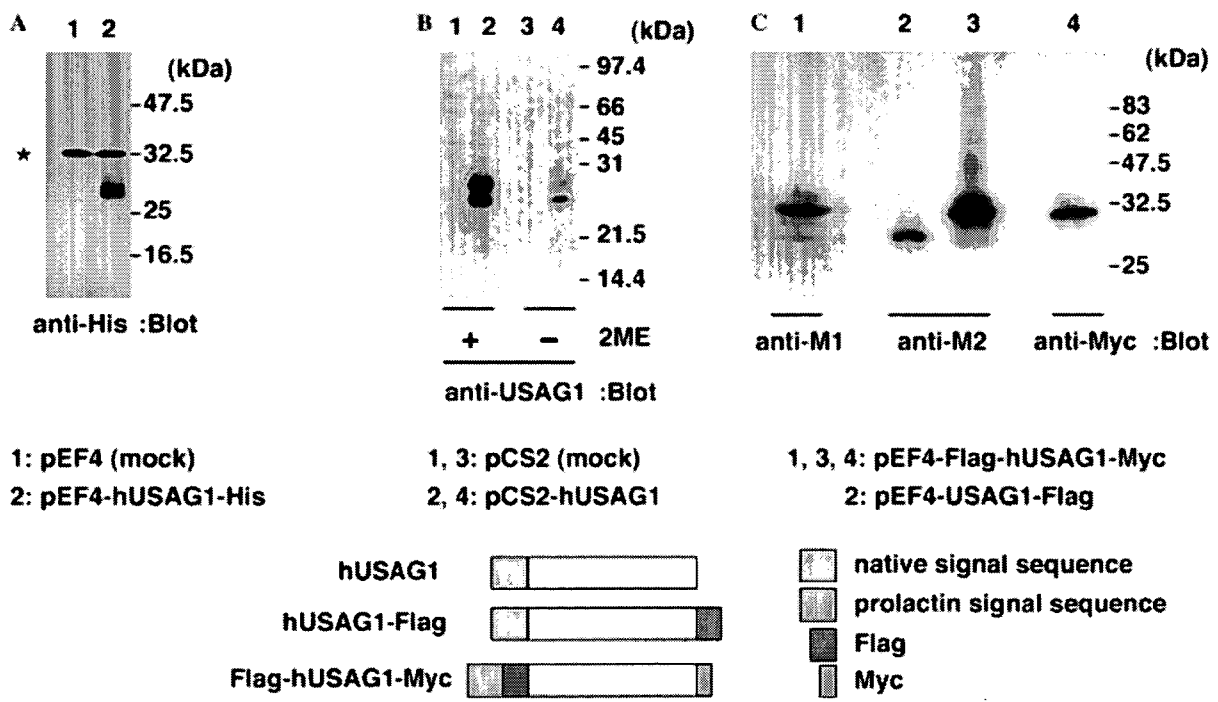


Fig. 2. Secretion of recombinant human USAG-1. (A) Culture supernatant from COS7 cells transfected with pEF4 mock vector (lane 1) or pEF4-hUSAG1-His (lane 2) was subjected to immunoblotting with anti-His antibody. Representative data from three independent experiments are shown. Non-specific signals are indicated by asterisks. (B) Lysates of COS7 cells transfected with pCS2 mock vector (lanes 1 and 3) and pCS2-hUSAG1 (lanes 2 and 4) separated by SDS–polyacrylamide gel electrophoresis under reducing (with 2-mercaptoethanol) (lanes 1 and 2) or non-reducing (without 2ME) (lanes 3 and 4) conditions were subjected to immunoblotting with anti-USAG-1 antibody. Representative data are shown from three independent experiments. (C) Lysates of COS7 cells transfected with pEF4-Flag-hUSAG1-Myc (lanes 1, 3, and 4) and pEF4-hUSAG1-Flag (lane 2) were subjected to immunoblotting with anti-Flag M1 (lane 1), anti-Flag M2 (lanes 2 and 3), and anti-Myc (lane 4) antibodies. Representative data are shown from three independent experiments.

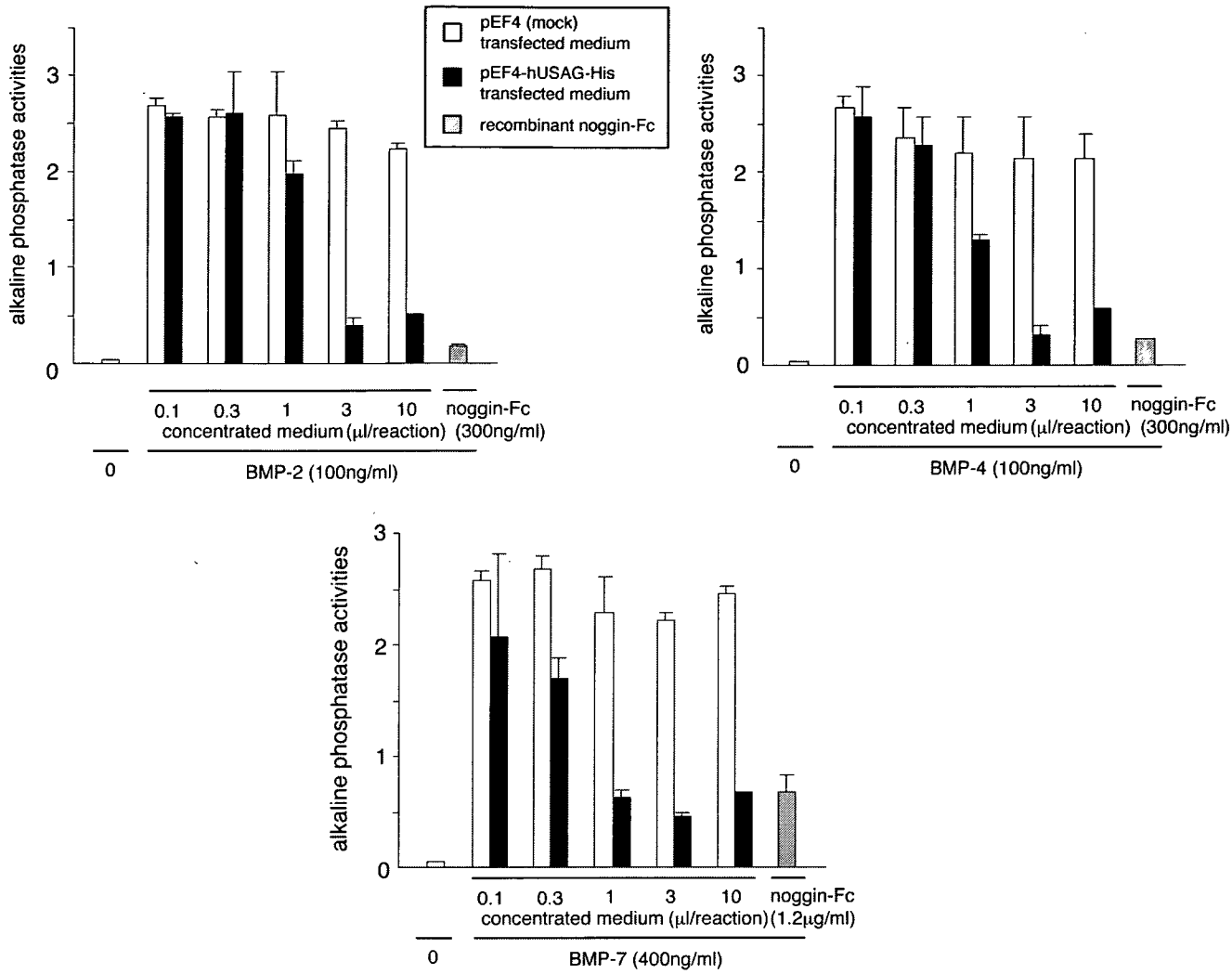


Fig. 3. Effects of USAG-1 on BMP-induced alkaline phosphatase activity in C2C12 cells. C2C12 cells were treated for 48 h with BMP-2 (100 ng/ml), BMP-4 (100 ng/ml) or BMP-7 (400 ng/ml), plus the indicated doses of concentrated culture medium from COS7 cells transfected with pEF4 mock vector (white bars) or pEF4-hUSAG1-His (black bars). Indicated doses of noggin-Fc were used as positive controls (gray bars). After treatment, cellular alkaline phosphatase activity was determined. Results are means ± SD for quadruplicate cultures. Representative data are shown from three separate experiments.

with different tags at its either terminus were expressed in COS7 cells. In the lysate of COS7 cells transfected with pEF4-USAG1-Flag (see Materials and methods), expressed protein is detectable with the anti-Flag M2 antibody (Fig. 2C), indicating that the tagged C-terminus of USAG-1 was not cleaved posttranslationally. In the lysates of COS7 cells transfected with pEF4-Flag-USAG1-Myc (see Materials and methods), protein was detectable either with the anti-Flag M1, anti-Flag M2 or anti-Myc antibody. Similar results were obtained with concentrated culture medium from these cells (data not shown), indicating that neither terminus of USAG-1 is cleaved during the transit through the secretory pathway.

*USAG-1 antagonizes the action of BMPs in C2C12 cells*

To determine whether USAG-1 can act as a BMP antagonist, we first examined the effects of USAG-1 on the activity of BMP-2, BMP-4, and BMP-7 inducing differentiation of C2C12 cells by measuring the alkaline phosphatase activity. BMP-2, -4, and -7 markedly stimulated the alkaline phosphatase activity in C2C12 cells as previously reported [23,24] (Fig. 3). Addition of culture supernatant from pEF4-USAG1-His-transfected COS7 cells inhibited BMP-2, -4 or -7-induced alkaline phosphatase activities in a dose-dependent manner, while addition of conditioned medium of mock transfected COS7 cells exerted no appreciable effects.

*USAG-1 inhibits endogenous BMP activity in Xenopus embryogenesis*

Next we examined whether USAG-1 antagonizes BMP activities in vivo in *Xenopus* embryos. Ventral injection of synthetic mRNA encoding a BMP antagonist is known to induce secondary axis and hyperdorsaliza-

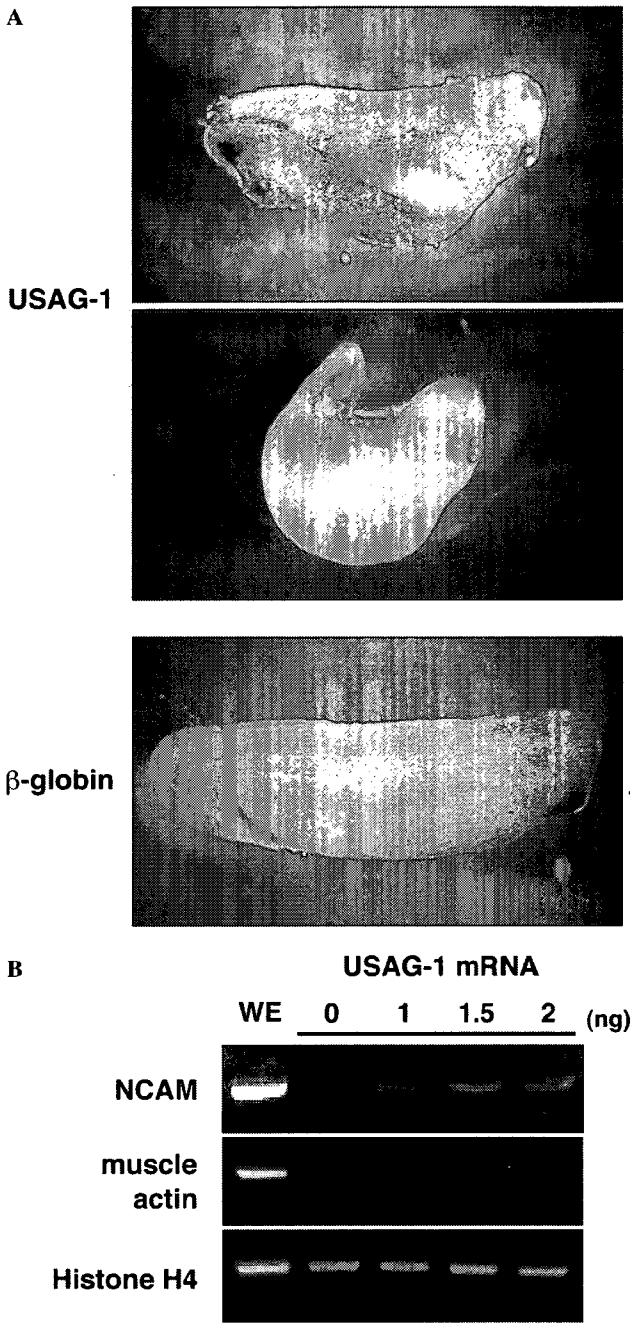


Fig. 4. USAG-1 inhibits endogenous BMP signaling in *Xenopus* embryos. (A) USAG-1 RNA (0.5 ng) was injected near the ventral midline of four-cell embryos. Typical resultant phenotypes are shown (top panel, secondary axis; middle panel, hyperdorsalized embryo). When 1 ng of β-globin RNA was injected, embryos developed normally (bottom panel). (B) Increasing doses of RNA encoding USAG-1 were injected near the animal pole of two-cell embryos. Animal caps were isolated from embryos at blastula stage 8 and cultured to stage 23. Total RNA was extracted from pooled caps and control embryos, and subjected to RT-PCR analysis. WE, whole embryo. Representative data are shown from three independent experiments.

tion in *Xenopus* embryos by blocking the ventralizing signal of BMPs [30]. We found that the injection of low doses of synthetic USAG-1 mRNA caused secondary axis formation and hyperdorsalized phenotype, in which the trunk and tail were severely reduced or lost (Fig. 4A, top and middle panels). In contrast, embryos developed normally when irrelevant mRNA (encoding β-globin) was injected (Fig. 4A, bottom). 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 secondary axis formation and hyperdorsalization (Table 1).

Table 1
The effects of USAG-1 on the formation of dorsalized phenotypes

| Injected RNA (pg) | | Dorsalized phenoptypes (%) | | | *N* |
|---|---|---|---|---|---|
| USAG-1 | β-Globin | Hyperdorsalization | Secondary axis | Total | |
| 0 | 0 | 0 | 0 | 0 | 50 |
| 0 | 500 | 0 | 0 | 0 | 28 |
| 0 | 1000 | 0 | 0 | 0 | 36 |
| 100 | 0 | 14 | 25 | 39 | 28 |
| 200 | 0 | 18 | 45 | 63 | 33 |
| 500 | 0 | 30 | 40 | 70 | 40 |
| 1000 | 0 | 41 | 36 | 77 | 56 |

Although these effects strongly suggest that USAG-1 acts as a BMP antagonist in vivo, it is known that similar effects are also observed when the activin signaling pathway is stimulated in *Xenopus* embryos [25]. Overexpression of activin induces the formation of mesoderm including Spemann organizer, and the induction of the organizer in turn forces dorsalization of *Xenopus* embryos. To determine whether the dorsalization phenotype we observed above represents a direct effect of inhibition of BMP signaling, or it is a consequence of mesodermal induction by activin signaling, we next examined the activities of USAG-1 mRNA on *Xenopus* animal-cap explants. Injection of mRNA for a BMP antagonist into the animal pole of *Xenopus* embryos induces neural differentiation of explants, as evidenced by the expression of neural markers such as NCAM [31,32], whereas the injection of activin is known to induce mesodermal markers such as muscle actin [25]. Injection of USAG-1 mRNA induced expression of NCAM in animal caps dose-dependently, but not that of muscle actin (Fig. 4B). These results indicate that USAG-1 directly antagonizes BMP signaling in *Xenopus* embryos.

### *USAG-1 directly binds BMP-2, -4, and -7*

Since many BMP antagonists are known to physically bind BMPs to neutralize their activities, we examined whether USAG-1 binds directly to BMP proteins by co-immunoprecipitation. Lysates of COS7 cells transfected with pEF4 mock vector or pEF4-hUSAG1-Flag (see Materials and methods) were incubated with the anti-Flag M2 affinity gel in the presence or absence of

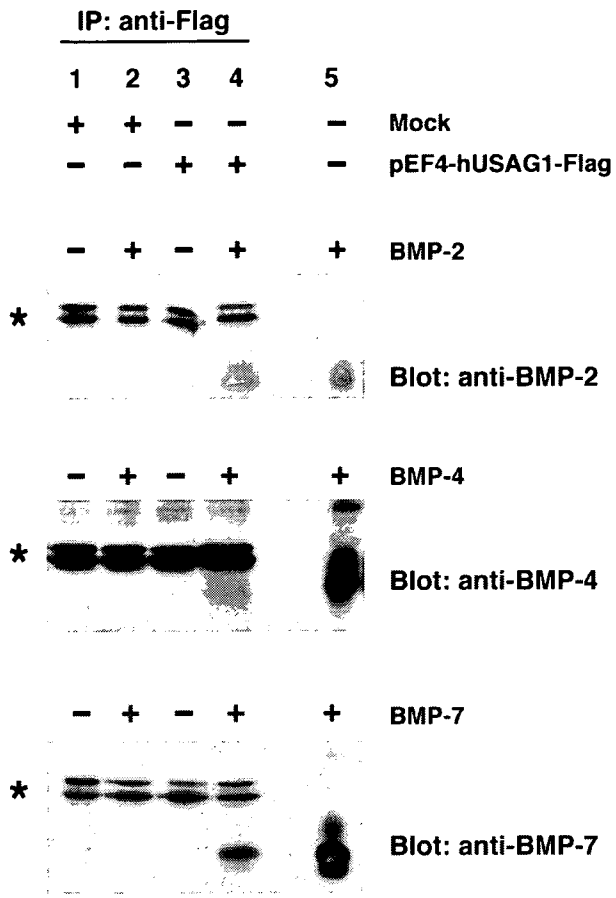


Fig. 5. USAG-1 binds BMP-2, -4, and -7. Lysates of COS7 cells transfected with pEF4 mock vector (lanes 1 and 2) and pEF4-hUSAG1-Flag (lanes 3 and 4) were incubated with anti-Flag M2 antibody affinity gel in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of BMP (450 ng). Gel was washed and subjected to immunoblotting with anti-BMP antibodies. Lane 5 shows the positive control for immunoblotting of BMP-2, -4, and -7 (50 ng/lane). Representative data are shown from three independent experiments.

recombinant BMP proteins. Gel was washed and subjected to immunoblotting with anti-BMP antibodies. We detected BMP-2, -4, and -7 co-precipitated with USAG-1-Flag, demonstrating that USAG-1 physically associates with these BMP proteins (Fig. 5).

*Tissue distribution of USAG-1 in fetal and adult mice*

Because USAG-1 inhibits the dorsalizing activities of BMPs in *Xenopus* embryos, we postulated that USAG-1 might play roles in embryogenesis and we examined the expression of USAG-1 in mouse embryos. Expression of USAG-1 mRNA was first detected on embryonic Day 11 (E11) in the whole mouse embryo and increased towards E17 (Fig. 6A). In situ hybridization to mouse embryos showed that, on E11.5, USAG-1 mRNA was expressed in several tissues including the first and second branchial arches, pharynx, and metanephros (Figs. 7A–C). On E17.5, strong USAG-1 mRNA expression was restricted to kidney tubules and ameloblasts in teeth

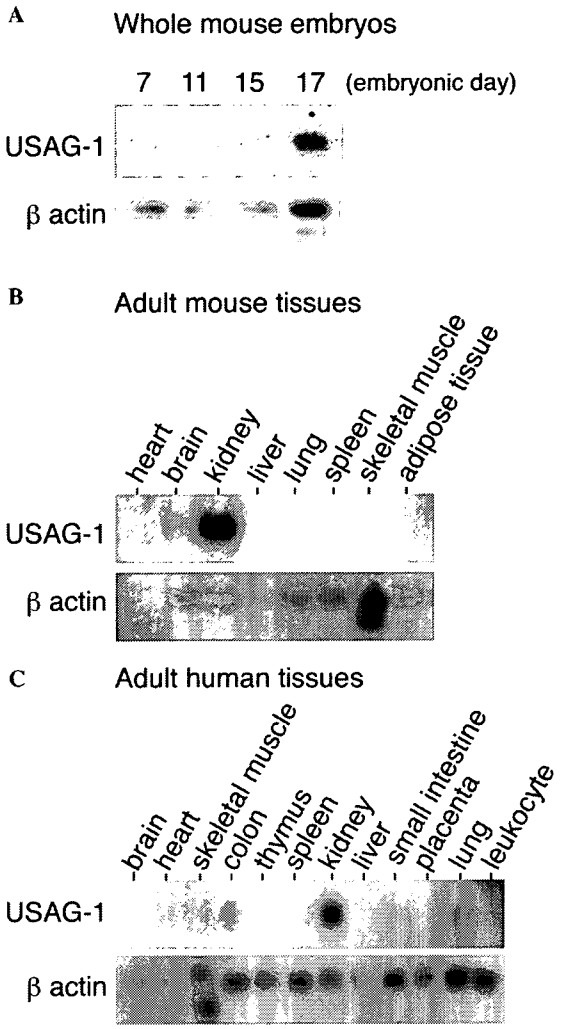


Fig. 6. Tissue distribution of USAG-1 mRNA. (A) Northern blot analysis of mouse whole embryos with embryonic day noted above each lane. (B,C) Northern blots of mouse (B) and human (C) tissues. Lower panels, membranes were re-hybridized with β-actin probe as internal control.

(Figs. 7D–F). In addition, moderate expression was observed in hair follicles (Fig. 7G), choroids plexus of the fourth cerebral ventricle (Fig. 7H), and ependymal cells in the lateral ventricle of the brain (Fig. 7I).

We also examined the distribution of USAG-1 mRNA in adult tissues. The expression was by far most abundant in the kidney, as shown by multi-tissue Northern blots of mice and humans (Figs. 6B and C). Weak expression was observed in the mouse brain and human lung. In situ hybridization to sections of adult mouse kidneys revealed a highly localized expression in the distal tubules (Figs. 7J–L). No expression was observed in glomeruli or blood vessels in the kidney.

*Effects of USAG-1 on Wnt1 signaling*

While the present study was being carried out, a *Xenopus* ortholog of USAG-1 (termed Wise) was

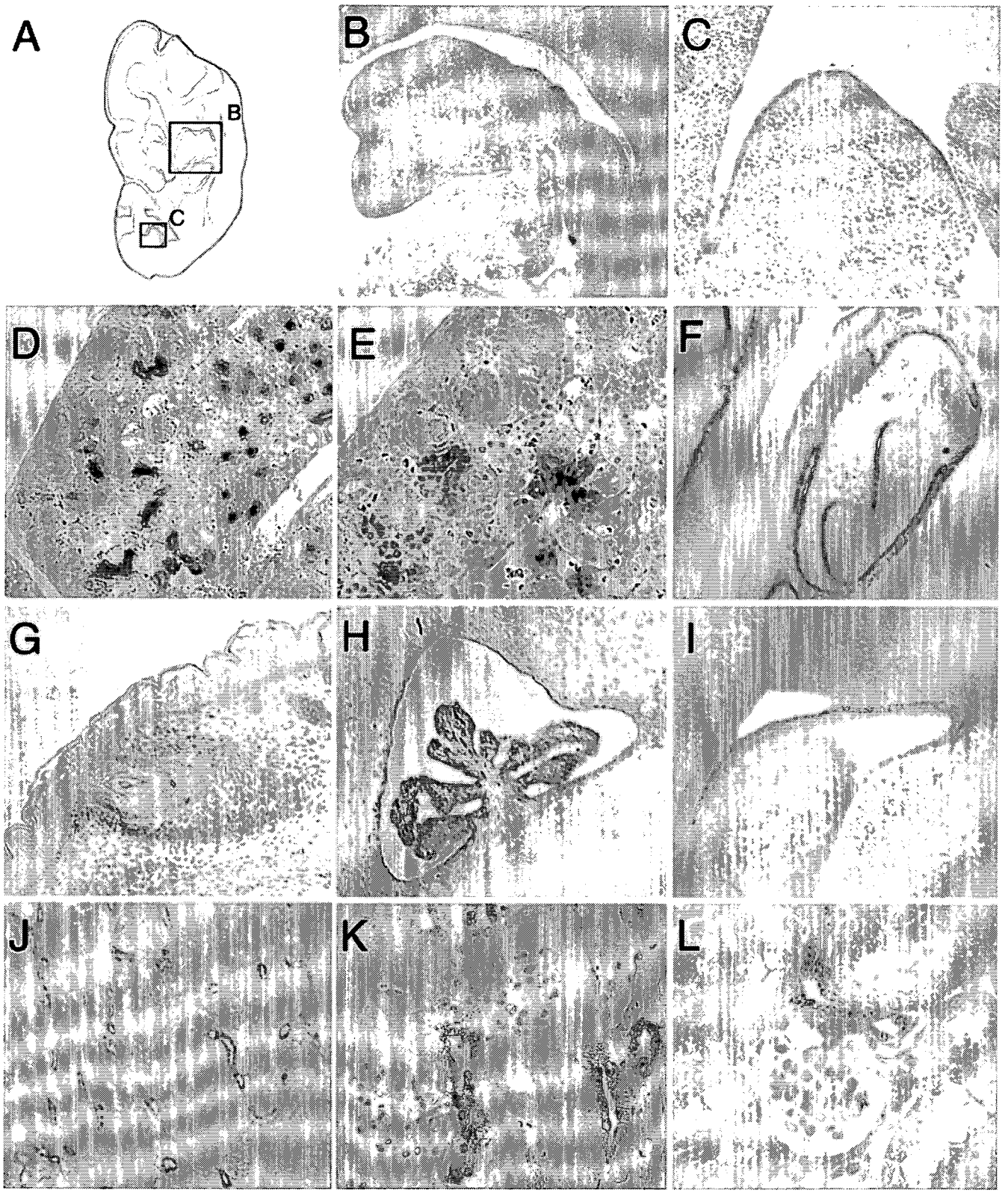


Fig. 7. In situ hybridization of mouse USAG-1 mRNA. (A) Schematic drawing of sagittal section of E11.5 mouse embryo, showing the approximate fields for (B) and (C). (B) USAG-1 is expressed in the first and second branchial arches and in pharynx at E11.5. (C) USAG-1 is also expressed in metanephros. In E17.5 mouse embryos, the expression of USAG-1 is prominent in kidney tubules [(D) lower magnification, (E) higher magnification], and in ameloblasts of teeth (F). Moderate expression is observed in hair follicles (G), choroids plexus in the fourth brain ventricle (H), and in ependymal cells of the lateral ventricle (I). In adult kidney, the expression of USAG-1 is confined to distal tubules [(J) lower magnification, (K,L) higher magnification].

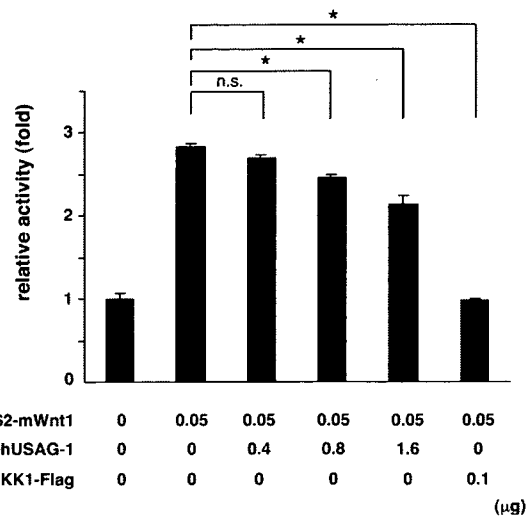


Fig. 8. Effect of USAG-1 on Wnt-1-induced β-catenin/TCF-regulated transcription. 293T cells were transfected with pTOP-tk-luciferase reporter plasmid and pRL-tk *Renilla* reporter plasmid, plus pCS2-hUSAG1, pCS2-mWnt1, and/or pcDNA-hDKK1-Flag as designated. Luciferase activities were measured after 24 h and expressed relative to the samples not transfected with pCS2-mWnt1. Results are means ± SD for triplicate wells. Representative data are shown from three independent experiments. $^*P < .0001$; n.s., not significant; by Fisher's PLSD.

reported to function as a context-dependent activator and inhibitor of Wnt signaling by directly binding LRP6 [33]. Therefore, we examined the possible effect of human USAG-1 on the Wnt1-induced β-catenin/TCF-regulated transcription, using a luciferase reporter assay as described previously [27]. Co-transfection of pCS2-hUSAG1 slightly but dose-dependently inhibited the luciferase activities induced by Wnt1. However, the effect of USAG1 was considerably weaker compared to that of the canonical Wnt antagonist DKK1 (Fig. 8).

## Discussion

In this study, we have shown at the cellular and organismal levels that USAG-1 inhibits BMP actions. We have found that the expression of USAG-1 mRNA increases towards the late stage of mouse embryogenesis, and it is primarily confined to distal tubules of the kidney in adult mice. Avsian-Kretchmer and Hsueh [34] very recently reported the human ortholog of USAG-1 in their genome-wide search for cystine-knot proteins. Based on the sequence homology to sclerostin [20], they suggested the possibility that USAG-1 is a BMP antagonist. Our present results are consistent with their hypothesis and define USAG-1 as a novel BMP antagonist in the kidney. Furthermore, during the preparation of the present manuscript, Laurikkala et al. [35] independently reported that the mouse and human ortholog of USAG-1 (termed Ectodin) acts as a BMP antagonist in tooth enamel knot. Our present observations are also consistent with their results. The primary structures of USAG-1 and sclerostin are rather dissimilar to other known BMP antagonists. USAG-1 and sclerostin are secreted as monomers, while many other BMP antagonists form dimers. The expression of USAG-1 and sclerostin is also relatively confined to a certain tissue, i.e., USAG-1 in the kidney and sclerostin in bones, whereas other BMP antagonists tend to be widely expressed in many tissues. We thus propose that USAG-1 and sclerostin represent a distinct family of tissue-specific BMP antagonists.

The expression of USAG-1 mRNA in adult mouse kidney is restricted to the epithelial cells of distal collecting tubules. Previous studies showed the expression of BMP-7 in the tubular epithelial cells of all segments, although staining was most intense in distal convoluted tubules and collecting ducts [36]. The type II receptor for BMP-7 is expressed in the renal cortex as well as in the medulla, though the injection of radiolabeled BMP-7 revealed that majority of labeled BMP-7 accumulated in glomeruli and the adjacent distal convoluted tubules [37]. Thus, the cellular distribution of BMP-7 and its receptor is partially overlapping with that of USAG-1; it is attractive to speculate that USAG-1 interacts with BMP-7 in the distal convoluted tubules to modulate the local BMP functions.

Our preliminary studies have shown that renal expression of USAG-1 is upregulated in disease models such as nephrosclerosis in Dahl salt-sensitive hypertensive rats (data not shown). It has been shown that renal expression of BMP-7 is reduced in acute and chronic renal injuries, and the reduction plays an important role in progression of the disease [36]. Therefore, we speculate that increased expression of USAG-1 in these pathological situations might inhibit the actions of remaining BMP-7 available and may further worsen the injuries to kidney tubules.

In embryogenesis, BMP-7 has an essential role in metanephric development [10,11,38]. Analysis of *BMP-7* null mice has shown that BMP-7 expression is required for coordinating the branching morphogenesis of the ureteric bud and for renal epithelium formation from condensing mesenchyme [10,11]. USAG-1 mRNA in the metanephros at E11.5 is confined to the epithelial lining (Fig. 7C), while BMP-7 is expressed in the ureteric bud and the surrounding mesenchymal condensates [39]. BMP-4, also known to play a role in kidney organogenesis, is expressed in the mesenchymal cells surrounding the Wolffian duct but not in the metanephric mesenchyme at E10.5 [40]. Therefore, the spatial pattern of USAG-1 expression in these embryonic stages is not closely overlapping with that of BMPs, and the possible role of USAG-1 in the early renal organogenesis remains to be determined. However, in the later stage of renal development, both USAG-1 and BMP-7 localize within the tubules, suggesting the possibility that USAG-1 interferes with the activities of BMP-7 there. Precise

evaluation of USAG-1 expression through developmental stages will provide further insights into the interaction between USAG-1 and BMPs in kidney organogenesis.

Among the known BMP antagonists, Gremlin is also expressed in the kidney [16] and has been considered to play a role in the progression of certain kidney diseases [41]. However, there are important differences between Gremlin and USAG-1: first, target molecules of Gremlin are considered to be BMP-2 and BMP-4 [18], whereas USAG-1 antagonizes BMP-7 as well. Second, Gremlin is expressed in mesangial cells in the glomerulus [16], while USAG-1 is expressed in epithelial cells of distal tubules. Finally, according to phylogenetic analyses [34], the distance between Gremlin and USAG-1 is considerably far. We speculate that these two BMP antagonists play distinct roles in the development and progression of kidney diseases.

In later developmental stages, USAG-1 is highly expressed also in ameloblasts of teeth. Ameloblasts secrete enamel matrix extracts, of which the mineralization remains incomplete until apathetic crystals accumulate [42]. It has recently been reported that BMPs in enamel extracts induce mineralization of teeth and exogenous administration of recombinant noggin inhibits these activities in tissue cultures [43]. Thus far, however, no endogenous BMP antagonist has been described in developing tooth tissues. We postulate that USAG-1 secreted from ameloblasts may modulate the activities of BMPs and regulate mineralization of enamel matrix extracts. Indeed, a very recent report seems to support this hypothesis [35].

USAG-1 is also expressed in hair follicles of E17.5 mouse embryos. It has been reported that noggin is expressed in hair follicles and plays a critical role in the induction of hair follicle by modulating the activities of BMPs [44,45]. USAG-1 might also share some roles with noggin in hair follicle induction.

Rat USAG-1 was originally reported as a gene preferentially expressed in the receptive rat endometrium [19]. We examined the expression of USAG-1 mRNA in the uterus of 6-week-old female Wistar rats at random, without synchronizing their stage of estrous cycle (data not shown). The levels of expression in the uterus varied from undetectable to almost comparable to the levels in the kidney, probably depending on the estrous cycle.

In partial disagreement with a recent report on the *Xenopus* homolog of USAG-1 [33], we have found in the present study that human USAG-1 inhibits the Wnt1 action in a TCF-mediated transcriptional reporter assay only slightly, an effect considerably weaker than that of the established Wnt antagonist DKK1 in the same assay. Close relationships between the Wnt and BMP pathways have been recently reported: for instance, noggin and DKK1 cooperate in head induction [46], while the expression of DKK1 is regulated by BMP-4 in limb development [47]. Furthermore, a BMP antagonist called Cerberus has a binding site for Wnt proteins that is distinct from the BMP binding site, and antagonizes Wnt activities by directly binding Wnt [48]. The effect of USAG-1 on the Wnt pathway requires further investigation.

## Acknowledgments

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# Uterine sensitization-associated gene–1 (USAG-1), a novel BMP antagonist expressed in the kidney, accelerates tubular injury

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**Dialysis dependency is one of the leading causes of morbidity and mortality in the world, and once end-stage renal disease develops, it cannot be reversed by currently available therapy. Although administration of large doses of bone morphogenetic protein–7 (BMP-7) has been shown to repair established renal injury and improve renal function, the pathophysiological role of endogenous BMP-7 and regulatory mechanism of its activities remain elusive. Here we show that the product of *uterine sensitization-associated gene–1* (*USAG1*), a novel BMP antagonist abundantly expressed in the kidney, is the central negative regulator of BMP function in the kidney and that mice lacking USAG-1 (*USAG1*$^{-/-}$ mice) are resistant to renal injury. *USAG1*$^{-/-}$ mice exhibited prolonged survival and preserved renal function in acute and chronic renal injury models. Renal BMP signaling, assessed by phosphorylation of Smad proteins, was significantly enhanced in *USAG1*$^{-/-}$ mice with renal injury, indicating that the preservation of renal function is attributable to enhancement of endogenous BMP signaling. Furthermore, the administration of neutralizing antibody against BMP-7 abolished renoprotection in *USAG1*$^{-/-}$ mice, indicating that USAG-1 plays a critical role in the modulation of renoprotective action of BMP and that inhibition of USAG-1 is a promising means of development of novel treatment for renal diseases.**

## Introduction

Despite a significant increase in understanding of the pathophysiology of renal diseases, the incidence of end-stage renal disease (ESRD) is still increasing. Tubular damage and interstitial fibrosis are the final common pathway leading to ESRD (1, 2), irrespective of the nature of the initial renal injury, and the degree of tubular damage parallels the impairment of renal function (2). Once tubular damage is established, it cannot be reversed or repaired by currently available treatment, and renal function deteriorates to renal failure, which is often life threatening (3). If we can come up with an agent that can reverse established tubular damage, it would significantly reduce the need for dialysis. Bone morphogenetic protein–7 (BMP-7) is a promising candidate for such an agent, because it is reported to protect the kidney from renal injury (4–8). BMP-7 is known to play essential roles in kidney development, because BMP-7–null mice die shortly after birth due to severe renal hypoplasia (9, 10). BMP-7 is also abundant in the adult kidney, especially in distal tubule epithelial cells (11, 12). Recently, several reports indicated that the expression of BMP-7 is decreased in renal disease models (5, 6, 13–16) and that administration of recombinant BMP-7 at pharmacological doses repairs chronic renal injury (4–8). However, the pathophysiological role and regulatory mechanism of endogenous BMP-7 remain elusive.

The local activity of endogenous BMP is controlled not only by regulation of its expression, but also by certain classes of molecules termed BMP antagonists (17). BMP antagonists function through direct association with BMP, thus inhibiting the binding of BMP to its receptors. *Uterine sensitization-associated gene–1* (*USAG1*) encodes a secreted protein and was initially found as a gene of unknown function whose expression was upregulated in sensitized endometrium of the rat uterus (18). Recently, Avsian-Kretchmer et al. suggested USAG-1 as a candidate for a novel BMP antagonist using bioinformatic analysis (19). Furthermore, Laurikkala et al. demonstrated USAG-1 to be a BMP antagonist expressed in teeth (20).

We independently identified USAG-1 to be a novel BMP antagonist, abundantly expressed in the kidney (21). The expression of USAG-1 is abundant in renal tubules and teeth in late embryogenesis and in adult tissues it is by far most abundant in the kidney, especially in the distal tubule with a pattern similar to that of BMP-7. From these findings, we hypothesized that USAG-1 might regulate the renoprotective action of BMP-7 in the adult kidney.

To evaluate this hypothesis, we generated *USAG1*-knockout (*USAG1*$^{-/-}$) mice and induced acute and chronic renal disease models in which renal tubules, but not glomeruli, were mainly damaged.

**Nonstandard abbreviations used:** BMP-7, bone morphogenetic protein–7; EMT, epithelial-mesenchymal transition; MCP-1, monocyte chemotactic protein-1; PTEC, proximal tubule epithelial cell; USAG1, uterine sensitization-associated gene–1; UUO, unilateral ureteral obstruction.

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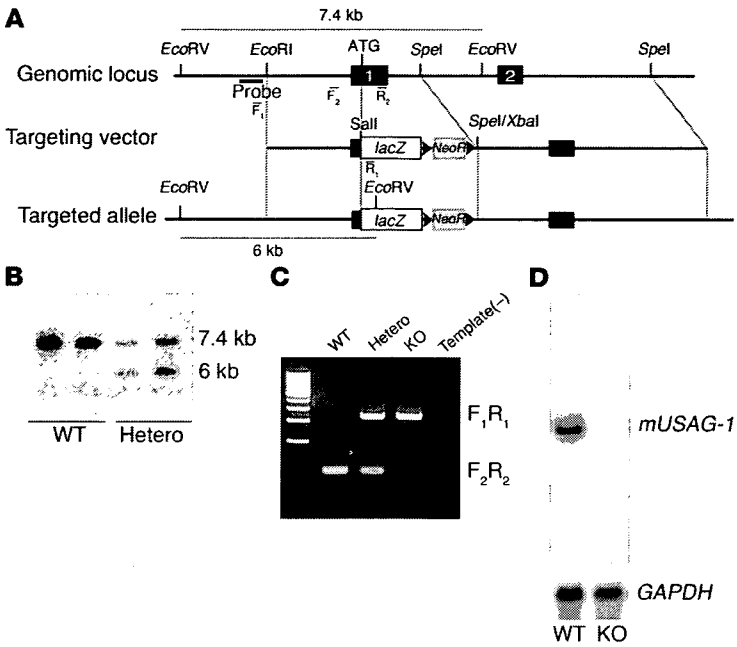


**Figure 1**
Generation of *USAG1*$^{-/-}$ mutation by gene targeting. **(A)** *USAG1*–null allele was generated by homologous recombination in ES cells. Exon 1 (black box) and part of the intron were replaced with a *lacZ* gene (white box) and the *Neo*R cassette (gray box). **(B)** Analysis of *USAG1*$^{+/+}$ (WT) and correctly targeted heterozygous (Hetero) ES cell clones by Southern blot analysis using 5′ genomic probe (thick black line in **A**). **(C)** PCR genotyping of $F_2$ littermates. Template(–) is the negative control. **(D)** Northern blot analysis of *USAG1* mRNA in the kidney of *USAG1*$^{+/+}$ and *USAG1*$^{-/-}$ (KO) mice.

## Results

*Generation and analysis of USAG1*$^{-/-}$ *mice.* *USAG1*$^{-/-}$ mice were generated by deleting the first exon including the transcription initiation codon, the signal peptide, and the following 46 amino acids (Figure 1). *USAG1*$^{-/-}$ mice were born at the ratio expected according to Mendel's law of heredity and were viable, fertile, and appeared healthy except that they exhibited supernumerary teeth, both in the incisors and molars, and fused teeth in the molar region (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI25445DS1). Although there was variation in the sites of extra teeth and fused teeth, this tooth phenotype was fully penetrant. Food consumption was not disturbed by this tooth phenotype in *USAG1*$^{-/-}$ mice (data not shown).

*Attenuated acute tubular injury in USAG1*$^{-/-}$ *mice.* To induce acute tubular injury, we utilized a cisplatin nephrotoxicity model (22–24). Administration of a nephrotoxic agent, cisplatin, to wild-type littermates caused acute tubular injury that resulted in severe renal failure. Within the first 3 days, 54% of wild-type mice died, while 92% of *USAG1*$^{-/-}$ mice survived the period (Figure 2A). The renal function of *USAG1*$^{-/-}$ mice on day 3 was significantly preserved compared with that in wild-type littermates (Figure 2B). Histological examination of the kidneys of wild-type mice on day 3 showed severe proximal tubular damage, while this change was markedly reduced in *USAG1*$^{-/-}$ mice (Figure 2, C and D). Expression of E-cadherin, a marker for tubular epithelial integrity (25), was markedly reduced in the kidneys of wild-type mice, while its expression was preserved in *USAG1*$^{-/-}$ mice (Figure 2E). Tubular apoptosis, a characteristic feature of tubular injury in cisplatin nephrotoxicity (23), was also significantly reduced in *USAG1*$^{-/-}$ mice (Figure 2F). As reported previously (24), cisplatin administration resulted in upregulation of TNF-α, IL-1β, monocyte chemotactic protein-1 (MCP-1), TGF-β1, and type IV collagen expression in the kidney of wild-type mice. However, the induction of these genes was completely abolished in *USAG1*$^{-/-}$ mice (Figure 2G). Infiltration of macrophages and monocytes in the kidney was also significantly reduced in *USAG1*$^{-/-}$ mice (Figure 2H), in accordance with the reduction of MCP-1 expression (Figure 2G). Expression of BMP-7 was comparable between wild-type mice and *USAG1*$^{-/-}$ mice before and after injection of cisplatin (Figure 2G).

*Renal fibrosis is reduced in USAG1*$^{-/-}$ *mice.* As a model of chronic renal injury, we performed unilateral ureteral obstruction (UUO) (26, 27) in both *USAG1*$^{-/-}$ mice and wild-type mice, and the kidneys were harvested 14 days after the operation. In wild-type mice, the obstructed kidney showed dilatation/degeneration of renal tubules and interstitial fibrosis, whereas the normal architecture was preserved in *USAG1*$^{-/-}$ mice, except for mild dilatation of tubules (Figure 3, A and B). Expression of E-cadherin was markedly reduced in the kidneys of wild-type mice, while its expression was preserved in *USAG1*$^{-/-}$ mice (Figure 3C). Furthermore, expression of α-SMA, a marker of tubulointerstitial myofibroblasts (28), was upregulated in the interstitium of the obstructed kidney of wild-type mice, while high expression of α-SMA was restricted to vascular smooth muscle cells in *USAG1*$^{-/-}$ mice (Figure 3D). Since expansion and fibrosis of the renal interstitium is another characteristic feature of UUO (6), we examined the deposition of type IV collagen, which is a normal component of the tubular basement membrane. The basement membranes of neighboring tubules are adjacent to each other in the normal kidney. In the obstructed kidney of wild-type mice, expansion of the interstitial component increased the distance between adjacent basement membranes, and type IV collagen produced by interstitial myofibroblasts was aberrantly expressed in the interstitium. However, in the obstructed kidney of *USAG1*$^{-/-}$ mice, the distance between the basement membranes was significantly smaller than that in wild-type mice (Figure 3E). Expression of TNF-α, IL-1β, MCP-1, TGF-β1, and type IV collagen was markedly upregulated on day 14 in the obstructed kidney of wild-type mice. In contrast, the induction of these genes was significantly attenuated, by 33%, 46%, 37%, 75%, and 23%, respectively, in *USAG1*$^{-/-}$ mice (Figure 3F). Expression of BMP-7 in the obstructed kidney was comparable in wild-type mice and *USAG1*$^{-/-}$ mice.

*BMP signaling is enhanced in USAG1*$^{-/-}$ *mice.* To evaluate whether the reduction in renal injury in *USAG1*$^{-/-}$ mice is attributable to enhanced BMP signaling, phosphorylation of Smad1/5/8 in the kidney was examined in both models (Figure 4). After the induction of kidney disease models, phosphorylation of Smad1/5/8 was hardly detected in wild-type mice, while in *USAG1*$^{-/-}$mice, the phosphorylation was preserved in the nuclei of tubular epithelial cells (Figure 4A). To examine the specificity of the antibody against phospho-Smad1/5/8, we performed double immunostaining using anti-phospho-Smad1/5/8 antibody and anti-phospho-Smad2/3, and found that most of the nuclei positive for phospho-Smad1/5/8 were negative for phospho-Smad2/3 (Figure 4B), indicating the

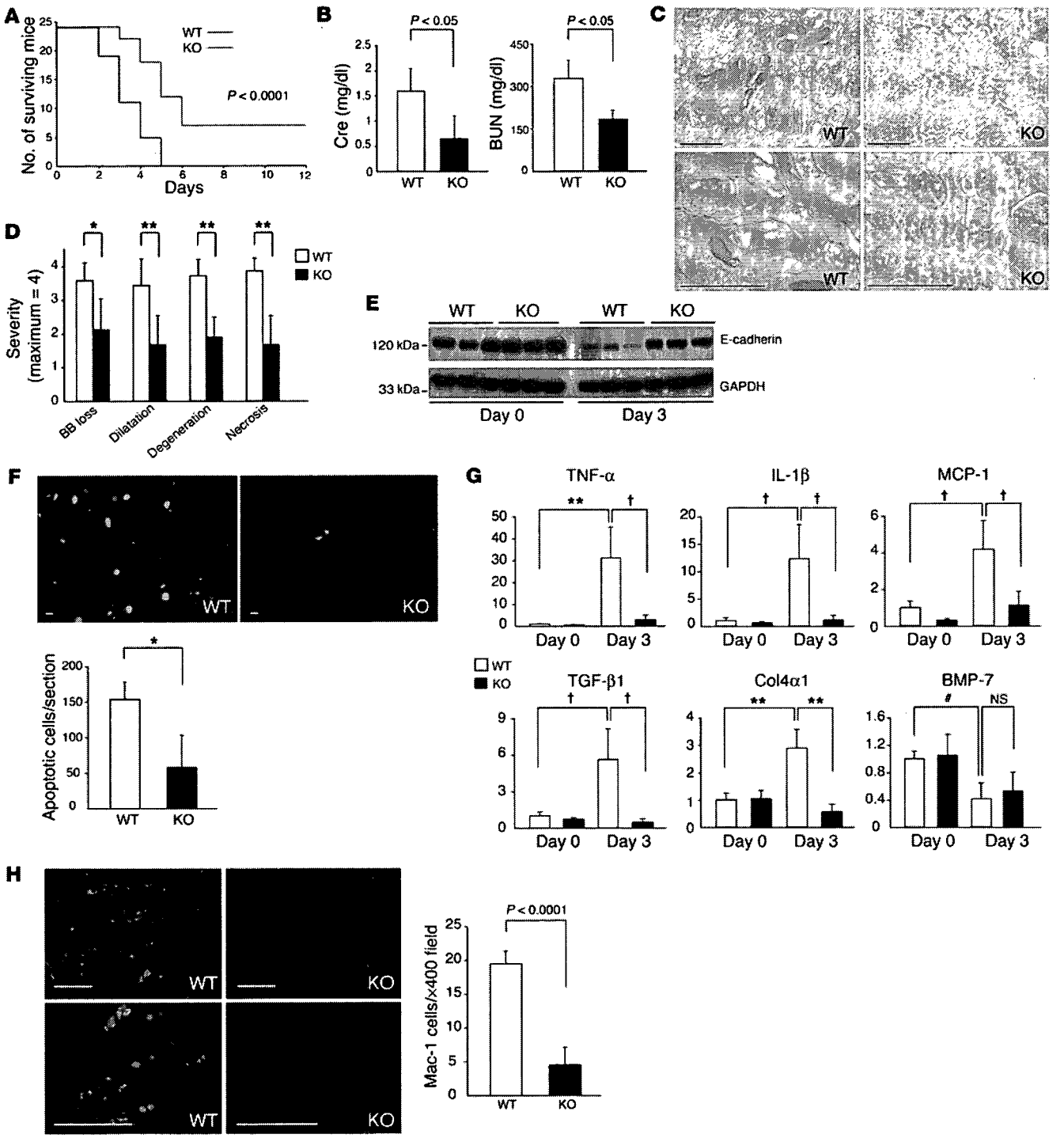


**Figure 2**

*USAG1*$^{-/-}$ mice showed less renal injury in cisplatin nephrotoxicity. (**A**) Survival curves of wild-type mice (black line) and *USAG1*$^{-/-}$ mice (red line) after cisplatin administration ($n$ = 24). (**B**) Serum creatinine (Cre) and blood urea nitrogen (BUN) levels at 3 days after injection of cisplatin ($n$ = 6). (**C**) Representative renal histological findings in wild-type mice and *USAG1*$^{-/-}$ mice on day 3. Scale bars: 100 μm. (**D**) Semiquantitative evaluation of morphologic kidney damage, expressed as relative severity on a scale from 0 to 4 ($n$ = 6). Morphological findings were scored according to proximal tubule brush border loss (BB loss), tubule dilatation (Dilatation), tubule degeneration (Degeneration), and tubule necrosis (Necrosis). $^{*}P < 0.01$; $^{**}P < 0.001$. (**E**) E-cadherin expression in cisplatin nephrotoxicity. Kidney lysates were subjected to immunoblotting with anti–E-cadherin antibody. Representative data from 4 independent experiments are shown. (**F**) TUNEL staining of kidneys on day 3 of cisplatin nephrotoxicity. The number of TUNEL-positive cells per section was counted in transverse sections ($n$ = 6). Scale bars: 10 μm. (**G**) Gene expression in cisplatin nephrotoxicity. Gene expression was determined by real-time RT-PCR. In each experiment, expression levels were normalized to the expression of GAPDH and expressed relative to mice on day 0. $n$ = 4–6 for each experiment. $^{\dagger}P < 0.005$; $^{\#}P < 0.02$. Col4α1, collagen type IV α 1. (**H**) Infiltration of Mac-1–positive cells after cisplatin injection. The number of Mac-1–positive cells per field was counted in 10 consecutive fields ($n$ = 6). Scale bars: 100 μm.