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BMP-2 Suppresses Renal Interstitial Fibrosis by Regulating Epithelial–Mesenchymal Transition

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ABSTRACT

Dysregulation of epithelial-to-mesenchymal transition (EMT) may contribute to renal fibrogenesis. Our previous study indicated that bone morphogenetic protein-2 (BMP-2) significantly reversed transforming growth factor (TGF)-β1-induced renal interstitial fibrosis. In this study, we examined the underlying mechanism and elucidate the regulation of EMT process under BMP-2 treatment. Cultured renal interstitial fibroblast (NRK-49F) was treated with TGF-β1 (10 ng/ml) with or without BMP-2 (10–250 ng/ml) for 24 h. The expression of α-smooth muscle actin (α-SMA), E-cadherin, fibronectin, or Snail transcriptional factors was analyzed by immunofluorescence staining or Western blotting. Cell migration was analyzed by wound-healing assay. NRK-49F treated with TGF-β1 induced significant EMT including upregulatioin of α-SMA, fibronectin, and snail proteins and down-regulation of E-cadherin. Interestingly, co-treatment with BMP-2 dose-dependently reversed TGF-β1-induced cellular fibrosis, cell migration, and above EMT change. The above effect was closely correlated with Snail since BMP-2 dose- and time-course dependently induced a significant decrease in the level of Snail. Moreover, Snail siRNA significantly reversed TGF-β1-induced increases in the level of α-SMA and fibronectin (intracellular and extracellular). We suppose that BMP-2 have the potential to attenuate TGF-β1-induced renal interstitial fibrosis by attenuating Snail expression and reversing EMT process. J. Cell. Biochem. 112: 2558–2565, 2011.

KEY WORDS: EMT; BMP-2; TGF-β1; FIBROSIS; SNAIL

Transforming growth factor (TGF)-β1 and its downstream signaling molecules have been shown to play an essential role in epithelial–mesenchymal transition (EMT) [Zeisberg et al., 2003; Xu et al., 2010]. Both in vitro and in vivo studies have demonstrated that TGF-β1 can initiate and complete the entire EMT process [Zvaifler, 2006; Dudas et al., 2009]. Bone morphogenetic protein-2 (BMP-2), a member of the TGF-β1 superfamily, has been implicated in the maintenance of the normal health of the kidney, skeleton, and vascular system. Our preliminary study showed that BMP-2 appears to help attenuate renal fibrosis both in vitro and in vivo [Yang et al., 2009]. However, there is little literature discussing the underlying mechanism under the treatment of BMP-2 and elucidating the interactions with EMT process.

E-cadherin is a trans-membrane glycoprotein and a marker for differentiated epithelial phenotype. It acts as a "gate keeper" of epithelial phenotype and its loss would cause an early event during EMT. EMT is a conversion process from an epithelial to a mesenchymal phenotype and a process in several disease states, including renal fibrosis [Huang and Huang, 2005]. Aberrant activation of EMT in the kidney is sufficient to induce renal fibrosis [Smith et al., 2009].

Snails are zinc-finger type transcription factors, which have been proven to behave like master genes for EMT [Hardy et al., 2007; Olmeda et al., 2007] as they are able to induce the full process in renal fibrosis [Boutet et al., 2006; Nieto, 2009]. One hallmark of EMT is down-regulation of the adherens junction protein E-cadherin, a process mediated by transcription factors such as the zinc fingers Snail and Slug. However, little literature discussed the interactions of Snail pathways in the pathogenesis of renal interstitial fibrosis. Thus, in this study, we elucidate the expression of pro-epithelial

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markers and Snails expression and investigating the role of BMP-2 in the regulation of pathogenesis in renal interstitial fibrosis.

MATERIALS AND METHODS

CELL CULTURE

NRK-49F (CRL-1570; American Type Culture Collection, Manassas, VA), a normal *Rattus norvegicus* (rat) kidney cell line was cultured in Dulbecco's modified Eagle's medium (Hyclone Labs, Logan, UT) supplemented with 5% bovine calf serum (BCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone Labs, Logan, UT) at 37°C in 5% CO_2. The cells were trypsinized by 0.025% trypsin–EDTA (Hyclone Labs, Logan, UT), typically seeded at approximately 70% confluence in complete medium that contained 5% BCS for 24 h. NRK-49F cells were cultured on 25 T flasks in 0.5% BCS 24 h and treatment with TGF-β1 (10 ng/ml; R&D Systems) with or without exogenous BMP-2 (R&D Systems).

ENZYME-LINKED IMMUNOASSAY

An ELISA was used to evaluate the expression of secreted fibronectin. To quantify fibronectin in the supernatant, conditioned culture medium was collected and centrifuged at 2,000 rpm for 5 min to remove particulates. The clear supernatant was collected and concentrated. The concentrated supernatant was stored at −80°C until use. Immediately before performing the ELISA, the sample was acidified by 1 N HCl followed by adding NaOH to equilibrate the sample to the original pH. A commercial sandwich ELISA kit was used for the detection of fibronectin (Assaypro, St. Charles, MO). The protocols were performed according to the manufacturer's instructions. The absorbance (450 nm) for each sample was analyzed by an ELISA reader. After the absorbance for fibronectin was assayed, the concentration was determined by interpolating against the standard curve.

IMMUNOFLUORESCENCE

Conditioned cells were grown on chamber slides. After washing with phosphate-buffered saline (PBS), cells were fixed with 2.5% paraformaldehyde for 20 min. Cells were permeablized with 5% Triton-X 100 for 15 min then blocked with 10% bovine serum albumin for 1 h. E-cadherin (ab53033; Abcam, Cambridge, UK), α-smooth muscle actin (α-SMA, ab5694; Abcam, Cambridge, UK), fibronectin (ab23751; Abcam, Cambridge, UK), and Snail (ab85931; Abcam, Cambridge, UK) were reconstituted in 1% bovine serum albumin and incubate the cells for 1 h. Then, the cells were incubated with secondary antibodies (goat polyclonal anti-rabbit FITC, ab6717; Abcam, Cambridge, UK) for additional 1 h. After a series of PBS washing procedure, 4,6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnology) was used to stain nuclei. Finally, chamber slides were then analyzed by an Olympus fluorescence microscope (CK41).

shRNA TRANSFECTION

Snail shRNA plasmid was purchased from Santa Cruz Biotechnology. Snail shRNA plasmid is a pool of 3′ target-specific lentiviral vector plasmid encoding 19–25 nt (plus hairpin) shRNAs designed to knock down the expression of Snail. NRK-49F cells were cultured on 6-well plates in 5% BCS 24 h. After medium replacing with serum free for 1 h, cells were treated by Snail shRNA (2 μg/ml) using Fugene6^{TM} (Rhoch) and incubated for 6 h. Stable transfection was obtained after selection with puromycin (3 μg/ml) for 3 months.

WESTERN BLOT ANALYSIS

Western blotting was used to evaluate the expression of protein level for E-cadherin, Snail, Slug, β-actin, fibronectin, and α-SMA. In brief, cells were lysed by lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM dithiothreitol, 50 μg phenylmethylsulfonyl fluoride). The crude protein lysate was resolved by 7.5, 10, or 12.5% SDS–PAGE. After protein transfer to a polyvinylidene difluoride membrane, the polyvinylidene difluoride membrane was blocked with 10% (w/v) nonfat milk in Tris-buffered saline for 24 h at 4°C. The primary antibodies used were as follows: Anti-E-cadherin (sc-7870), anti-α-smooth muscle α-Actin (sc-32251), Fibronectin (sc-9068), β-actin (Sigma, St. Louis, MO), Snail (Rabbit mAb #3879; Cell Signaling Biotechnology System, Beverly, MA), Slug (Rabbit mAb #9585). After hybridization at 37°C, the blots were washed and hybridized with 1:4,000 (v/v) dilutions of goat anti-rabbit IgG or horseradish peroxidase-conjugated secondary antibody (Calbiochem, Darmstadt, Germany). The signals were captured by adding enhanced chemoluminescent reagent. β-actin was used as an internal loading control.

WOUND-HEALING ASSAY

To determine cell motility ability, NRK-49F cells (2×10^5 cells/ml) were seeded in six-well tissue culture plate and grown to 80–90% confluence. After aspirating the medium, the center of the cell monolayers was scraped with a sterile micropipette tip to create a denuded zone of constant width. Subsequently, cellular debris was washed with PBS, and cells were incubated with TGF-β1 (10 ng/ml) in the absence or presence of BMP-2 (50 ng/ml). The wound closure was monitored and photographed at 0, 6, 12, and 24 h with Motic AE-21 inverted microscope and TUCSEN camera. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Migrated cells across the white lines were counted in six random fields from each triplicate treatment.

STATISTICS

Results were expressed as mean ± SEM. The unpaired Student's *t* test was used for comparison between two groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

EFFECT OF BMP-2 ON RENAL INTERSTITIAL FIBROBLAST

BMP-2 (10, 50, or 250 ng/ml) dose-dependently reversed TGF-β1 (10 ng/ml)-induced increase in the level of secreted fibronectin in NRK-49F (Fig. 1A). Transcriptional factor Snail initiates EMT process and promotes the accumulation of extracellular matrix [Zvaifler, 2006; Nieto, 2009]. Thus, the expression of Snail

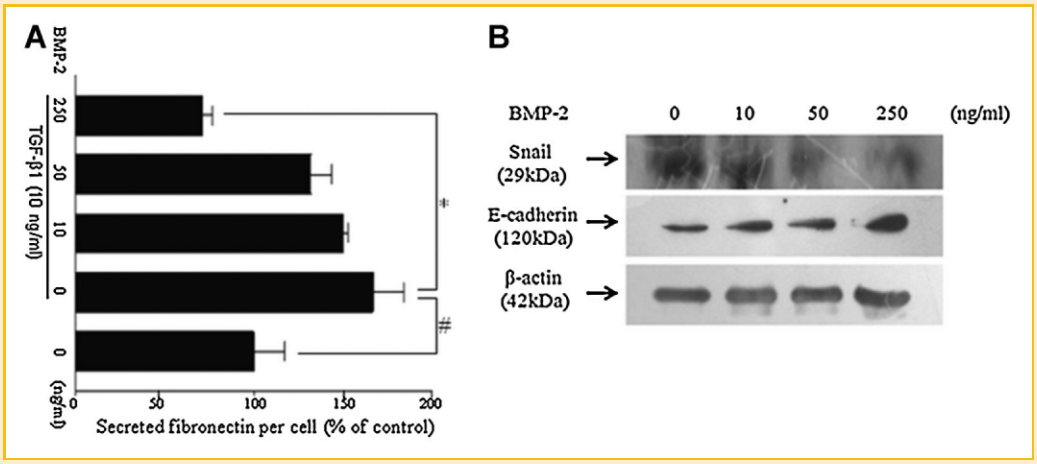

Fig. 1. Effects of BMP-2 on fibronectin, Snail, and E-cadherin expression in NRK-49F. A: Cells were treated with 10 ng/ml of TGF-β1 and co-treated with BMP-2 dose-dependently (10–250 ng/ml) in 0.5% bovine calf serum for 24 h. The secreted fibronectin was measured by ELISA. B: Cells were treated with BMP-2 (0–250 ng/ml) in 0.5% bovine calf serum for 24 h. The expression level of Snail and E-cadherin proteins were analyzed by Western blot. β-actin was used as a loading control. Results are presented as mean ± SEM of three samples from each condition and normalized against cell numbers. Experiments were repeated twice and similar results were observed. $^{\#}P < 0.05$ vs. control. $^{*}P < 0.05$ vs. 0 ng/ml of BMP-2 in the presence of 10 ng/ml of TGF-β1.

and E-cadherin were examined under the treatment of BMP-2. It is evident that BMP-2 dose-dependently (10, 50, or 250 ng/ml) suppressed the expression of Snail. Simultaneously, the expression of E-cadherin was significantly increased, instead (Fig. 1B). Thus, BMP-2 appears to help attenuate renal cellular fibrosis partly by suppressing EMT pathway since the Snail proteins was significantly down-regulated. This is the first demonstration showing the relationship between BMP-2 and EMT in renal fibroblasts.

EFFECTS OF SNAIL PROTEINS ON RENAL INTERSTITIAL FIBROBLAST

To ensure the significance of Snail proteins, Snail-shRNA transfected NRK cells were generated by using Fugene6^{TM} transfection system. After puromycin selection up to 3 months, Snail-silenced cells were selected as shown in Figure 2. It is evident that the expression of Snail proteins was significantly silenced by Snail shRNA (Fig. 2A). In addition, we demonstrated that expression of Snail was closely correlated to the expression of fibronectin (both intracellular and extracellular) and EMT pathway in renal interstitial fibroblast cells (Fig. 2B and C).

EFFECTS OF BMP-2 ON SNAILS, EMT, AND CELL MIGRATION

According to the above observations (Fig. 2), Snail is essential for the accumulation of fibronectin and induction of EMT since silencing the expression of Snail will retard the progression of cellular fibrosis in NRK-49F. In addition, we demonstrated that 10 ng/ml of TGF-β1 time course-dependently (0, 8, 16, and 24 h) induced a significant increase in Snail and α-SMA. Again, these effects have been dramatically attenuated by BMP-2 (50 ng/ml) especially at the time point of 24 h (Fig. 3). Moreover, migration assay was performed since the regulation of EMT has been reported to be closely correlated with cell migration. As shown in Figure 4, NRK-49F was incubated with TGF-β1 (10 ng/ml) in the presence or absence of BMP-2 (50 ng/ml). An apparent and gradual increase of cells in the denuded zone was observed in the cells of control group (0, 6, 12, and 24 h) under inverted microscope. According to a quantitative assessment, TGF-β1 (10 ng/ml) induced a significant increase in cell migration at 6 h of stimulation. More importantly, BMP-2 (50 ng/ml) dramatically reversed TGF-β1-induced cell migration as early as 6 h after treatment. The results demonstrated BMP-2 has the potential to retard TGF-β1-induced cell migration in NRK-49F cells.

EFFECTS OF BMP-2 ON TGF-β1-INDUCED EXPRESSION OF SNAIL AND EMT MARKERS

In Figure 5, TGF-β1 (10 ng/ml) induced a significant increase in both Snail types (i.e., Snail (Snail1) and Slug (Snail2)) in normal rat kidney interstitial fibroblast cells. Importantly, BMP-2 dose-dependently (0, 10, 50, or 250 ng/ml) reversed TGF-β1-induced increase in the expression of both Snail and Slug proteins. In addition, BMP-2 dose-dependently reversed TGF-β1-induced increase in fibronectin expression and EMT character (i.e., BMP-2 restored the expression of E-cadherin and attenuated the expression of α-SMA induced by TGF-β1). As shown in immunofluorescence analysis (Fig. 6), E-cadherin was expressed on the margin of cells showing the expression of epithelial character in control group. 10 ng/ml of TGF-β1 dramatically induced the downregulation of E-cadherin. However, 50 ng/ml of BMP-2 significantly restored the expression of E-cadherin compared to control. Similarly, expression of α-SMA, fibronectin, and Snail were examined. We showed that 10 ng/ml of TGF-β1 significantly induced the expression of α-SMA, fibronectin, and Snail in the cytoplasm in NRK-49F cells; however, 50 ng/ml of BMP-2 significantly attenuated the above mesenchymal character induced by TGF-β1. Since Snail mediates early induction of EMT process, we suggest that BMP-2 might exert its fibrosis-antagonizing effects by regulating Snail proteins. According to Figure 7, we hereby propose that loss of epithelial character (i.e.,

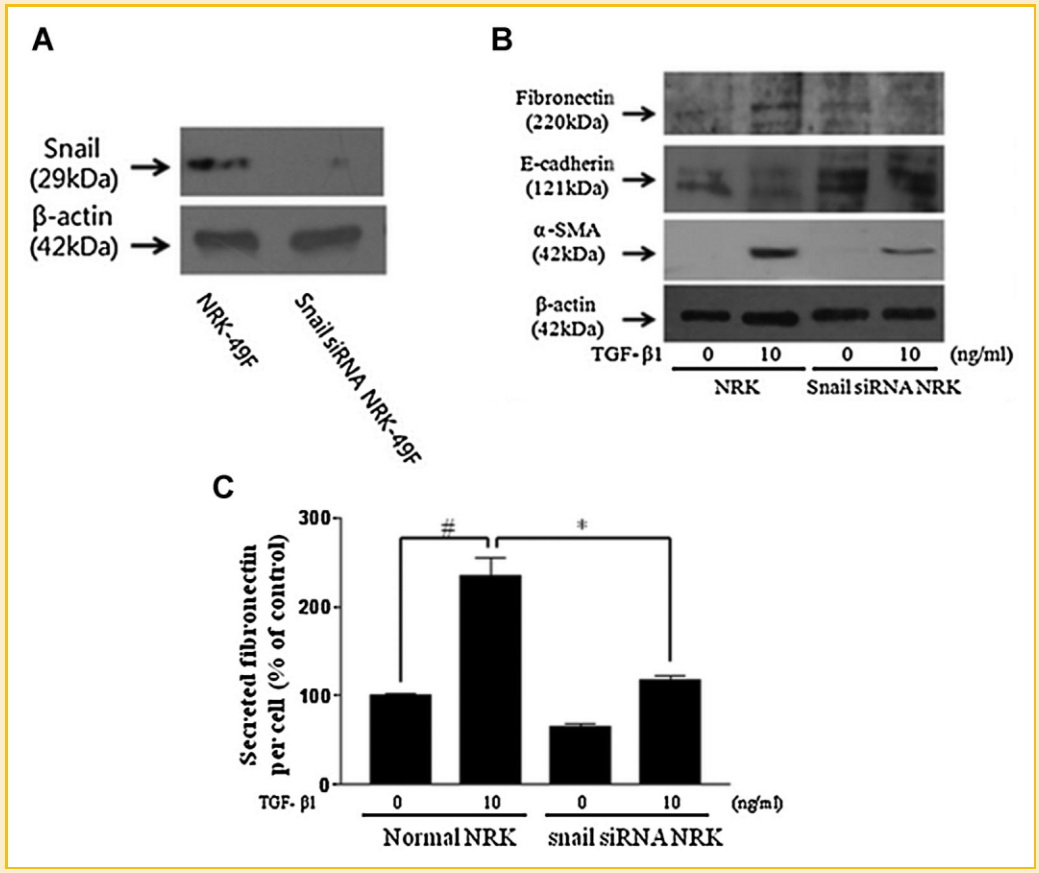

Fig. 2. Effects of Snail silencing on the expression of fibronectin, E-cadherin, and α-SMA in NRK-49F cells. A: Cells were stable transfected by Snail shRNA (2 μg) in serum free medium for 6 h. The positive transfected cells were selected by puromycin (3 μg/ml) for 3 months. Whole cell lysates from shRNA transfected cells and its control was subjected to immunoblot assay for Snail and β-actin. B: Snail shRNA stable transfected cells were treated with TGF-β1 (10 ng/ml) in the presence of 0.5% bovine calf serum for 24 h. The fibronectin, E-cadherin, and α-SMA proteins were analyzed by Western blot. β-Actin was used as a loading control. C: Fibronectin level of the conditioned culture medium from conditioned cells of Figure 2B was measured by ELISA. Results were presented as mean ± SEM of three samples from each condition and normalized against cell number. Experiments were repeated twice and similar results were observed. $^{\#}P < 0.05$ vs. control (0 ng/ml TGF-β1), $^{*}P < 0.05$ vs. control (10 ng/ml TGF-β1). The experiment was repeated twice and similar results were obtained.

downregulation of E-cadherin) and gain of mesenchymal marker (i.e., upregulation of α-SMA and fibronectin) induced by TGF-β1 can be reversed or restored by BMP-2 in renal interstitial fibroblast cells. More importantly, Snail proteins play a pivotal role in above biological effects of BMP-2 as demonstrated in Figure 2.

DISCUSSION

Based on previous studies, BMP-2 appears to be implicated in the pathogenesis of renal diseases. Gambaro et al. showed that abnormal urine calcium-oxalate supersaturation triggers inflammation in the long Henle's loop cells [Gambaro et al., 2004]. This in turn induces differentiation of these cells toward the osteogenic lineage and induces synthesis of BMP-2. Nett et al. showed that gene expression of vascular BMP-2 was almost doubled in NOD mice after 6 weeks compared with nondiabetic controls [Nett et al., 2006]. Moreover, BMP-2 has been demonstrated to be related to nephropathy [Stelnicki et al., 1998], but so far there is no clear-cut evidence elucidating the interactions between and BMP-2 and renal fibrosis.

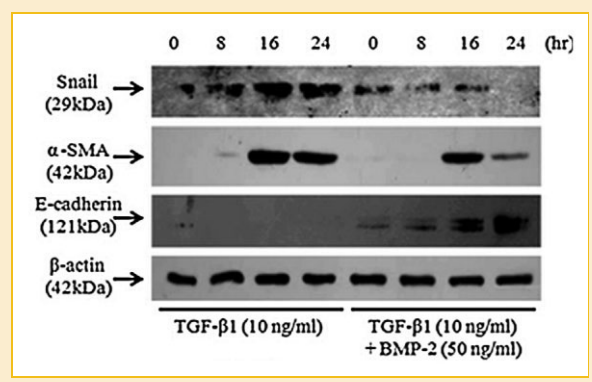

Fig. 3. Time-course effects of BMP-2 on TGF-β1-regulated Snail, α-SMA, and E-cadherin in NRK-49F cells by immunocytochemistry assay. Cells were treated with TGF-β1 (10 ng/ml) in the presence of concentrations (50 ng/ml) of BMP-2 with 0.5% bovine calf serum for the indicated time points (0, 8, 16, or 24 h). The expression of Snail, α-SMA, and E-cadherin were analyzed by Western blot. β-actin was used as a loading control. The relative abundance of Snail, α-SMA, and E-cadherin was expressed as a percentage normalized to β-actin control. Experiments were repeated twice and similar results were observed.

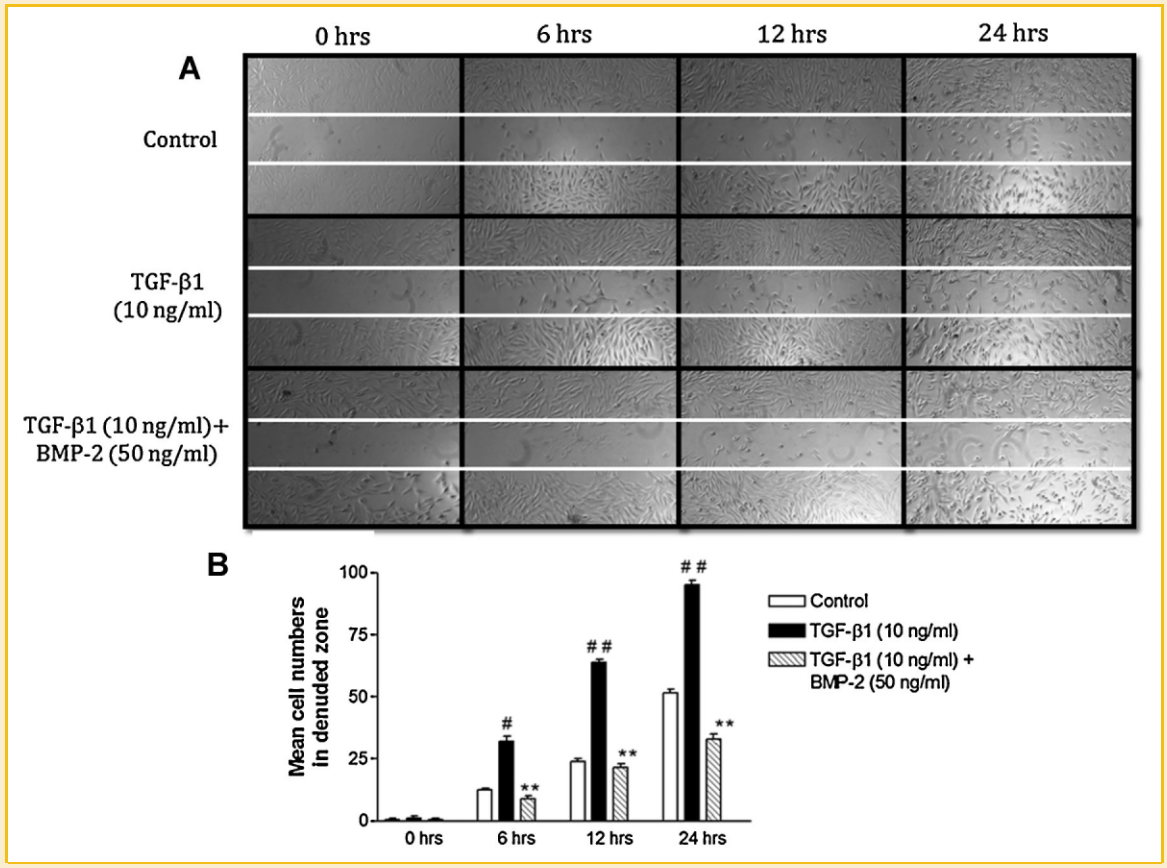

Fig. 4. Effects of BMP-2 on cell migration in normal rat kidney fibroblast. A: Cells were incubated with TGF-β1 (10 ng/ml) in the absence or presence of BMP-2 (50 ng/ml) for various periods of time (0, 6, 12, or 24 h) as indicated. Then the conditioned cells were subject to migration analysis as described in "Material and methods" section. B: Mean cell numbers in respective denuded zone were counted and expressed as mean ± SEM of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the control; $^{**}P < 0.01$ compared with the TGF-β1 (10 ng/ml) treating control in respective time point.

High affinity-specific binding proteins for BMP-2 were identified not only on osteoblastic cells but also on fibroblasts, kidney epithelial cells, and kidney [Iwasaki et al., 1995]. Moreover, expression of the BMP transcripts and presence of the BMP proteins in kidney organ suggests a regulatory role for BMP [Ozkaynak et al., 1991]; however, so far little literature has focused on the regulatory effects on BMP-2 on kidney. According to our previous study, BMP-2 plays a protective role against renal cellular fibrosis [Yang et al., 2009]. BMP-2 antagonizes TGF-β1-induced renal fibrogenic signals partly by enhancing the catabolism of type I TGF-β receptors in renal interstitial fibroblast cells. In addition, once-daily treatment with BMP-2 (70 μg/kg) for 7 days significantly reduces the collagen staining intensity in interstitial and tubular areas of the kidney from 6-week-old male SD rats subjected with unilateral ureteral obstruction procedures. The above observations imply that the regulation of BMP-2 is highly correlated with the pathogenesis of interstitial nephritis or renal fibrosis; however, further investigation is required to elucidate underlying mechanism.

TGF-β has long been well studied as a key regulator in up-regulating matrix production in nearly most types of renal fibrosis. During kidney fibrosis, conversion of renal cells into myofibroblasts/fibroblasts is considered unfavorable because it leads to disruption of the polarization and increased fibrotic scar formation. Therefore, EMT in the kidney is of significant interest as a therapeutic target. Zeisberg et al. [2003] showed that systemic administration of BMP-7 can result in the regression of established lesions in the kidney and improved renal function and this occurs by inhibiting EMT. However, the role of BMP-2 in the pathogenesis of renal fibrosis remains unclear.

According to previous studies, the relationship between BMP-2 and EMT may be relevant to the pathogenesis of fibrosis. Luna-Zurita et al. [2010] demonstrated that embryonic BMP-2-Snail interactions were relevant to endocardial EMT conversion and adult cardiac valve disease. Chen et al. [2010] demonstrated that BMP-2 induced EMT and invasiveness in a human pancreatic cancer cell line, accelerating invasion of cancer cells via the PI3K/AKT pathway. Kang et al. [2010] demonstrated that BMP-2 induced a full EMT characterized by Snail induction, E-cadherin delocalization, and down-regulation, and up-regulation of mesenchymal and invasiveness markers in gastric cancer cells. In endocardial cells and some cancer cells (e.g., pancreatic and gastric cancer), BMP-2 appears to drive EMT conversion. However, in renal interstitial cells, the role of BMP-2 remains unclear.

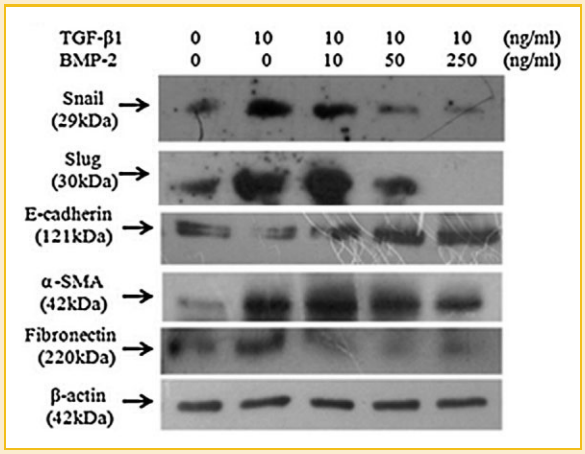

Fig. 5. Effects of BMP-2 on TGF-β1-regulated Snail, Slug, E-cadherin, α-SMA, and fibronectin expression in NRK-49F cells. NRK-49F cells were treated with TGF-β1 (10 ng/ml) in the presence of different concentrations (0, 10, 50, or 250 ng/ml) of BMP-2 with 0.5% bovine calf serum for 24 h. Expression of Snail, Slug, E-cadherin, α-SMA, and fibronectin were analyzed by Western blot. β-actin was used as a loading control. The results are presented as mean ± SEM of two samples from each condition. Experiments were repeated twice and similar results were observed.

NRK-49F cells were used in this study due to their basal but steady epithelial character exhibited in unconditioned media. In Figures 1B, 2B, 5, and 6, expression of the epithelial marker E-Cadherin is significant and basally expressed. In addition, TGF-β treatment in NRK-49F cells induced significant loss of E-Cadherin marker, dramatic increase in EMT inducer (i.e., Snail), and accumulation of extracellular matrix (i.e., fibronectin) as shown in revised Figures 5 and 6. The above parallel observation showed that NRK model was appropriate in this type of study.

EMT is a feature of migratory cellular processes in all stages of life, including wound healing and tissue fibrosis. A review of the current literature has revealed that many signal transducers may regulate each other in a hierarchical pattern where Snail (formerly Snail1) and Slug (formerly Snail2) are initially induced, leading to the activation of family members of some transcriptional factors contributing to the process of EMT. Activation of Snail, which is not only a mesenchymal marker but also a negative regulator of E-cadherin, is closely relevant to EMT process during renal fibrosis [Hardy et al., 2007; Fedorova et al., 2009; Smith et al., 2009]. Snail is an initiation factor for EMT process. Some studies showed that TGF-β1 acts through inducing Smad signal pathway and then trans-inducing the expression of Snails to alter the differential character

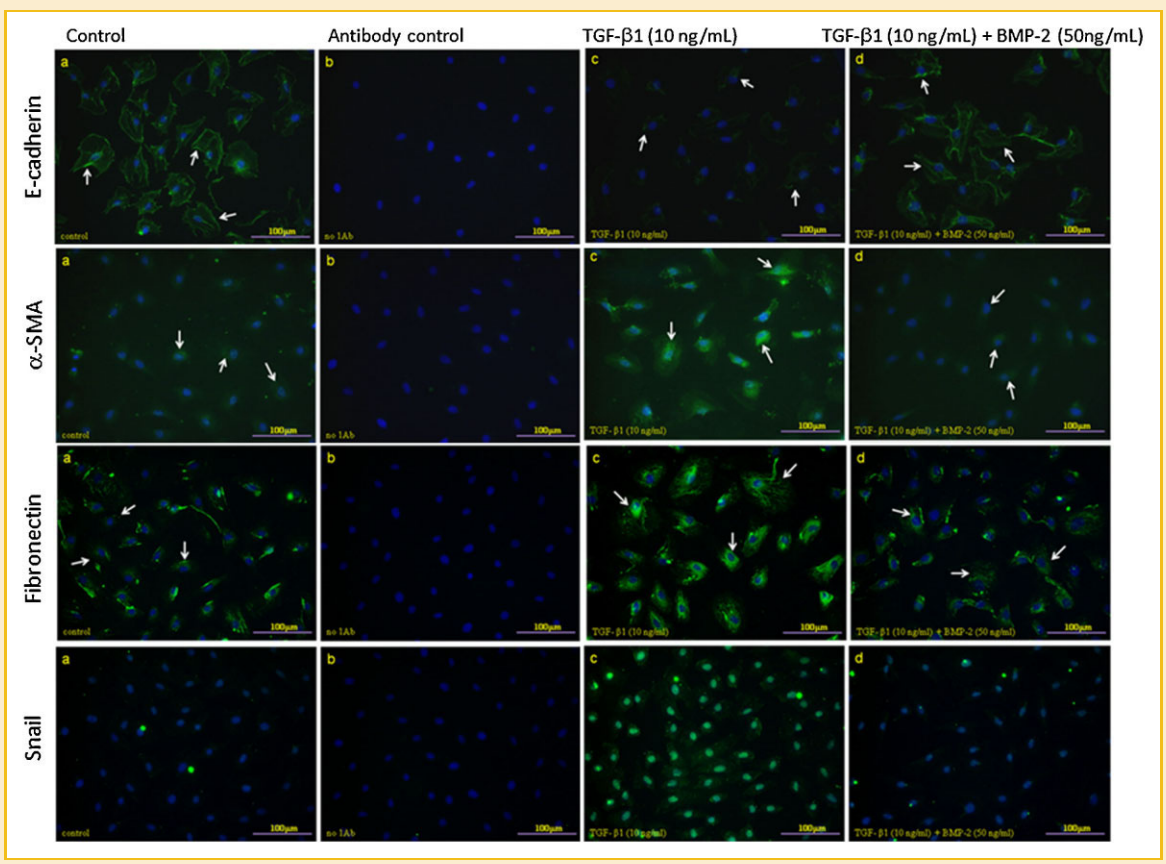

Fig. 6. Effects of BMP-2 on E-cadherin, α-SMA, fibronectin, and Snail in NRK-49F cells by immunocytochemistry assay. Cells were treated with TGF-β1 (10 ng/ml) in the presence or absence of BMP-2 (50 ng/ml) with 0.5% bovine calf serum. E-cadherin, α-SMA, fibronectin, and Snail were assayed by immunofluorescence. a: Control. b: Antibodies control (i.e., without adding primary antibody). c: TGF-β1 (10 ng/ml) treating group. d: BMP-2 (50 ng/ml) combining TGF-β1 (10 ng/ml) treating group. Magnifications scale is 200×. The bar indicates 100 μm in length.

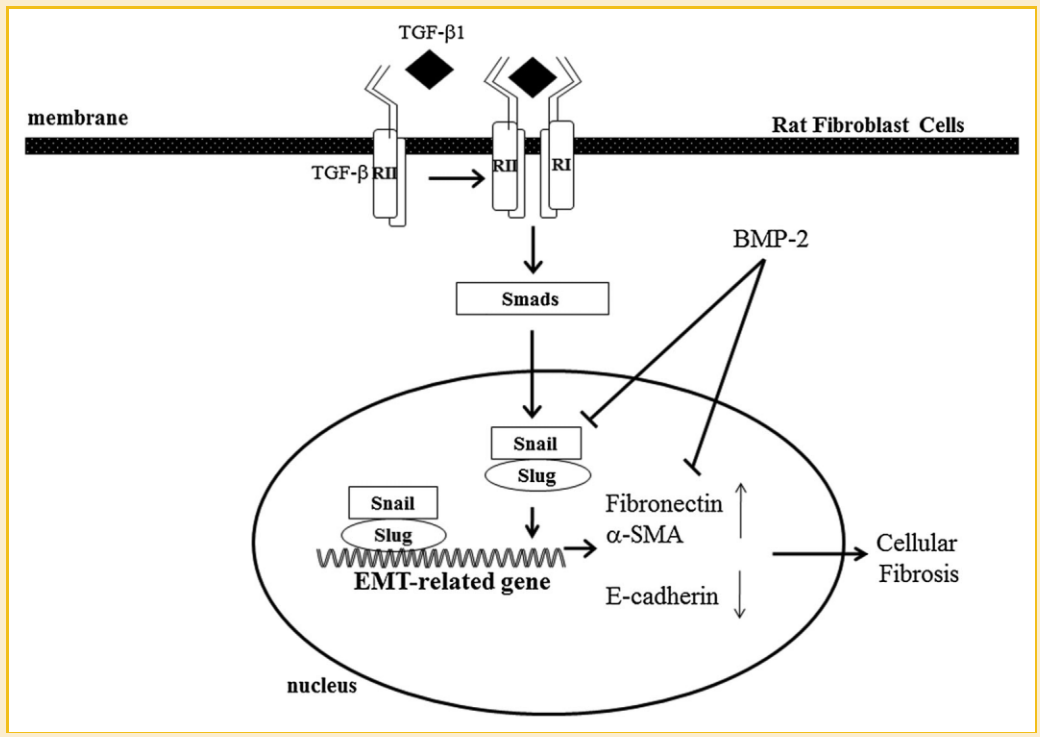

Fig. 7. A postulated mechanism underlying BMP-2 regulated cellular fibrosis-induced by TGF-β1. TGF-β1 transmits fibrogenic signals through Smads and Snail/Slug signaling pathway, which transactivated EMT-related genes such as fibronectin and α-SMA and down-regulated epithelial marker (e.g., E-cadherin). In this study, we demonstrated that BMP-2 attenuated TGF-β1-induced cellular fibrosis and mesenchymal character by down-regulating Snail/Slug proteins, which is a key protein driving the progress of EMT.

toward mesenchymal state [Tan et al., 2006; Nieto, 2009]. About the regulation of Smads pathway under BMP-2 treatment demonstrated that BMP-2 significantly reversed TGF-β-induced increase in Smad4 and decrease in Smad7 [unpublished observation]. Thus, BMP-2 appears to exert the anti-fibrotic effects by regulating not only EMT but also Smad signaling.

This notion is consistent with the results shown in this study. We demonstrated that BMP-2 significantly down-regulated the expression of Snail in renal interstitial fibroblast cells concomitantly with the induction of the expression of E-cadherin (Fig. 1). Moreover, we showed that Snail-silencing cells attenuated TGF-β1-induced increase in fibronectin and restored TGF-β1-inducing changes in EMT markers as shown in Figure 2. In other words, silencing of Snail blocked TGF-β1-induced EMT and cellular fibrosis in renal fibroblast. Thus, regulating Snail per se is able to regulate EMT process and is capable of attenuating renal interstitial fibrosis-induced by TGF-β1. Thus, this observation suggests that modulating the expression of Snail proteins might be a potential strategy against renal interstitial fibrosis.

According to Figure 5, BMP-2 dose-dependently reversed TGF-β1-induced increase in Snail (i.e., Snail1), Slug (i.e., Snail2), α-SMA, and fibronectin secretion. However, BMP-2 dose-dependently reversed TGF-β1-induced decrease in E-cadherin. In addition, time course-dependent experiment showed that BMP-2 exerted the consistent effects reversing TGF-β1-induced EMT as early as 8 h after cotreatment with TGF-β1 (Fig. 3). These observations demonstrate that BMP-2 has the potential to reverse TGF-β1-induced EMT and cellular fibrosis. However, these observations seem different with other studies performed in endocardial cells and some cancer cells (e.g., pancreatic and gastric cancer), which showed that BMP-2 tends to enhance Snail expression and promote EMT conversion. BMP-2 tends to attenuate the expression of Snail and to suppress the process of EMT in renal interstitial fibroblast. In other words, in renal interstitial fibroblast BMP-2 exerted favorable effects (i.e., fibrosis inhibitory effects) by down-regulating Snail proteins. These dramatic differences of biological effects of BMP-2 might be due to the distinct differentiation character in distinct organs.

According to immunofluorescence study, TGF- β1 (10 ng/ml) significantly induced the expression of α-SMA, fibronectin, and Snail in the cytoplasm in NRK-49F cells. More importantly, BMP-2 (50 ng/ml) significantly attenuated the above fibrotic character induced by TGF-β1 as shown in Figure 6. Since Snail mediates early EMT induction in renal interstitial fibroblast, we supposed that BMP-2 exerted its fibrosis-antagonizing effects by regulating Snail proteins. So far, there is no clear evidence showing the interactions of BMP-2-Snail signaling pathway with renal interstitial fibrosis.

In this study, we showed that TGF-β1 induced significant EMT conversion including upregulating mesenchymal markers (e.g., α-SMA and fibronectin) and down-regulating epithelial marker (e.g., E-cadherin). More importantly, we hereby demonstrated that BMP-2 has the potential to reversed TGF-β1-induced EMT and cellular

fibrosis by regulating Snail and EMT (i.e., down-regulating α-SMA and up-regulating E-cadherin). This study provides an encouraging possibility of the therapeutic application of BMP-2 in the treatment of renal fibrosis in the future; however, this requires further investigation.

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