

# Blockade of Smad signaling by 3'-deoxyadenosine: a mechanism for its anti-fibrotic potential

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*Cordyceps militaris* has been used in Eastern countries for the treatment of various diseases including chronic kidney diseases. However, there are no reports that identified its active entities and molecular mechanisms underlying its therapeutic effectiveness. 3'-Deoxyadenosine is a major nucleoside derivative isolated from *C. militaris*. Some reports suggested that both *C. militaris* and 3'-deoxyadenosine have anti-inflammatory and anti-fibrotic effects. In the present report, we investigated whether and how 3'-deoxyadenosine interferes with fibrogenic processes in the kidney. For this purpose, we examined effects of 3'-deoxyadenosine on the expression of collagens triggered by transforming growth factor- $\beta$  (TGF- $\beta$ 1) and bone morphogenetic protein-4 (BMP-4), especially focusing on the regulation of Smad signaling *in vitro* and *in vivo*. We found that 3'-deoxyadenosine suppressed expression of collagens induced by TGF- $\beta$ 1 and BMP-4 dose dependently. This suppression occurred at the transcriptional level and was correlated with blunted activation of the CAGA box and the BMP-responsive element. The suppressive effect on the TGF- $\beta$ /BMP signaling was mediated mainly by adenosine transporter and partially by the A3 adenosine receptor, but not A1/A2 adenosine receptors. 3'-Deoxyadenosine reduced levels of both phosphorylated and total Smad proteins (Smad1, 2 and 3) dose dependently. It was mainly ascribed to transcriptional suppression, but not to enhanced protein degradation and eIF2 $\alpha$ -mediated translational suppression. Consistent with the *in vitro* results, *in vivo* administration with 3'-deoxyadenosine reduced the levels of phosphorylated and total Smad proteins, as well as the levels of Smad mRNAs, in the kidney subjected to unilateral ureteral obstruction. It was associated with blunted induction of type I collagen and  $\alpha$ -smooth muscle actin, a decrease in the number of interstitial myofibroblasts and reduced fibrotic area. These results suggest that 3'-deoxyadenosine interferes with the TGF- $\beta$  and BMP signaling via downregulation of Smads, which may underlie the anti-fibrotic effect of this agent. 3'-Deoxyadenosine may be useful for therapeutic intervention in various TGF- $\beta$ -related fibrotic disorders.

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*Cordyceps militaris* is a medicinal mushroom that has been considered as an abundant source of bioactive substances. It possesses various biological activities including anti-inflammatory, anti-tumor, anti-microbial, anti-fibrotic and neuroprotective effects.<sup>1</sup> Because of this reason, *C. militaris* has been used in Eastern countries for the treatment of various diseases, including chronic kidney diseases.<sup>2</sup> However, there are few reports that showed pharmacological mechanisms underlying its clinical utility and therapeutic effectiveness.

Tubulointerstitial fibrosis is an end-stage feature of a wide range of chronic kidney diseases. Members of the

transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily have important roles in the progression of tissue fibrosis by inducing epithelial-to-mesenchymal transition, chronic inflammation, activation of interstitial myofibroblasts and accumulation of extracellular matrix.<sup>3,4</sup> In particular, prototypic TGF- $\beta$ 1 and bone morphogenetic protein-4 (BMP-4) have important roles in the regulation of tissue fibrosis and sclerosis.<sup>5–7</sup> During the past decades, the signal transduction pathways triggered by the TGF- $\beta$  superfamily have been extensively investigated. TGF- $\beta$ s and BMPs bind to their receptors and activate Smad2/3 and Smad1/5/8,

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respectively.<sup>8</sup> These receptor-activated Smads (R-Smads) are phosphorylated directly by receptor kinase and induce association and formation of heterotrimeric complex of two R-Smads and a common Smad protein (eg, Smad4), leading to their translocation to the nucleus.<sup>9,10</sup> Smad3 and Smad4 preferentially bind to a specific DNA sequences, namely the CAGA box, whereas Smad1 binds to the BMP-responsive element (BRE), leading to transcriptional induction of their target genes including collagens.<sup>11,12</sup>

Recent studies indicated potential roles of adenosine in renoprotection from ischemic injury. In the kidney, adenosine acts through adenosine receptors and has a critical role in regulating renal inflammation and ischemic kidney disease.<sup>13</sup> Four adenosine receptor subtypes, including A1, A2A, A2B and A3, are detectable in the kidney. Activation of these receptors has both beneficial and deteriorative effects on renal pathophysiology, as recently reviewed by Yap and Lee.<sup>14</sup>

3'-Deoxyadenosine, also called cordycepin, is an analogue of adenosine and was identified as a major bioactive constituent in *C. militaris*<sup>15,16</sup> and possesses a wide range of biological effects, including anti-cancer, anti-inflammatory and anti-mitogenic effects.<sup>17-19</sup> Nan *et al.*<sup>20</sup> reported that biopolymers produced by *C. militaris* inhibited experimental liver fibrosis in rats. A recent report indicated that 3'-deoxyadenosine could affect Smad2/3 signaling, but underlying mechanisms are largely unknown.<sup>21</sup> In the present study, we examine the anti-fibrogenic potential of 3'-deoxyadenosine in the kidney, especially focusing its effect on the Smad signaling pathways. Our current results reveal for the first time that 3'-deoxyadenosine interferes with TGF- $\beta$  and BMP signaling via downregulation of Smads *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Reagents

3'-Deoxyadenosine, adenosine, 1,3-dipropyl-8-(*p*-sulphophenyl)xanthine (DPSPX; A1/A2 adenosine receptor antagonist), MRS1523 (A3 adenosine receptor antagonist), 2-chloro-N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (Cl-IB-MECA; adenosine A3 receptor agonist), S-(4-Nitrobenzyl)-6-thioinosine (NBTI; adenosine transport inhibitor) and actinomycin D were purchased from Sigma-Aldrich Japan (Tokyo, Japan). ATP, ADP and chloroquine diphosphate were obtained from Wako Pure Chemical (Osaka, Japan), salubrinal (inhibitor of eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) dephosphorylation) was from Tocris Bioscience (Ellisville, MO, USA), MG132 was from Peptide Institute (Osaka, Japan), and human TGF- $\beta$ 1 and human recombinant BMP-4 were from R&D Systems (Minneapolis, MN, USA). Throughout the study, 5  $\mu$ g/ml of 3'-deoxyadenosine was generally used for experiments, because this concentration is non-toxic to NRK-52E cells and has the maximum biological effects, including suppression of endoplasmic reticulum stress-induced apoptosis and inhibition of NF- $\kappa$ B activation.<sup>22,23</sup>

### Cell Culture

The rat renal tubular epithelial cell line NRK-52E was purchased from American Type Culture Collection (Manassas, VA, USA). SM43 rat mesangial cells were established as described previously.<sup>24</sup> Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS). Experiments were performed in the presence of 1% FBS.

### Northern Blot Analysis

Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described before.<sup>22</sup> cDNAs for procollagen  $\alpha$ 1(IV),<sup>25</sup> procollagen  $\alpha$ 1(I),<sup>26</sup>  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA),<sup>27</sup> Smad1, Smad2 and Smad3 (provided by Dr Keiji Miyazawa, University of Yamanashi) were used to prepare radio-labeled probes. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 28S ribosomal RNA were used as loading controls. Densitometric analysis was performed using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

### Western Blot Analysis

Western blot analysis was performed as described before.<sup>28</sup> Levels of phosphorylated and total proteins of Smad1, Smad2 and Smad3 were assessed using anti-phospho Smad1 (Ser206) antibody, anti-Smad1 antibody, anti-phospho Smad2 (Ser465/467) antibody, anti-Smad2 antibody, anti-phospho Smad3 (Ser423/425) antibody and anti-Smad3 antibody (Cell Signaling, Beverly, MA, USA). Anti-phospho eIF2 $\alpha$  (Ser51) antibody was purchased from Cell Signaling, and anti-eIF2 $\alpha$  antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The level of  $\beta$ -actin and  $\alpha$ -SMA was assessed using anti- $\beta$ -actin antibody (Sigma-Aldrich, Japan) and anti- $\alpha$ -SMA antibody (Dako, Tokyo, Japan). Blots were visualized using the enhanced chemiluminescent system (Amersham Biosciences, Buckinghamshire, UK). Densitometric analysis was performed using ImageJ Software.

### Reporter Assay

Using electroporation, NRK-52E cells were transiently transfected with p(CAGA)<sub>12</sub>-MLP-Luc (provided by Dr Atsuhito Nakao, University of Yamanashi)<sup>11</sup> or pBRE-Luc (provided by Dr Keiji Miyazawa, University of Yamanashi),<sup>12</sup> treated with TGF- $\beta$ 1 or BMP-4 in the absence or presence of 3'-deoxyadenosine and subjected to luciferase assay. In some experiments, pcDNA3.CD2-heIF2 $\alpha$ -S51A encoding a dominant-negative mutant of eIF2 $\alpha$  (eIF2 $\alpha$ -DN) (provided by Dr David Ron, University of Cambridge)<sup>29</sup> or pcDNA3.1-GADD34 coding for growth arrest and DNA damage gene 34 (GADD34) (provided by Dr Takeshi Omasa, Osaka University)<sup>30</sup> was co-transfected to evaluate involvement of eIF2 $\alpha$ . pGL2-F-1005/+93Smad1 that introduces a luciferase gene under the control of the Smad1 gene promoter (provided by Dr Yuewen Gong, University of Manitoba)<sup>31</sup> was also used to evaluate the effect of

3'-deoxyadenosine. pSV40-Luc that introduces a luciferase gene under the control of the simian virus 40 (SV40) promoter (provided by Dr Takahiro Taira, University of Yamanashi) was used for control experiments. Using GeneJuice (Novagen, Madison, WI, USA), NRK-52E cells were also transfected with p(CAGA)<sub>12</sub>-MLP-Luc or pBRE-Luc together with A2B adenosine receptor siRNA (sense, 5'-AACAGUAAAGACCGUGCCA-3'; antisense, 3'-UUGUCAU UUCUGGCACGGU-5') (Takara, Shiga, Japan), A3 adenosine receptor siRNA (Silencer Select Pre-designed siRNA; Ambion, Austin, TX, USA) or control siRNA (sense, 5'-GCUGCAA UCGAUUGAUAGC-3'; antisense, 3'-CGACGUUAGCUAACU AUCG-5') (Takara). The cells were then treated with or without 3'-deoxyadenosine and subjected to luciferase assay.

### Luciferase Assay

Activity of luciferase was evaluated by Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.<sup>32</sup> Assays were performed in quadruplicate.

### Formazan Assay

The number of viable cells was assessed by formazan assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Assays were performed in quadruplicate.

### Animal Experiment

PBS or 3'-deoxyadenosine (5 mg/kg) was injected intraperitoneally into C57BL/6 mice (20–25 g body weight) on day 0, 1, 2 and 3. On day 1, unilateral ureteral obstruction (UUO) was performed, as described previously.<sup>33</sup> In brief, the proximal part of the left ureter was exposed under anesthesia and ligated completely with a 5.0 silk thread at two separate points (UUO groups; severe ligature). In sham-operated groups, the left ureter was exposed, and the incision was closed without ligation. Three to seven days after the UUO (day 3 and 7), left kidneys were removed and processed for histopathological analysis, immunohistochemistry and northern/western blot analyses.<sup>34</sup> In some experiments, partial UUO (mild ligature) was induced using a nylon thread (0.6 mm in diameter).<sup>35</sup> Individual groups contained 3–4 mice. Animal experiments were approved by the local institutional review board for the care of animal subjects.

### Histopathological Analysis and Immunohistochemistry

Kidneys were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4 °C, embedded in paraffin and subjected to tissue sectioning. The sections were stained by hematoxylin, periodic acid-Schiff or Masson's trichrome. Using Masson's trichrome staining, the area of fibrosis (blue area) was evaluated by LuminaVision V2.04 software (Mitani, Fukui, Japan). Immunohistochemical analysis was performed using anti- $\alpha$ -SMA antibody. Peroxidase reaction was performed using 3,3'-diaminobenzidine tetrahydrochloride.

### Statistical Analysis

Reporter assays were performed in quadruplicate. Northern and western blot analyses were repeated 2–3 times independently (or performed in triplicate or in quadruplicate), and individual data were subjected to densitometric analysis. Data were expressed as means  $\pm$  s.e. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test to compare data in different groups. A *P*-value < 0.05 was considered to indicate a statistically significant difference.

## RESULTS

### Suppression of TGF- $\beta$ and BMP-4 Signaling by 3'-Deoxyadenosine

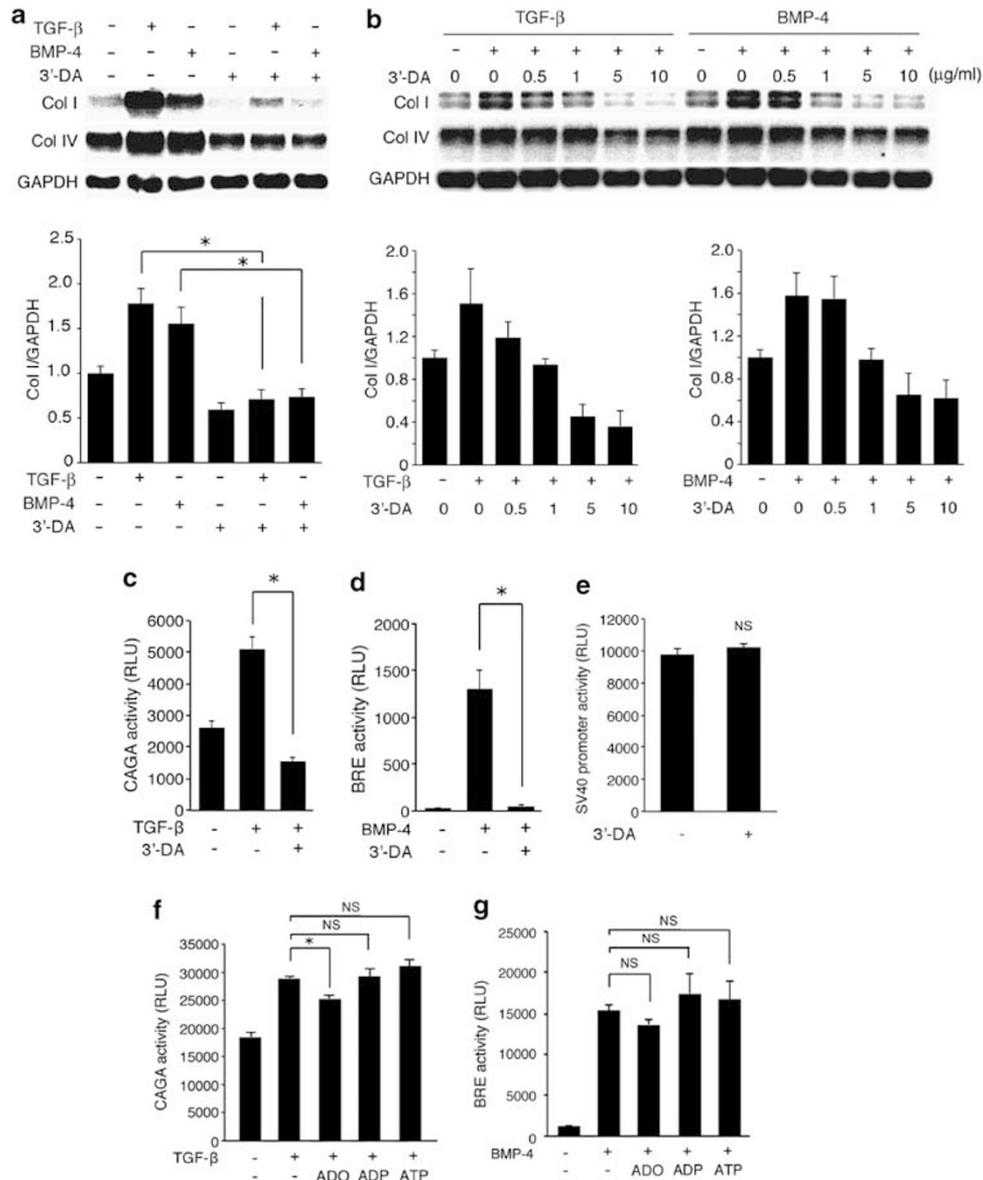
TGF- $\beta$  family members including TGF- $\beta$ 1 and BMP-4 regulate production of extracellular matrix, such as collagens.<sup>36</sup> To examine whether 3'-deoxyadenosine has the potential to influence TGF- $\beta$  signaling, we first examined effects of 3'-deoxyadenosine on TGF- $\beta$ 1- and BMP-4-induced collagen gene expression. When NRK-52E cells were treated with TGF- $\beta$ 1 or BMP-4, expression of type I collagen mRNA was upregulated. The induction of type IV collagen was only minimum. Treatment with 3'-deoxyadenosine suppressed transcriptional expression of both collagens (Figure 1a and Supplementary Figure S1), and the effects were dose dependent (Figure 1b and Supplementary Figure S2). Of note, treatment with 3'-deoxyadenosine also reduced basal levels of type I and type IV collagens (Figure 1a) that may be maintained by platelet-derived TGF- $\beta$ 1 contained in FBS.<sup>37</sup>

TGF- $\beta$ 1 and BMP-4 regulate expression of target genes via activation of the CAGA box and BRE.<sup>7</sup> We next tested effects of 3'-deoxyadenosine on the activation of the CAGA box and BRE by TGF- $\beta$ 1 and BMP-4. Reporter assay showed that TGF- $\beta$ 1 and BMP-4 significantly induced activation of the CAGA box and BRE, respectively. 3'-Deoxyadenosine blocked the activation of these responsive elements (Figures 1c and d). This suppressive effect was not ascribed to non-specific effects on transcription and/or translation, because activity of luciferase driven by the SV40 promoter was not influenced by the treatment with 3'-deoxyadenosine (Figure 1e).

3'-Deoxyadenosine is an analogue of adenosine. Other adenosine-related substances may mimic the effects of 3'-deoxyadenosine. To examine this possibility, effects of adenosine, ADP and ATP were tested. The results showed that, in contrast to 3'-deoxyadenosine, ATP and ADP did not inhibit TGF- $\beta$ 1- and BMP-4-induced activation of the CAGA box (Figure 1f) and BRE (Figure 1g), respectively. Adenosine slightly reduced activation of the CAGA box and BRE, whereas the suppressive effect was much less than that of 3'-deoxyadenosine (Figures 1f and g).

### Roles of Adenosine Receptors and Adenosine Transporter in the Suppression of TGF- $\beta$ and BMP-4 Signaling by 3'-Deoxyadenosine

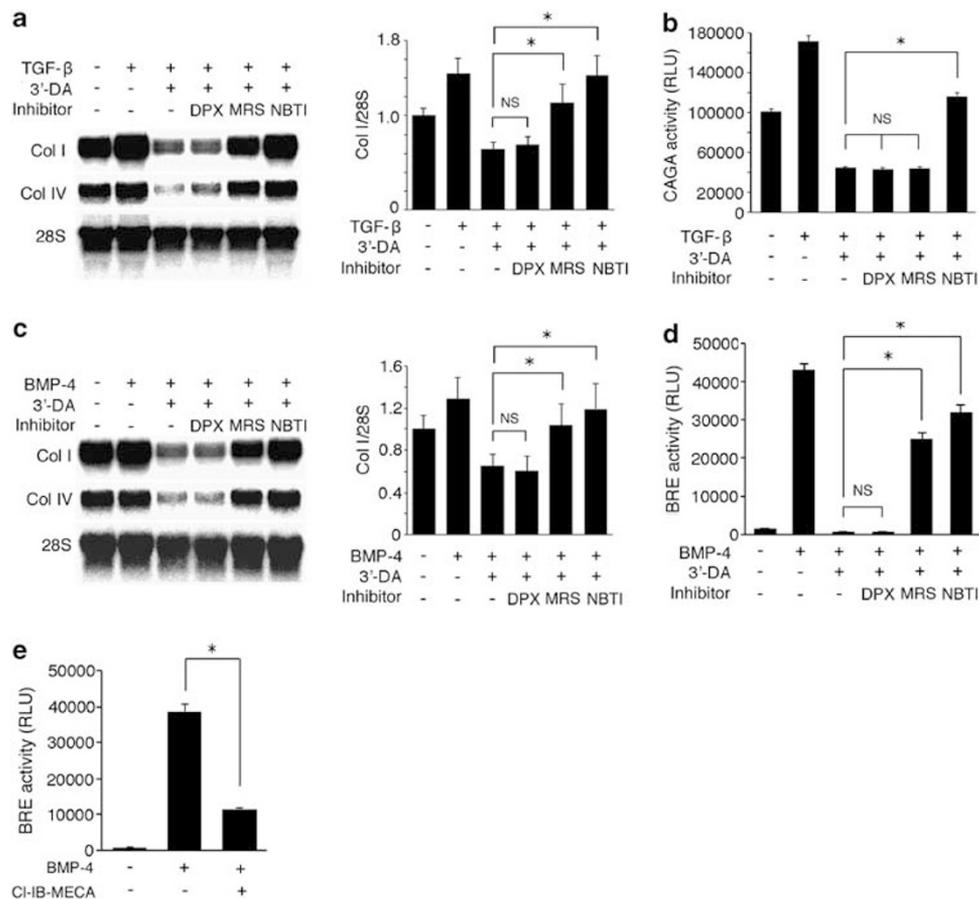
We previously reported that 3'-deoxyadenosine modulated stress responses via the A3 adenosine receptor and adenosine



**Figure 1** Suppression of TGF-β and BMP-4 signaling by 3'-deoxyadenosine (3'-DA). (a) NRK-52E cells were treated with 5 ng/ml TGF-β1 or 50 ng/ml BMP-4 in the absence (-) or presence (+) of 5 μg/ml 3'-DA for 24 h, and expression of type I collagen (Col I) and type IV collagen (Col IV) was evaluated by northern blot analysis. The level of GAPDH mRNA serves as a loading control. Quantitative assessment of Col I mRNA level normalized by the level of GAPDH is shown in the graph. (b) Cells were exposed to TGF-β1 or BMP-4 in the presence of serial concentrations of 3'-DA for 24 h and subjected to northern blot analysis. Quantitative assessment is shown in graphs. (c-e) Cells were transfected with p(CAGA)<sub>12</sub>-MLP-Luc (c), pBRE-Luc (d) or pSV40-Luc (e), treated with indicated reagents for 24 h and subjected to chemiluminescent assay. (f, g) Cells were transfected with p(CAGA)<sub>12</sub>-MLP-Luc (f) or pBRE-Luc (g), treated with adenosine (ADO; 100 μM), ADP (100 μM) or ATP (100 μM) in the presence of TGF-β (f) or BMP-4 (g) for 24 h and subjected to chemiluminescent assay. Data are expressed as means ± s.e., and asterisks indicate statistically significant differences (P < 0.05). NS, not statistically significant; RLU, relative light unit.

transporter.<sup>22</sup> To elucidate mechanisms underlying the suppressive effects of 3'-deoxyadenosine on TGF-β1 and BMP-4 signaling, roles of adenosine receptors and adenosine transporter were examined. For this purpose, A1/A2 adenosine receptor antagonist DPSPX, A3 adenosine receptor antagonist MRS1523 and adenosine transport inhibitor NBTI were used. Northern blot analysis showed that the suppressive effect of 3'-deoxyadenosine on the induction of collagens by TGF-β1 was reversed by MRS1523 and NBTI

(Figure 2a and Supplementary Figure S3a). In contrast, DPSPX did not affect the suppressive effect of 3'-deoxyadenosine. The suppressive effect of 3'-deoxyadenosine on TGF-β1-triggered CAGA activation was also reversed by NBTI, whereas DPSPX and MRS1523 did not influence the effect of 3'-deoxyadenosine (Figure 2b). Similarly, the suppressive effect of 3'-deoxyadenosine on the induction of collagens by BMP-4 was reversed by MRS1523 and NBTI, whereas DPSPX did not affect the suppressive effect of



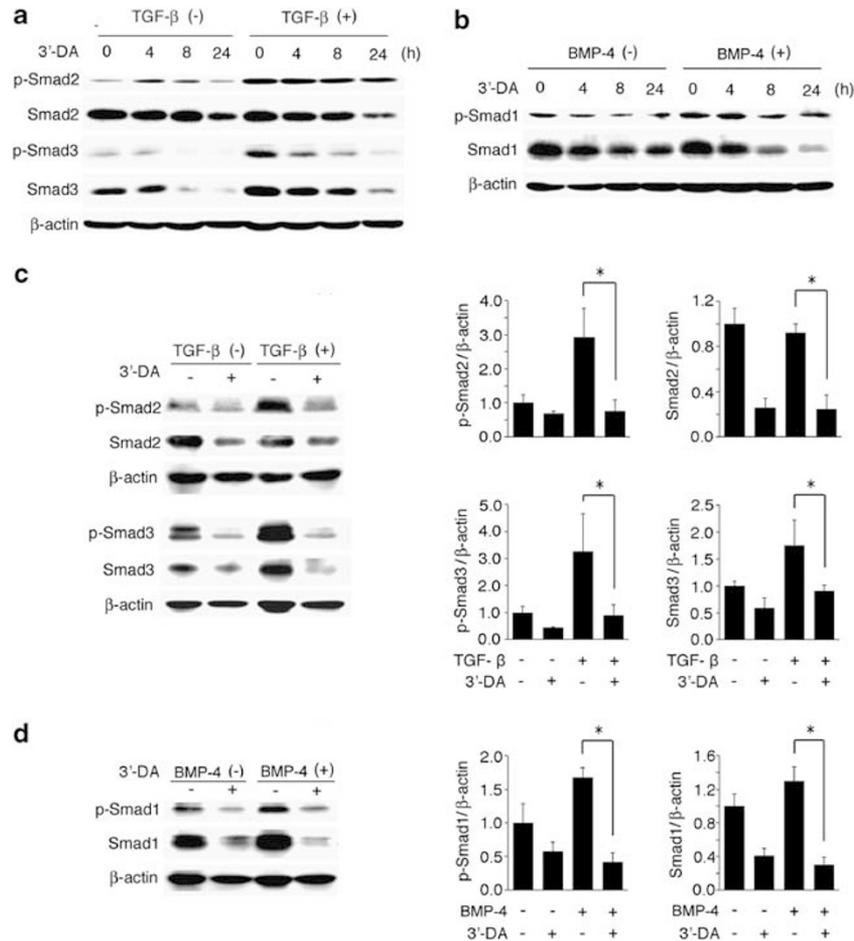
**Figure 2** Roles of adenosine receptors and adenosine transporter in the suppression of TGF- $\beta$  and BMP-4 signaling by 3'-deoxyadenosine (3'-DA). (**a, c**) NRK-52E cells were exposed to TGF- $\beta$ 1 (**a**) or BMP-4 (**c**) in the absence or presence of 3'-DA, DPSPX (DPX; 10 nM), MRS1523 (MRS; 5  $\mu$ M) or NBTI (10  $\mu$ M) for 24 h and subjected to northern blot analysis of Col I and Col IV. The level of 28S ribosomal RNA is shown at the bottom as a loading control. Quantitative analysis of Col I normalized by the level of 28S is shown in graphs. (**b, d**) Cells transfected with p(CAGA)<sub>12</sub>-MLP-Luc (**b**) or pBRE-Luc (**d**) were treated with indicated agents for 24 h and subjected to luciferase assay. (**e**) Cells transfected with pBRE-Luc were treated with BMP-4 with or without Cl-IB-MECA (10  $\mu$ M) for 24 h and subjected to chemiluminescent assay. Asterisks indicate statistically significant differences. NS, not statistically significant.

3'-deoxyadenosine (Figure 2c and Supplementary Figure S3b). The suppressive effect of 3'-deoxyadenosine on BMP-4-triggered BRE activation was also reversed by MRS1523 and NBTI, but not by DPSPX (Figure 2d). Treatment with agonist of adenosine A3 receptor Cl-IB-MECA significantly reduced BRE activation triggered by BMP-4 (Figure 2e). Consistent with these results using pharmacological inhibitors, knockdown of the A2B adenosine receptor by siRNA had little impacts on the suppressive effects of 3'-deoxyadenosine on TGF- $\beta$ 1- and BMP-4-induced activation of the CAGA box and BRE (Supplementary Figures S4a and S4b, left). In contrast, knockdown of the A3 receptor partially reversed the suppressive effect of 3'-deoxyadenosine on BMP-4-induced BRE activation, but not on TGF- $\beta$ 1-induced activation of the CAGA box (Supplementary Figures S4a and S4b, right).

### Reduction in the Level of Smad Proteins by 3'-Deoxyadenosine

Phosphorylation of Smad proteins has central roles in TGF- $\beta$  and BMP signaling.<sup>11</sup> To elucidate mechanisms underlying

the suppressive effects of 3'-deoxyadenosine on the TGF- $\beta$  and BMP-4 pathways, we tested effects of 3'-deoxyadenosine on the phosphorylation of Smad proteins. When cells were treated with TGF- $\beta$ 1, phosphorylation of Smad2 and Smad3 was observed. The basal and inducible phosphorylation of Smad3 was substantially suppressed by the treatment with 3'-deoxyadenosine (Figure 3a and Supplementary Figure S5). Phosphorylation of Smad2 was also inhibited modestly by 3'-deoxyadenosine. Interestingly, levels of total Smad2 and Smad3 proteins, especially Smad3, were depressed by the treatment with 3'-deoxyadenosine (Figure 3a and Supplementary Figure S5). Similarly, when cells were treated with BMP-4, phosphorylation of Smad1 was observed, and the basal and phosphorylated Smad1 was reduced by the treatment with 3'-deoxyadenosine (Figure 3b, and Supplementary Figure S6). Like Smad3, the level of total Smad1 was also reduced by the treatment with 3'-deoxyadenosine. The suppressive effects of 3'-deoxyadenosine on Smads were not specific to NRK-52E cells, and similar effects were also observed in rat mesangial cells (Figures 3c and d).



**Figure 3** Reduction in the levels of Smad proteins by 3'-deoxyadenosine (3'-DA). (a, b) NRK-52E cells were pretreated with 3'-DA for indicated time periods, stimulated with or without TGF- $\beta$ 1 (a) or BMP-4 (b) for 1 h and subjected to western blot analysis of phosphorylated and total Smad proteins. The level of  $\beta$ -actin is shown at the bottom as a loading control. (c, d) SM43 cells were pretreated with 20  $\mu$ g/ml 3'-DA for 8 h, stimulated with TGF- $\beta$ 1 (c) or BMP-4 (d) for 1 h and subjected to western blot analysis of Smad proteins. Quantification of individual signals normalized by the level of  $\beta$ -actin is presented as graphs. Asterisks indicate statistically significant differences.

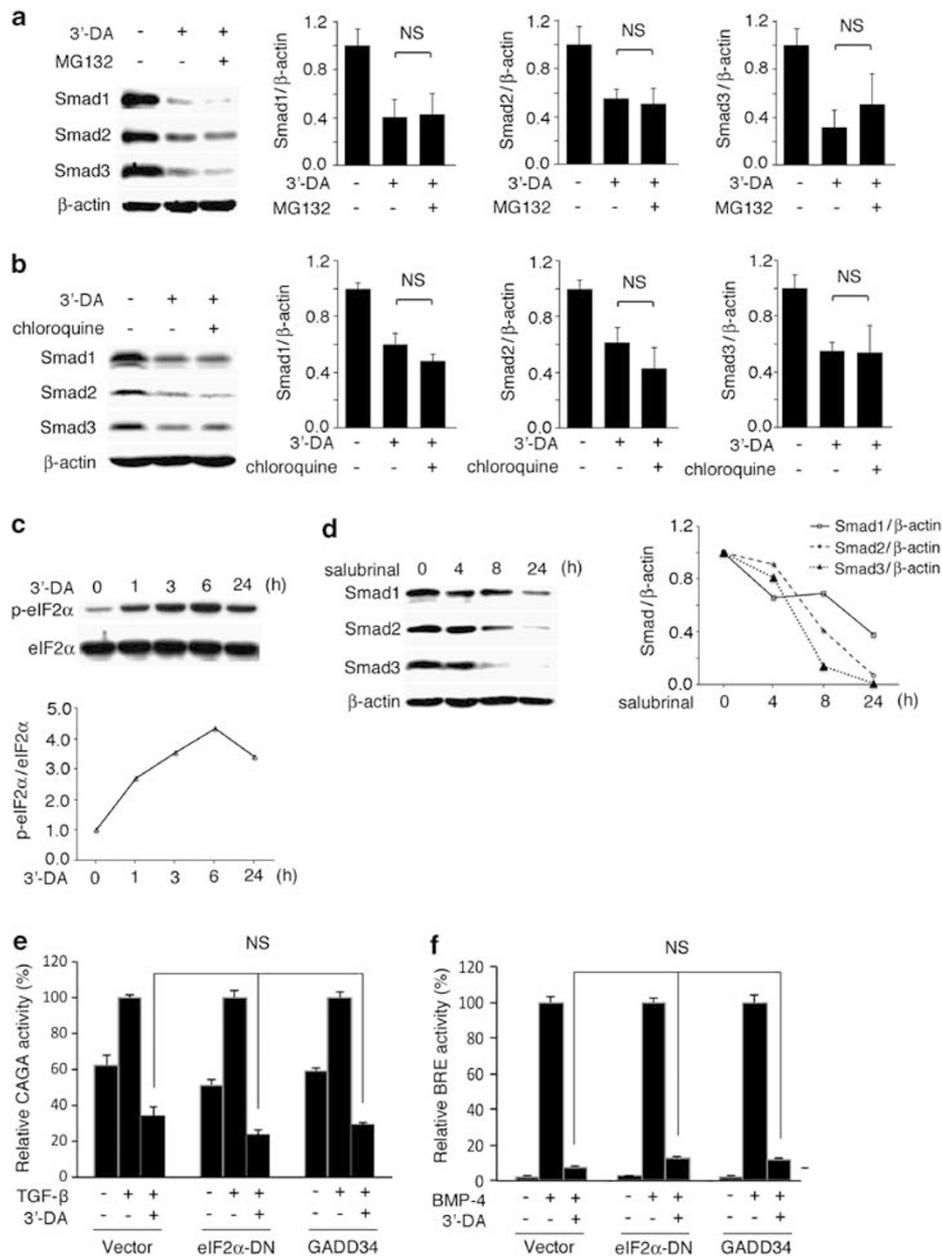
Consistent with the lack of effects of adenosine on TGF- $\beta$ 1- and BMP-4-induced activation of the CAGA box and BRE (Figures 1f and g), TGF- $\beta$ 1- and BMP-4-induced phosphorylation of Smad1, Smad2 and Smad3 were not affected by adenosine. The levels of total Smad proteins were also unaffected by the treatment with adenosine (Supplementary Figures S7a and S7b).

#### Lack of eIF2 $\alpha$ -mediated Translational Suppression for the Effect of 3'-Deoxyadenosine

The down regulation of Smad proteins may be caused by enhanced protein degradation or translational suppression. To examine the former possibility, cells were treated with 3'-deoxyadenosine in the absence or presence of proteasome inhibitor MG132 or lysosome inhibitor chloroquine. Western blot analysis showed that both MG132 and chloroquine failed to reverse the inhibitory effects of 3'-deoxyadenosine on Smad proteins (Figures 4a and b). Of note, the concentration of MG132 was sufficient to inhibit proteasome function, because MG132 efficiently inhibited degradation of connexin

43 in NRK-52E cells (our unpublished data). Similarly, the concentration of chloroquine was sufficient to inhibit autophagy (data not shown).

As we recently reported,<sup>22</sup> 3'-deoxyadenosine rapidly induces phosphorylation of eIF2 $\alpha$  that causes translational suppression (Figure 4c). Induction of the eIF2 $\alpha$  pathway may be involved in the downregulation of Smads by 3'-deoxyadenosine. Indeed, treatment with salubrinal, an inhibitor of eIF2 $\alpha$  dephosphorylation, reduced the levels of all Smad proteins (Figure 4d). However, activation of eIF2 $\alpha$  was found not to be the primary mechanism, because functional knockdown of eIF2 $\alpha$  by a eIF2 $\alpha$ -DN or GADD34 that dephosphorylates eIF2 $\alpha$  did not affect the suppressive effect of 3'-deoxyadenosine on TGF- $\beta$ 1-induced activation of the CAGA box (Figure 4e). Similarly, functional inhibition of eIF2 $\alpha$  by eIF2 $\alpha$ -DN or GADD34 did not reverse the suppressive effect of 3'-deoxyadenosine on BMP-4-induced activation of BRE (Figure 4f). Of note, transfection with eIF2 $\alpha$ -DN significantly inhibited activation of CCAAT/enhancer-binding protein via the eIF2 $\alpha$  pathway.<sup>38</sup> Transfection



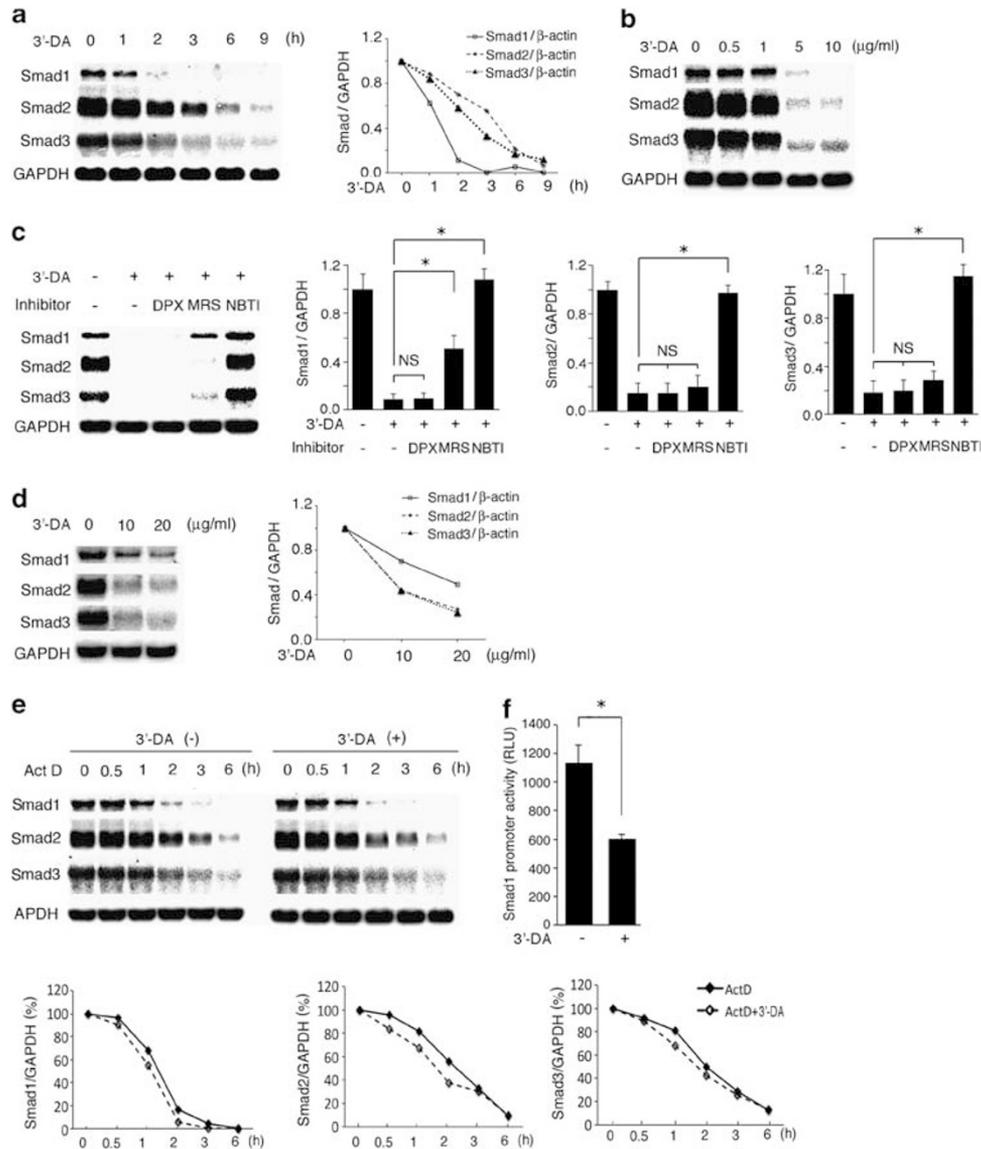
**Figure 4** Lack of eIF2 $\alpha$ -mediated translational suppression for the effect of 3'-deoxyadenosine (3'-DA). **(a, b)** NRK-52E cells were treated with 3'-DA for 9 h with or without 10  $\mu$ M MG132 **(a)** or 20  $\mu$ M chloroquine **(b)** and subjected to western blot analysis of Smad proteins. Quantification of individual signals is shown in graphs. **(c)** Cells were treated with 3'-DA for indicated time periods and subjected to western blot analysis of phosphorylated eIF2 $\alpha$ . The protein level of total eIF2 $\alpha$  is shown as a loading control. Quantification of phosphorylated eIF2 $\alpha$  normalized by the level of total eIF2 $\alpha$  protein is shown. **(d)** Cells were treated with 50  $\mu$ M salubrinal for indicated time periods and subjected to western blot analysis of Smad proteins. Quantification of Smad proteins normalized by the level of  $\beta$ -actin is shown. **(e, f)** NRK-52E cells were transfected with p(CAGA)<sub>12</sub>-MLP-Luc **(e)** or pBRE-Luc **(f)** together with empty vector (Vector), eIF2 $\alpha$ -DN or GADD34, treated with TGF- $\beta$ 1 **(e)** or BMP-4 **(f)** in the absence or presence of 3'-DA for 24 h and subjected to luciferase assay. NS, not statistically significant.

with GADD34 also significantly reversed suppressive effects of salubrinal on p21<sup>CIP1/WAF1</sup> (our unpublished data).

### Transcriptional Suppression of Smads by 3'-Deoxyadenosine

As described, the downregulation of Smad proteins by 3'-deoxyadenosine was ascribed neither to enhanced

degradation nor to translational suppression via eIF2 $\alpha$ . To examine another possibility, we tested an effect of 3'-deoxyadenosine on the steady-state level of *Smad* mRNAs. Northern blot analysis revealed that 3'-deoxyadenosine substantially reduced the levels of *Smad* mRNAs in both time- and dose-dependent manners (Figures 5a and b). Consistent with the results of reporter assays for CAGA and



**Figure 5** Transcriptional suppression of *Smads* by 3'-deoxyadenosine (3'-DA). (a) NRK-52E cells were treated with 3'-DA for indicated time periods and subjected to northern blot analysis for *Smad* mRNAs. The levels of *Smad1*, *Smad2* and *Smad3* mRNAs were normalized by the level of *GAPDH*, and the kinetics is shown in the graph. (b) Cells were treated with 3'-DA for indicated concentrations for 9 h and subjected to northern blot analysis of *Smads*. (c) Cells were treated with 3'-DA in the presence of DPSPX, MRS1523 or NBTI for 9 h and subjected to northern blot analysis. Quantification of individual signals is shown in graphs. (d) SM43 cells were treated with 3'-DA for 9 h and subjected to northern blot analysis. Quantification of individual signals is shown in the graph. (e) NRK-52E cells were pretreated with or without 3'-DA for 30 min, exposed to actinomycin D (Act D; 5 µg/ml) for 0–6 h and subjected to northern blot analysis. The levels of *Smad1*, *Smad2* and *Smad3* mRNAs were normalized by the level of *GAPDH*, and relative intensity of *Smad* mRNAs is shown in graphs. (f) Cells were transiently transfected with pGL2-F-1005/+93Smad1, treated with or without 3'-DA for 24 h and subjected to chemiluminescent assay. Asterisks indicate statistically significant differences. NS, not statistically significant; RLU, relative light unit.

BRE activity (Figures 2b and d), downregulation of *Smad* mRNAs by 3'-deoxyadenosine was reversed by the treatment with NBTI, whereas DPSPX had little impacts (Figure 5c). Downregulation of *Smad1* by 3'-deoxyadenosine was reversed partially by MRS1523, whereas downregulation of *Smad2* and *Smad3* was unaffected (Figure 5c). The suppressive effects of 3'-deoxyadenosine on *Smad* mRNAs were not specific to NRK-52E cells, and similar effects were also observed in rat mesangial cells (Figure 5d).

The downregulation of *Smad* mRNAs may be caused by transcriptional suppression or enhanced degradation of mRNAs. To examine the latter possibility, cells were treated with actinomycin D, an inhibitor of RNA synthesis, in the absence or presence of 3'-deoxyadenosine. Northern blot analysis showed that actinomycin D caused degradation-dependent decreases in *Smads*, and the degradation of *Smad* mRNAs was not reinforced by the treatment with 3'-deoxyadenosine (Figure 5e). This result suggested that

3'-deoxyadenosine downregulated *Smad* mRNAs via transcriptional inhibition. To confirm this conclusion, we examined an effect of 3'-deoxyadenosine on the activity of the *Smad1* gene promoter. The result showed that 3'-deoxyadenosine significantly suppressed *Smad1* promoter activity (Figure 5f), confirming our conclusion.

### **In Vivo Suppression of Smad Signaling by 3'-Deoxyadenosine**

To examine whether the level of Smads is depressed by 3'-deoxyadenosine *in vivo*, mice were subjected to severe UUO, an experimental model of renal fibrosis, with or without administration of 3'-deoxyadenosine. That is, sham-operated and ureter-ligated mice were treated everyday with PBS or 3'-deoxyadenosine, and after 3 days, kidneys were subjected to analyses. Northern blot analysis revealed that *Smad* mRNAs were induced by UUO, and the induction was suppressed by the treatment with 3'-deoxyadenosine (Figure 6a). Western blot analysis also showed that both phosphorylated and total Smad proteins (except for total Smad3) increased by UUO, and it was attenuated by administration with 3'-deoxyadenosine (Figure 6b). Consistent with this result, expression of type I collagen was markedly upregulated by UUO, and it was suppressed by the treatment with 3'-deoxyadenosine (Figure 6c). Quantitative analysis of fibrotic region confirmed reduction in fibrosis by the treatment with 3'-deoxyadenosine (Figure 6d). Similarly, western blot analysis also showed that the level of  $\alpha$ -SMA, a downstream target of Smad signaling and a marker of fibrogenesis,<sup>39,40</sup> was induced by UUO, and it was attenuated by 3'-deoxyadenosine (Figure 6e). This result was further confirmed using immunohistochemical analysis. As shown in Figure 6f,  $\alpha$ -SMA increased in the renal interstitium in the UUO model, and it was diminished by the treatment with 3'-deoxyadenosine (Figure 6f, left). The increase in the number of  $\alpha$ -SMA-positive myofibroblasts was also reduced by this agent (Figure 6f, right).

In our complete-ligature model of UUO, tissue damage is too severe, and 3'-deoxyadenosine failed to halt progression of renal fibrosis significantly, when examined at day 7 (data not shown). We therefore used a mild-ligature model of UUO<sup>34</sup> to examine the effect of this agent at a later time period. In this experimental model, induction of collagen and  $\alpha$ -SMA was mild, and treatment with 3'-deoxyadenosine modestly attenuated UUO-induced expression of type I collagen and  $\alpha$ -SMA even at day 7 (Supplementary Figure S8).

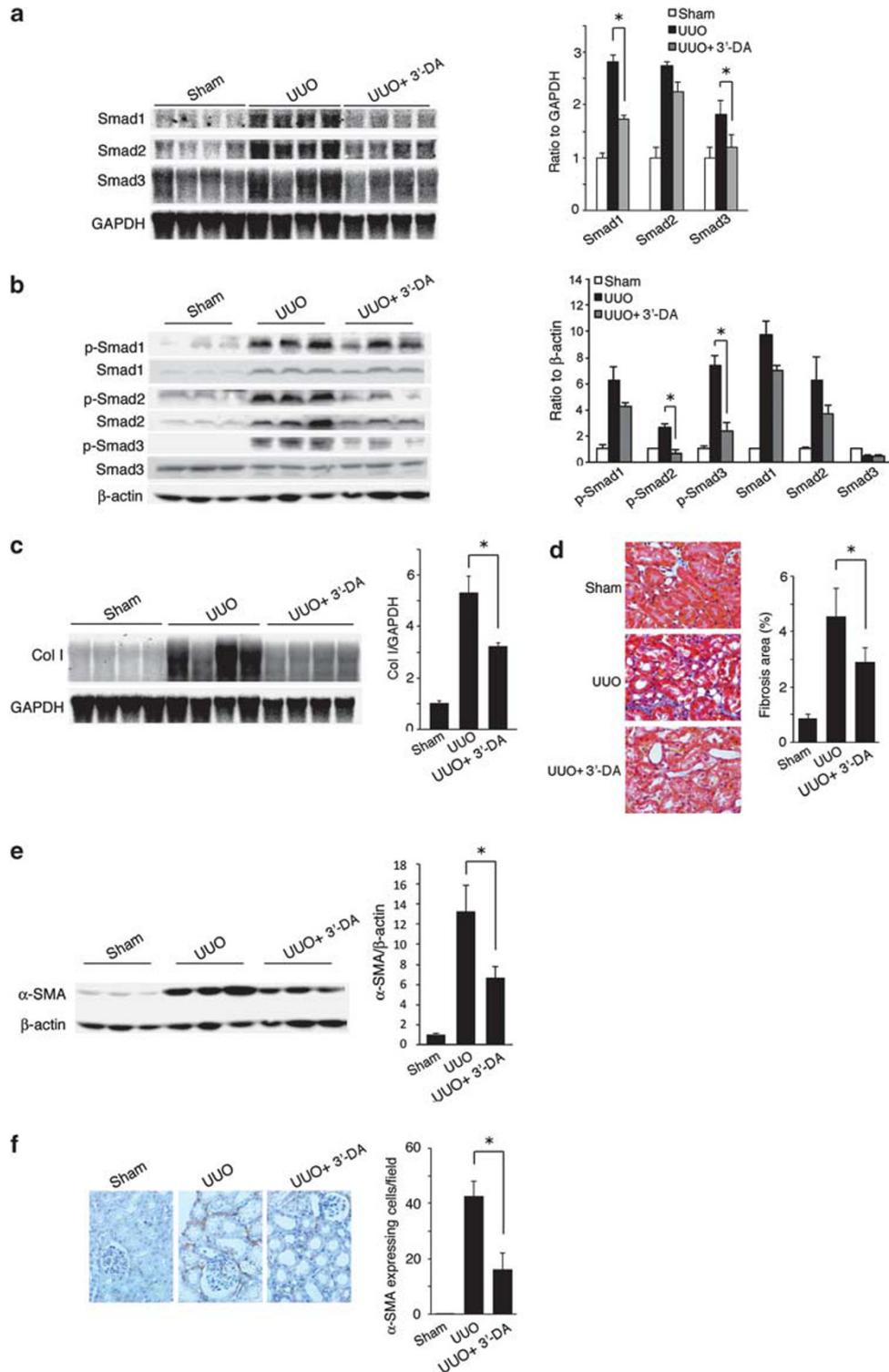
### **DISCUSSION**

In the present investigation, we demonstrated that 3'-deoxyadenosine interfered with the TGF- $\beta$  and BMP signaling via downregulation of phosphorylated Smad1, Smad2 and Smad3. We also elucidated that the decrease in the level of phosphorylated Smads was correlated with down regulation of total Smad proteins, which was caused by transcriptional suppression. Previous reports indicated that

3'-deoxyadenosine may inhibit transcriptional events via multiple mechanisms.<sup>41,42</sup> It inhibits mRNA polyadenylation, possibly, by acting as a chain terminator.<sup>42</sup> 3'-Deoxyadenosine may also inhibit incorporation of uridine into mRNA at high doses.<sup>41</sup> The suppression of *Smad* mRNAs by 3'-deoxyadenosine, observed in this report, may be through the similar mechanisms on mRNAs. However, based on our experience, individual mRNAs have different susceptibility and response to 3'-deoxyadenosine. For example, 3'-deoxyadenosine suppresses basal and inducible expression of an array of endogenous genes, whereas house-keeping gene promoter- and viral gene promoter-driven transcription may be paradoxically upregulated by this agent (manuscript in submission). In differentiating preadipocytes, 3'-deoxyadenosine rather upregulates expression of monocyte chemoattractant protein 1.<sup>22</sup> These results are consistent with a recent report showing different sensitivity of different mRNAs to 3'-deoxyadenosine.<sup>17</sup> The effect of 3'-deoxyadenosine on transcriptional events is not so simple as was previously proposed, and *Smad* mRNAs may be preferential targets of this agent to inhibit transcription.

In the present study, we showed that the inhibitory effects of 3'-deoxyadenosine on *Smad* mRNAs were mediated by adenosine transporter. A previous report suggested that 3'-deoxyadenosine 5'-triphosphate inhibited chromatin-associated poly(A) synthesis.<sup>43</sup> 3'-Deoxyadenosine 5'-triphosphate might be an active entity responsible for the inhibitory effects of 3'-deoxyadenosine. Conversion of 3'-deoxyadenosine to 3'-deoxyadenosine 5'-triphosphate occurs intracellularly. Transport of extracellular 3'-deoxyadenosine into the cells should be essential for the generation of 3'-deoxyadenosine 5'-triphosphate. It may be the reason why inhibition of adenosine transporter abrogated the suppressive effect of 3'-deoxyadenosine on *Smad* mRNAs. Interestingly, however, the suppressive effect of 3'-deoxyadenosine on *Smad1* was also mediated by the A3 adenosine receptor. Currently, it is unclear how activation of the A3 receptor causes suppression of Smad1 and BRE activation, but it is consistent with our recent report showing that, in addition to the adenosine transporter, the A3 receptor was also involved in the suppression of stress marker induction by 3'-deoxyadenosine under endoplasmic reticulum stress.<sup>22</sup>

Regarding the role of the A3 receptor, the majority of our current data are consistent; ie, it mediates the suppressive effect of 3'-deoxyadenosine on the Smad1-BRE signaling, but not its effect on the Smad2/3-CAGA box pathway. Only one exception is that inhibition of the A3 receptor by MRS1523 reversed the suppressive effect of 3'-deoxyadenosine on TGF- $\beta$ -triggered induction of collagens. We found that both MRS1523 and NBTI reversed the suppressive effects of 3'-deoxyadenosine on type I and type IV collagen mRNAs. It indicates that some signaling pathway other than Smad2/3-CAGA box is also involved in the induction of collagens by TGF- $\beta$  and that 3'-deoxyadenosine inhibits the putative signaling via ligation of the A3 adenosine receptor.



**Figure 6** *In vivo* suppression of Smad signaling by 3'-deoxyadenosine (3'-DA). **(a, b)** Sham-operated (Sham) and ureter-ligated (UUO) mice were treated daily with or without 3'-DA, and after 3 days, kidneys were subjected to northern blot analysis of *Smad* mRNAs **(a)** and western blot analysis of phosphorylated and total Smad proteins **(b)**. Quantification of individual signals normalized by the level of *GAPDH* **(a)** or  $\beta$ -actin **(b)** is presented in graphs. In the blots, individual lanes represent different animals. **(c)** Northern blot analysis of Col I expression in three groups. Quantification of Col I level normalized by the level of *GAPDH* is shown in the graph. **(d)** Histopathological analysis using Masson's trichrome staining (left) and quantification of fibrotic area (right). **(e, f)** Evaluation of  $\alpha$ -SMA expression by western blot analysis **(e)** and immunohistochemistry **(f)**. Quantitative assessment of  $\alpha$ -SMA level **(e)** and the number of  $\alpha$ -SMA-expressing interstitial cells **(f)** is shown in graphs. Asterisks indicate statistically significant differences.

In the present study, we showed that, compared with Smad1 and Smad3, the level of Smad2 protein was relatively preserved after the treatment with 3'-deoxyadenosine, although *Smad2* mRNA was similarly suppressed by this agent. A previous report suggested that Smad3, but not Smad2, was susceptible to ubiquitin/proteasome-dependent protein degradation.<sup>44</sup> It may result in decreased stability of Smad3 when compared with Smad2. It is worthwhile to note that Smad3 is considered as a dominant mediator for the induction of fibrogenic processes. For example, Smad3-deficient mice are resistant to interstitial fibrosis triggered by UO.<sup>45</sup> Using siRNA to achieve selective knockdown of Smad2 and Smad3, Phanish *et al.*<sup>46</sup> found that induction of connective tissue growth factor by TGF- $\beta$  was Smad3 dependent, but independent of Smad2 in renal tubular cells. 3'-Deoxyadenosine could interfere with the TGF- $\beta$  signaling through preferential suppression of Smad3, although there was some discrepancy between the *in vitro* data and *in vivo* results.

Smad signaling has been considered as a master switch for the induction of tissue fibrosis. Previous reports demonstrated that the Smad pathways were crucial for the induction of collagen genes.<sup>47,48</sup> Smads also mediate negative effects of TGF- $\beta$  on matrix metalloproteinases and its positive effects on tissue inhibitor of metalloproteinases, contributing to fibrogenesis.<sup>49,50</sup> In the present report, we demonstrated that *in vivo* administration with 3'-deoxyadenosine reduced the levels of phosphorylated and total Smad proteins, as well as *Smad* mRNAs in the kidney subjected to UO. It was associated with attenuated induction of interstitial-type collagen and  $\alpha$ -SMA, the downstream targets of Smad signaling, in the kidney. Our current results indicate that 3'-deoxyadenosine interferes with the profibrotic Smad signaling and could be useful for the treatment of fibrogenic disorders.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict interest.

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