Nephrol Dial Transplant (2008) 23: 1126–1134
doi: 10.1093/ndt/gfm786
Advance Access publication 28 November 2007

Original Article

NDT
Nephrology Dialysis Transplantation

Transforming growth factor-β1 decreases epithelial sodium channel functionality in renal collecting duct cells via a Smad4-dependent pathway

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Abstract

Background. Transformation growth factor-β1 (TGF-β1) inhibits transepithelial sodium transport and suppresses the epithelial sodium channel (ENaC) in many different types of epithelial cells; however, the molecular mechanism of this effect in the kidney is still not clear. The aim of this study was to examine the regulation of transepithelial sodium transport by TGF-β1 in renal cells.

Methods. We derived stable mouse cortical collecting duct cell lines that overexpressed Smad4 or N-termianl truncated Smad4, and studied the effects of TGF-β1 on them. The equivalent electrical current (I_{eq}) was taken as representing transepithelial current and the amiloride sensitive short circuit current (AmsIsc) as representing the ENaC activity. We used real-time PCR to quantify the expression of ENaC and measurement of the luciferase activity of cells transiently transfected with a mouse α-ENaC promoter to assess the α-ENaC promoter activity.

Result. The administration of TGF-β1 decreased I_{eq}, mainly as a result of the decrease of AmsIsc, and it correlated with inhibition of the α-ENaC mRNA expression. The overexpression of Smad4 led to a decrease in AmsIsc, α-ENaC mRNA and α-ENaC promoter activity, but the overexpression of the N-terminal truncated Smad4 did not induce these changes. The TGF-β1-induced reduction of AmsIsc was alleviated in the N-terminal truncated Smad4-overexpressed cells.

Conclusion. It appears that the N-terminus region of Smad4 is indispensable in Smad4-mediated inhibition of the transepithelial sodium transport. TGF-β1 may decrease the ENaC functionality via a Smad4-dependent pathway.

Keywords: TGF-β1; cortical collecting duct; epithelial sodium channel; short circuit current; Smad signalling pathway

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Introduction

One of the important functions of the kidney is to maintain extracellular fluid volume regulation. Most patients with renal failure have abnormalities of sodium homeostasis. The majority of them tend to retain sodium and have extracellular fluid overload, but some, especially those with tubulointerstitial and obstructive nephropathies, contrarily have sodium loss due to an increase of urinary sodium. Sodium transport is regulated by epithelial sodium channels (ENaC). It has been well established that ENaC contributes to the regulation of blood pressure through alterations in sodium balance and extracellular volume [1]. ENaC has been found in different types of epithelia including of the airway, sweat and salivary glands, colon, urinary bladder and renal collecting duct. Cortical collecting ducts (CCDs) are the segments of renal tubules that fine regulate sodium transport [2]. ENaC is a heteromultimeric complex composed of three subunits, α-, β- and γ-ENaC, and it is specifically inhibited by amiloride [2]. It has been demonstrated that α-ENaC alone, but not β- or γ-ENaC, is sufficient to produce small amiloride-sensitive currents [3]. Also, α-ENaC is a crucial ENaC subunit as α-ENaC knockout mice died within 48 h after birth due to respiratory distress caused by the failure of the α-ENaC-mediated clearance of pulmonary fluid [4].

The activation and regulation of ENaC expression depend mainly on aldosterone, which modulates the ENaC expression. Other factors, including hormones (such as vasopressin and insulin) or cytokines, epidermal growth factor and transforming growth factor-β1 (TGF-β1), can also alter the expression of ENaC and sodium pump function [5]. In a normal kidney, only a small amount of TGF-β can be detected, but it is prominently upregulated in many renal diseases such as diabetic nephropathy, obstructive nephropathy and tubulointerstitial nephritis [6]. It has been shown that α-ENaC is one of the targets regulated by TGF-β1. In human and rat alveolar cells, TGF-β1 can decrease transepithelial sodium transport by inhibiting the α-ENaC

expression through a mechanism dependent on ERK1/2 [7]. Tuyen *et al*. demonstrated that TGF-β1 can inhibit transepithelial sodium transport in M-1 mouse CCD cells [8,9]. Nevertheless, how TGF-β1 regulates the expression and function of α-ENaC remains unclear.

Furthermore, TGF-β1 interacts with its membrane-bound receptor and then activates the TGF-β1's downstream Smad signal pathway. Three classes of Smad have been identified and are categorized as receptor-activated Smads (Smad2 and Smad3), co-Smad (Smad4) and inhibitory Smad (Smad7). Despite the fact that the proteins of Smad are key participants in the classical TGF-β1 signalling pathways, other signalling pathways not dependent on Smad have also been shown to participate in the TGF-β signalling cascade, including several members of the superfamily of mitogen-activated protein kinase (MAPK) signalling pathways [10].

The aim of this study was to determine how TGF-β1 regulates transepithelial sodium transport in renal cortical collecting duct cells.

Material and methods

Reagents

Culture media (DMEM, HAM's F12) and the transfection reagent Lipofetamine-2000 were obtained from Invitrogen (Carlsbad, CA, USA). The permeable filters (0.4 μm pore size) were purchased from Costar (Cambridge, MA, USA) and amiloride from Sigma (Sigma Chemical Co., St Louis, MO, USA). The p38MAPK inhibitor (SB203580), the ERK1/2 kinase inhibitor (PD98059) and the JUN kinase inhibitor (SP600125) were obtained from Calbiochem (La Jolla, CA, USA) and Zeocin (phleomycin D1) from Invitrogen (Carlsbad, CA, USA).

Cell culture

We cultured mpkCCDcl$_4$ cells on porous filters in modified DM medium [DMEM:Ham's F12, 1:1 vol/vol; 60 nM sodium selenate; 5 μg/ml transferrin; 2 mM glutamine; 50 nM dexamethasone; 1 nM triiodothyronine; 10 ng/ml epidermal growth factor (EGF); 5 μg/ml insulin; 20 mM D-glucose; 2% foetal calf serum and 20 mM HEPES, pH 7.4] at 37°C in a 5% CO_2/95% O_2 environment [11]. The medium was changed every 2 days until the cells became confluent. The cells were then serum-deprived for 18 h (overnight) prior to further use.

Generation of wild-type or N-terminus truncated Smad4 overexpressing cell lines

Flag-epitope (DYKDDDDK) cDNA was attached to coding sequences of the N-terminus of full length human Smad4 (553 amino acid) (access No. BC002379) obtained by PCR from human genomic DNA using a standard technique [12]. Flag-tagged N-terminus truncated Smad4 (Smad4ΔN) was generated by deleting the first 222 amino acids of Smad4. Flag-tagged Smad4 or Smad4ΔN fragments were sub-cloned in the pcDNA4/TO vector (Promega, Madison, WI, USA). All resultant constructs were verified by DNA sequencing. Wild-type Smad4 or Smad4ΔN was introduced into mpkCCDcl$_4$ cells using lipofetamine-2000 (Invitrogen) according to the manufacturer's instructions. The cells that stably overexpressed the wild-type or N-terminus truncated Smad4s were drug selected using Zeocin (Invitrogen). Single colonies were isolated for culture, and the presence of protein of Smad4s was confirmed by western blot analysis.

Generation of the recombinant adenoviruses

The recombinant adenoviruses carrying the genes of Flag-tagged wild-type Smad4 or Smad4ΔN were generated using AdEasy^{TM} kits (Stratagene, La Jolla, CA, USA). Briefly, Flag-tagged wild-type Smad4 or Smad4ΔN cDNAs were isolated from plasmids using restriction enzymes. These cDNAs were subsequently inserted into shuttle vectors (p-Shuttle-CMV). A shuttle vector that did not carry the gene of interest was used as control. The shuttle DNA was then linearized and purified. The linearized DNA and the adenovirus vector were cotransfected into BJ5183 cells to produce recombinant adenoviral-plasmid. The recombinants were selected using kanamycin and identified with restriction digestion. Purified recombinant adenoviral-plasmid DNA was linearized and transfected into human embryonic kidney cells (HEK293) for viral packing. The resulting viral titers were measured using QuickTiter adenovirus titer immunoassay kits according to manufacturer's instructions (Cell Biolabs, San Diego, CA, USA). These adenoviruses were then used to infect mpkCCDcl$_4$ cells during a 48-h exposure before further use.

Electrophysiological studies

TGF-β1 was added to the apical or basal side of the porous filters on which the mpkCCDcl$_4$ cells were grown (as described above), and transepithelial voltage (*VT*) and electrical resistance (*R*) were measured regularly using a voltohmmeter (EVOM, World Precision Instruments, Saratosa, FL, USA). The transepithelial equivalent electrical current (I_{eq}) was calculated using Ohm's law $VT = I \times R$ [11].

To further confirm the results, short circuit current methods were also used to measure the sodium transport current. Cells cultivated in Snapwell porous filters (Costar) were mounted on a Ussing-type diffusion chamber connected to a voltage clamp apparatus (Physiological Instruments, San Diego, CA, USA). The cell layers were bathed on both sides simultaneously with an 8 ml serum-deprived medium warmed to 37°C and continuously gassed with a 95% O_2/5% CO_2 mixture to keep the pH at 7.4. Isc (μA/cm^2) was measured by clamping the open-circuit *VT* to 0 mV for 1 s [13].

Real-time PCR

Real-time PCR was performed following a method previously described [14]. Briefly, total RNA was isolated from mpkCCDcl$_4$ cells and reverse-transcribed to DNA. Real-time PCR was performed on an ABI-Prism 7700 using

SYBR Green I as a double-stranded DNA-specific dye according to the manufacturer's instructions (PE-Applied Biosystems, Cheshire, Great Britain). The glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA expression was simultaneously measured as an internal control. Primers (α-ENaC: sense 5-ACC GCA TGA AGA CGG CC-3 and anti-sense 5-CCA GTA CAT CAT GCC GAA GGT-3; β-ENaC: sense 5-GCC AGT GAA GAA GTA CCT CC-3 and anti-sense 5-CCT GGG TGG CAC TGG TGA A-3; γ-ENaC: sense 5-CAC TGG TCG AAG CGG AAA-3 and anti-sense 5-GCA CAG TCA GAG) were constructed to be compatible with a single RT-PCR thermal profile (95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min). The number of cycles to the threshold of detecting fluorescence was monitored in real time using ABI-Prism 7700 (PE-Applied Biosystems). The ENaC isoform mRNA expression was expressed relative to the GAPDH mRNA expression and the magnitude (in folds) of changes in the gene expression were determined in comparison with the control.

Western blot analysis

Total cellular protein extraction was performed as described previously [14]. Equal amounts of proteins were mixed with the equal volumes of reducing SDS sample buffer and boiled for 5 min at 95°C. Protein samples were resolved on a 10% SDS-PAGE and then electroblotted on nitrocellulose membranes (Bio-Rad). After the electroblotting, non-specific binding was blocked with a 5% non-fat milk/phosphate-buffered saline (PBS) solution. The membrane was then incubated overnight with primary antibodies at 4°C followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence (Amersham, UK) as previously described [14].

Immunofluorescent staining

The mpkCCDcl$_4$ cells, after recombinant virus infection for 48 h, were fixed in formaldehyde and blocked with 1% BSA (bovine serum albumin) for 30 min. Cells were then stained with rabbit anti-Flag primary antibody (1:100) for 1 h (Cayman Chemical, Ann Arbor, Michigan, USA) and FITC-conjugated goat anti-rabbit secondary antibody (Sigma, St. Louis, MO, USA) (1:100) for 1 h. The cell nuclei were counter-stained with DAPI (4,6-diamidino-2 phenylindoledihydrichloride, 1mM) (Sigma) for 5 min. Immunostained cells were examined under an Olympus BX 50 immunofluorescence microscope.

Luciferase activity assay

The pGL-3/mα-ENaC or p3TPLux plasmids were used to transiently transfect mpkCCDcl$_4$. The pGL-3/mα-ENaC promoter is a plasmid containing the luciferase gene under the control of a mouse α-ENaC promoter (donated by Dr André Dagenais, University of Montreal). The p3TPLux reporter is a plasmid containing a portion of a plasminogen activator inhibitor-1 gene promoter, which includes the Smad-binding sequences of TGF-β inducible elements linked to a luciferase reporter. The p3TPLux is commonly used to test a transcriptional regulation by TGF-β1 (a gift from Dr Liliana Attisano, University of Toronto) [7,15,16]. The β-Glycosidase plasmids used as an indicator of transfection efficiency were transfected simultaneously into the cells with p3TPLux plasmids. After transfection and TGF-β1 stimulation, cells were lysed by 1X reporter lysis buffer (Promega) at room temperature for 15 min and mixed with Bright-Glo luciferase assay buffer. The resulting luminescence was measured using a luminometer (MLX micro titer plate luminometer, Dynex Ltd, Billingshurst, UK). The activity of the p3TPLux reporter was adjusted by the β-glycosidase activity, which was determined using assay kits from Promega.

Cell toxicity assay

We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake assay to measure cell toxicity. Cells grown in a 96-well plate were washed with PBS in which 100 μl of 5 mg/ml of MTT was dissolved. After incubation at 37°C for 4 h, the formazan product was generated and subsequently solubilized overnight at 37°C by adding 100 μl of SDS lysis buffer (20% w/v SDS, 50% v/v dimethylformamide, 2% v/v acetic acid, 0.06% v/v hydrochloric acid) and the absorbance at 600 nm was read using a 96-well plate reader (Dynex Ltd, Billingshurst, UK).

Statistical analysis

All data are presented as means ± SE. Statistical analysis was performed using analysis of variance with Tukey's *post hoc* test. A value of $P < 0.05$ was considered to represent a significant difference.

Results

TGF-β1 decreases the transepithelial electrical current in CCD cells

To assess if TGF-β1 can alter the transepithelial electrical current, mpkCCDcl$_4$ were seeded on the top of the filter and grown to confluence followed by administration of TGF-β1 to both the apical and basal sides of the filter. The measurement of I_{eq} in these cells demonstrated that exposure to TGF-β1 decreases I_{eq} in a dose-dependent manner (0.1 to 10 ng/ml) after 8 h of incubation (Figure 1a). This decrease of I_{eq} was also time dependent with a nadir between 16 and 24 h following stimulation with TGF-β1. There was no significant difference between the I_{eq} of the control cells and that of the cells treated with 0.1 ng/ml of TGF-β1. In contrast, the I_{eq} was significantly decreased following a 24-h stimulation with higher concentration of TGF-β1 (0.5 to 10 ng/ml) when compared with the control cells (Figure 1a). We have previously demonstrated that TGF-β1 induces disassembly of cell–cell adhesion in a polarized manner [10]. To examine whether TGF-β1 regulated I_{eq} in a polarized manner, in this experiment TGF-β1 (1 ng/ml) was

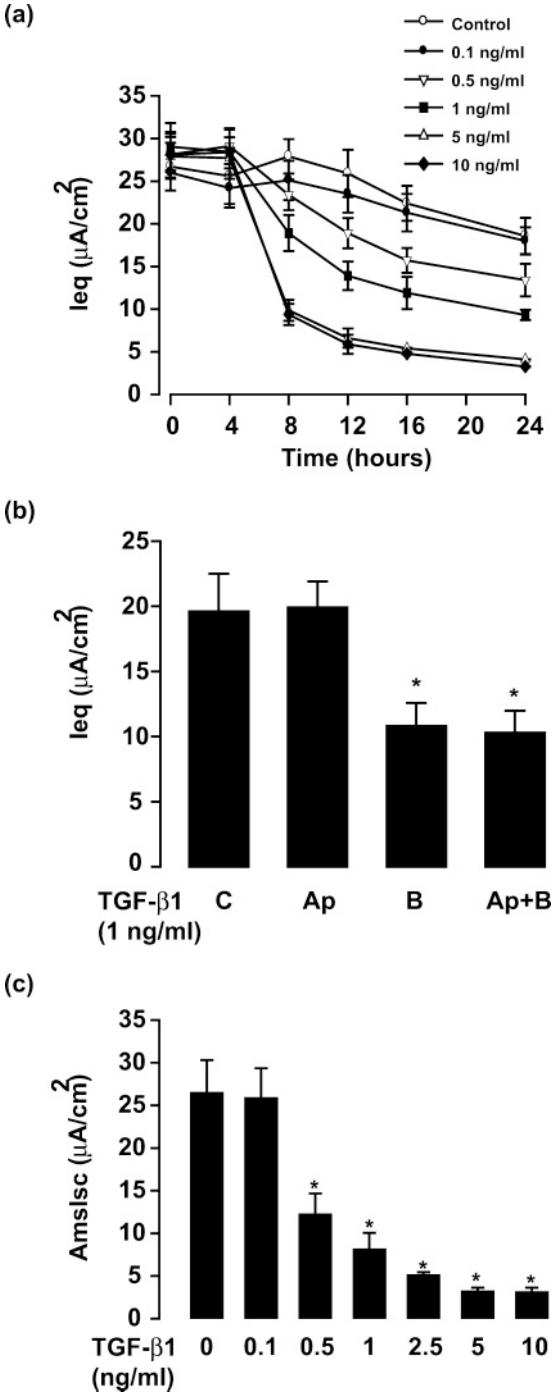

Fig. 1. TGF-β1 decreased I_{eq} and AmsIsc in mpkCCDcl$_4$. The mpkCCDc1$_4$ cells were seeded on porous filters and grown to confluence. I_{eq} and AmsIsc were determined as described in the Materials and methods section. (**a**) TGF-β1 added to both sides of filters decreased I_{eq} in a time- and dose-dependent manner ($n = 12$, $^*P < 0.001$ TGF-β1 versus control). (**b**) Application of TGF-β1 (1 ng/ml) to the basal (B) or both (Ap+B) of the filters for 24 h led to a significant reduction of I_{eq}. Apical application (Ap) alone did not decrease the I_{eq}. The I_{eq} of cells without treatment (C) was used as control ($n = 6$, $^*P < 0.001$ TGF-β1 versus control). (**c**) TGF-β1 administered to the basal side of the filters for 24 h caused a dose-dependent inhibition of AmsIsc ($n = 6$, $^*P < 0.001$ TGF-β1 versus control).

added to the apical or basal sides of the filters for 24 h. Our data showed no effect on I_{eq} when TGF-β1 was administrated to the apical side of the filter but a remarkable reduction of I_{eq} with TGF-β1 added to the basal side of the filter (Figure 1b). Therefore, TGF-β1 was thereafter administrated only to the basal side of the filter.

To determine if the TGF-β1-mediated decrease of transepithelial sodium transport was amiloride-sensitive, we first incubated the cells for 24 h either in the absence or in the presence of TGF-β1 (0.1 ng/ml) for 24 h. Subsequently, we administered amiloride (10^{-5} M) to the apical side of the filter for 10 min. The data show that TGF-β1 dose-dependently decreased AmsIsc (Figure 1c). The AmsIsc of the control cells versus that of cells stimulated with 0.1ng/ml of TGF-β1 did not differ statistically. The AmsIsc under stimulation by TGF-β1 at concentrations of 0.5, 1, 2.5, 5 and 10 ng/ml were significantly lower than that of the control. This decrease in AmsIsc was nearly equal to the reduction of I_{eq} induced by TGF-β1, suggesting that the decrease of I_{eq} mediated by TGF-β1 is caused mainly by the decreased transepithelial sodium transport.

TGF-β1 inhibited α-ENaC expression in CCD cells

To assess if alteration of the ENaC expression was involved in the decrease of I_{eq} mediated by TGF-β1, we used real-time PCR. The results reveal that TGF-β1 (at 1 and 5 ng/ml) significantly decreased the α-ENaC expression; however, the expressions of β-ENaC and γ-ENaC were unaltered (Figure 2a). To further clarify the role of the α-ENaC expression in the decrease of transepithelial sodium transport mediated by TGF-β1, we stimulated the mouse α-ENaC promoter (pGL-3/mα-ENaC luciferase promoter) with TGF-β1. This led to a significant reduction of the baseline luciferase activity of the α-ENaC promoter (Figure 2b). These results implied that alteration of the α-ENaC expression was involved in the TGF-β1-mediated inhibition of transepithelial sodium transport.

The inhibition of the transepithelial electric current by TGF-β1 was independent of MAPK signalling pathways

As MPAK pathways in many cell types are important downstream signal cascades of TGF-β1, we first tried to determine if the TGF-β1-mediated inhibition of the transepithelial electric current was dependent on MAPK signalling pathways. For this, the mpkCCDcl$_4$ cells were stimulated with TGF-β1 (5 ng/ml) for 24 h either in the absence or in the presence of a p38MAPK inhibitor (SB203580), a JNK inhibitor (SP600125) or an ERK1/2 inhibitor (PD98059). The results demonstrate that, whereas administering SB203580 (20 μM) reduced the basal phosphorylation of p38MAPK (Figure 3b, upper panel), it did not affect the TGF-β1-induced decrease of I_{eq} (Figure 3a). Similarly, SP600125 (40 μM) decreased the basal phosphorylation of JNK (Figure 3b, middle panel) but did not rescue the inhibition of I_{eq} mediated by TGF-β1 (Figure 3a). Finally, although PD98059 (50 μM) inhibited the basal

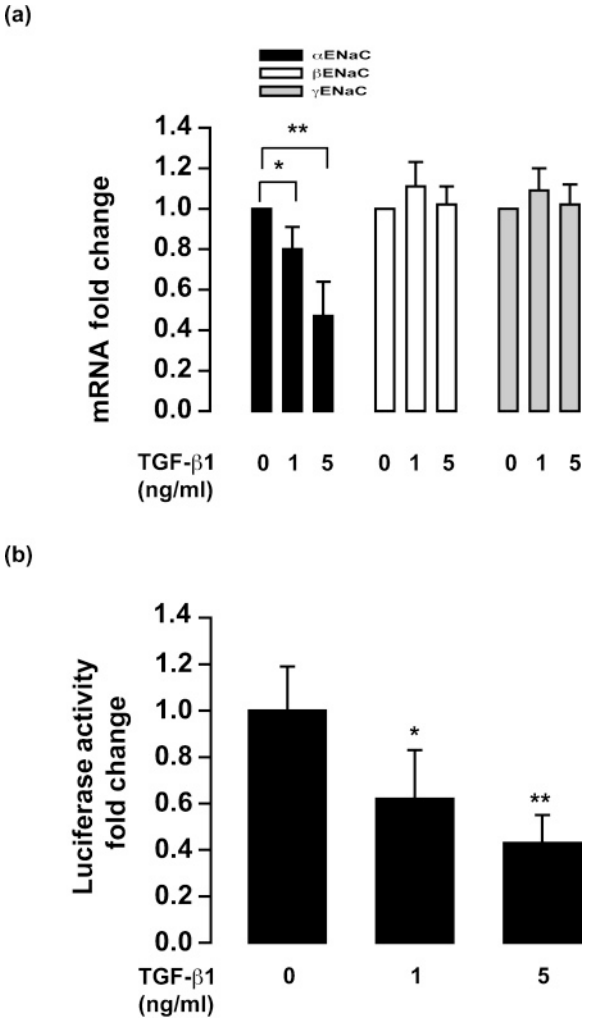

Fig. 2. TGF-β1 reduced the α-ENaC mRNA expression and promoter activity. (**a**) Administration of TGF-β1 (0, 1 or 5 ng/ml) to the basal side of the filters for 24 h dose-dependently decreased α-, but not β- or γ-ENaC mRNA expression of mpkCCDcl$_4$ as manifested by real-time PCR. (**b**) TGF-β1 (0, 1 or 5 ng/ml) administered to the basal side of the filters for 24 h dose-dependently inhibited the pGL-3/mα-ENaC promoter-luciferase activity in a dose-dependent manner ($n = 6$, $^*P < 0.05$, $^{**}P < 0.01$ TGF-β1 versus control).

ERK1/2 activity (Figure 3b, lower panel), it had no significant influence on the decrease of I_{eq} mediated by TGF-β1 (Figure 3a). These data indicated that TGF-β1-induced decrease of the transepithelial electrical current was independent of p38MAPK, JNK and ERK signalling pathways.

Smad4 overexpression altered the transepithelial electric current

The Smad signalling pathway is a classical downstream signal pathway of TGF-β1. To determine whether the pathway affected the transepithelial electrical current, we generated plasmids carrying the Flag-tagged Smad4, or the Flag-tagged N-terminus truncated Smad4 as described in the Materials and methods section (Figure 4a). These plasmids, together with the p3TPLux reporter plasmid, were transiently overexpressed in mpkCCDcl$_4$ cells, and the luciferase activity was measured in them; we observed the inhibitory effect of the N-terminus truncated Smad4 on the p3TPLux reporter activity. As shown in Figure 4b, TGF-β1 (1 ng/ml) increased the activity of the p3TPLux reporter up to 8.6-fold, and the wild-type Smad4 overexpression in the presence of TGF-β1 (1 ng/ml) further enhanced the activity of p3TPLux up to 10.5-fold. The overexpression of the N-terminus truncated Smad4 in the presence of TGF-β1 significantly decreased the activation of p3TPLux induced by TGF-β1, because only a 3.9-fold increase of the p3TPLux activity was detected (Figure 4b).

To determine if the Smad signalling pathway was implicated in the alteration of the transepithelial electrical current, either wild-type Smad4 or N-terminus truncated Smad4 was stably overexpressed in mpkCCDcl$_4$ cells and the mock-transfected cells were used as a negative control. The overexpression of the wild-type and N-terminus truncated Smad4 was confirmed by western blot analysis (Figure 4c). The data showed that I_{eq} of the confluent Smad4-overexpressing cells was reduced to 70% of that of control cells (22.1 ± 6.3 versus 31.5 ± 7.4 μm/cm^2, $P < 0.05$, $n = 6$) (Figure 5a). There was no difference in I_{eq} between the N-terminus truncated Smad4-overexpressing cells and the control cells (30.6 ± 6.8 versus 31.5 ± 7.4 μm/cm^2, $P > 0.05$, $n = 6$). Real-time PCR demonstrated that the expression of α-ENaC mRNA in the wild-type Smad4-overexpressing cells was significantly less than that in the control cells. In contrast, the α-ENaC mRNA expression in the N-terminus truncated cells was similar to that in the control cells (Figure 5b). To further confirm the result of real-time PCR study, the wild-type, N-terminus truncated stably overexpressing Smad4 cells or the control cells were transfected with a mouse α-ENaC promoter (pGL-3/mα-ENaC) for 24 h, following which, we measured the luciferase activity in these cells. In line with the result of the real-time PCR, the α-ENaC-luciferase reporter activity was reduced in the wild-type Smad4-overexpressing cells compared with that in the control cells (Figure 5c). The luciferase activity in the N-terminus truncated Smad4-overexpressing cells, however, was not different from that in the control cells. These data suggested that the overexpression of Smad4 led to a reduction of the transepithelial electric current as well as of the α-ENaC mRNA expression, and that N-terminus of Smad4 was indispensable for the function of Smad4 in these circumstances.

Smad4 plays an essential role in the TGF-β1-mediated inhibition of the transepithelial electric current

Measuring AmsIsc after stimulation of the wild-type and N-terminus truncated Smad4s and control cells with 1 ng/ml of TGF-β1 for 24 h revealed that, in a fashion compatible with our finding that the Smad4-overexpression led to a decrease in I_{eq}, the AmsIsc of the Smad4-overexpressing cells was significantly lower than that of the control cells (Figure 6). In accordance with the finding that the deletion of N-terminus led to a loss of the Smad4-mediated reduction of I_{eq}, the AmsIsc of the N-terminus truncated Smad4 cells was significantly higher than that of the wild-type Smad4 cells, and it was not statistically different from

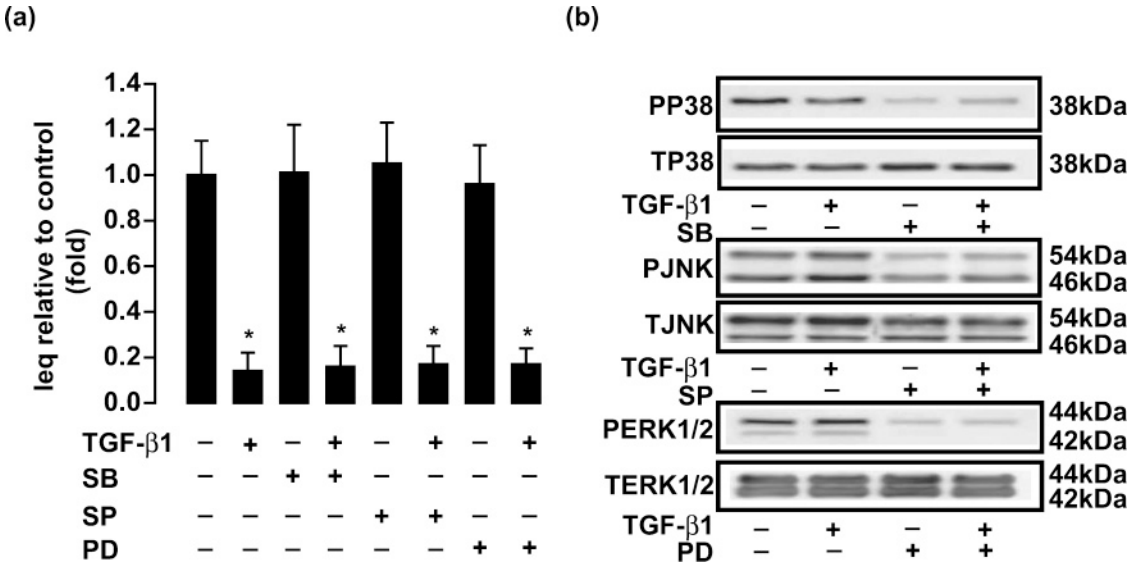

Fig. 3. The MAPK kinase inhibitors did not affect the TGF-β1-induced reduction in I_{eq}. The mpkCCDc1$_4$ cells were treated with either SB203580 (SB, 20 μM), SP600125 (SP, 40 μM) or PD98059 (PD, 50 μM) in both sides of porous filters in the presence (+) or absence (−) of TGF-β1 (5 ng/ml) in the lower chamber of the filters for 24 h. (**a**) I_{eq} was measured ($n = 6$, $^*P < 0.001$). (**b**) Cell lysates were subjected to western blot analysis to determine phosphorylation form (p) or total (t) MAPKs. One of the three individual experiments is presented. MAPK kinase inhibitors inhibited the expression of phorsphorylation form the MAPK expression but did not inhibit TGF-β1-associated I_{eq} reduction.

that of the control cells. In the presence of TGF-β1, the mock-transfected control cells, like normal mpkCCDcl$_4$ cells, still displayed the TGF-β1-induced decrease of AmsIsc. The AmsIsc of the Smad4 cells was significantly reduced by the administration of TGF-β1 and it was significantly lower than that of the control cells stimulated with TGF-β1. The AmsIsc of the N-terminus truncated Smad4 cells was significantly reduced after exposure to TGF-β1. More importantly, this TGF-β1-mediated inhibitory effect was significantly alleviated when compared with that effect in either the wild-type Smad4-overexpressing cells or the control cells, indicating that the N-terminus of Smad4 is essential for the TGF-β1-mediated inhibition of α-ENaC functionality.

To confirm on unselected cell populations the results shown above that were obtained with clonal cell lines, mpkCCDcl$_4$ cells were infected with the recombinant adenoviruses to overexpress wild-type or N-terminus truncated Smad4 for 2 days, which were followed by stimulation with TGF-β1. The successful infection of mpkCCDcl$_4$ cells was first measured by immunofluorescent staining, revealing more than 90% of the cells being infected (Figure 7a). The overexpression of Flag-tagged wild-type or N-terminus truncated Smad4 was further proved by western blot analysis (Figure 7b). Compared with the AmsIsc of the mock-infected cells, the AmsIsc in the cells infected with adenoviruses overexpressing Smad4 was decreased (27.8 ± 3.2 versus 21.3 ± 1.9 μm/cm^2, $P < 0.05$, $n = 7$), whereas the AmsIsc was unaltered in the cells infected with adenoviruses overexpressing N-terminus truncated Smad4 (27.8 ± 3.2 versus 27.2 ± 3.9 μm/cm^2, $P > 0.05$, $n = 7$) (Figure 7c). After stimulation by TGF-β1 (1 ng/ml) for 24 h, the AmsIsc of the cells with TGF-β1 stimulation was decreased in comparison with that of the mock-infected cells stimulated with TGF-β1 (27.8 ± 3.2 versus 14.1 ± 1.4 μm/cm^2). The overexpression of wild-type Smad4 by the adenovirus-mediated gene transfer in cells stimulated with TGF-β1 led to further reduction of the AmsIsc when compared with that of the mock-infected cells stimulated with TGF-β1 (14.1 ± 1.4 versus 10.1 ± 0.9 μm/cm^2, $P < 0.05$, $n = 7$). More importantly, the TGF-β1-induced reduction of the AmsIsc was attenuated when the cells overexpressed N-terminus truncated Smad4 following the adenovirus-mediated gene transfer (18.2 ± 1.8 μm/cm^2). These results, using the adenovirus-mediated gene transfer methods, were similar to those obtained from stable cell lines that overexpressed wild-type or N-terminus truncated Smad4, confirming, therefore, the crucial role of N-terminus Smad4 in the TGF-β1-mediated inhibition of α-ENaC functionality.

The inhibitory effect of TGF-β1 on the α-ENaC expression and functionality was not caused by cell toxicity

The result of the MTT test did not detect a significant difference in cell toxicity between mpkCCDcl$_4$ cells incubated in the presence or the absence of various concentrations of TGF-β1 (0.1 to 10 ng/ml) for 24 h.

Discussion

It has been shown that TGF-β1 can reduce sodium transport as well as the α-ENaC mRNA expression in type II alveolar cells [7]. Recently, it was also demonstrated that TGF-β1 decreases the transepithelial equivalent current in M-1 mouse renal cortical collecting ducts [9]. The present study confirmed that TGF-β1 decreased the transepithelial equivalent current in immortalized mouse cortical collecting duct cells (mpkCCDcl$_4$). Furthermore, we discovered that the TGF-β1-mediated reduction of the transepithelial equivalent current was mainly caused by a decrease of

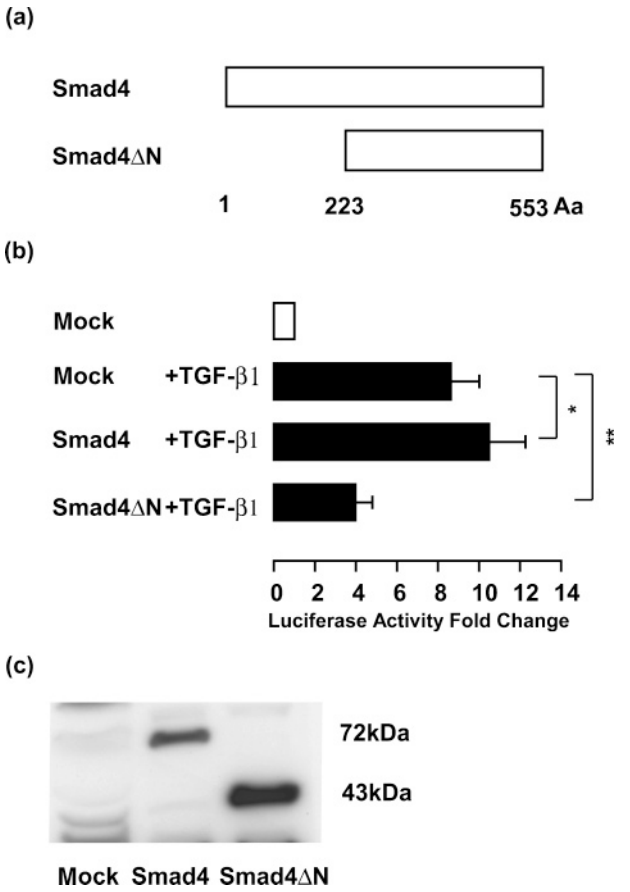

Fig. 4. Functionality of the wild-type or N-terminus truncated Smad4-overexpressing cell lines. (**a**) Schematic representation of the wild-type and N-terminus truncated Smad4s. (**b**) The enhancement of the luciferase activity of p3TPLux reporter after a 24-h incubation in TGF-β1 (1 ng/ml) in mpkCCDcl$_4$ co-transfected with an empty vector (mock) was lower than that in cells co-transfected wild-type (Smad4) plasmid, but higher than in cells co-transfected with N-terminus truncated Smad4 (Smad4ΔN) plasmid. The data are represented as mean ± SE. Three separate experiments and $n = 6$ in each experiment are evaluated ($^*P < 0.01$, $^{**}P < 0.001$ TGF-β1 versus mock). (**c**) The confirmation of the Flag-tagged Smad4 expression in each cell line was demonstrated by western blot analysis.

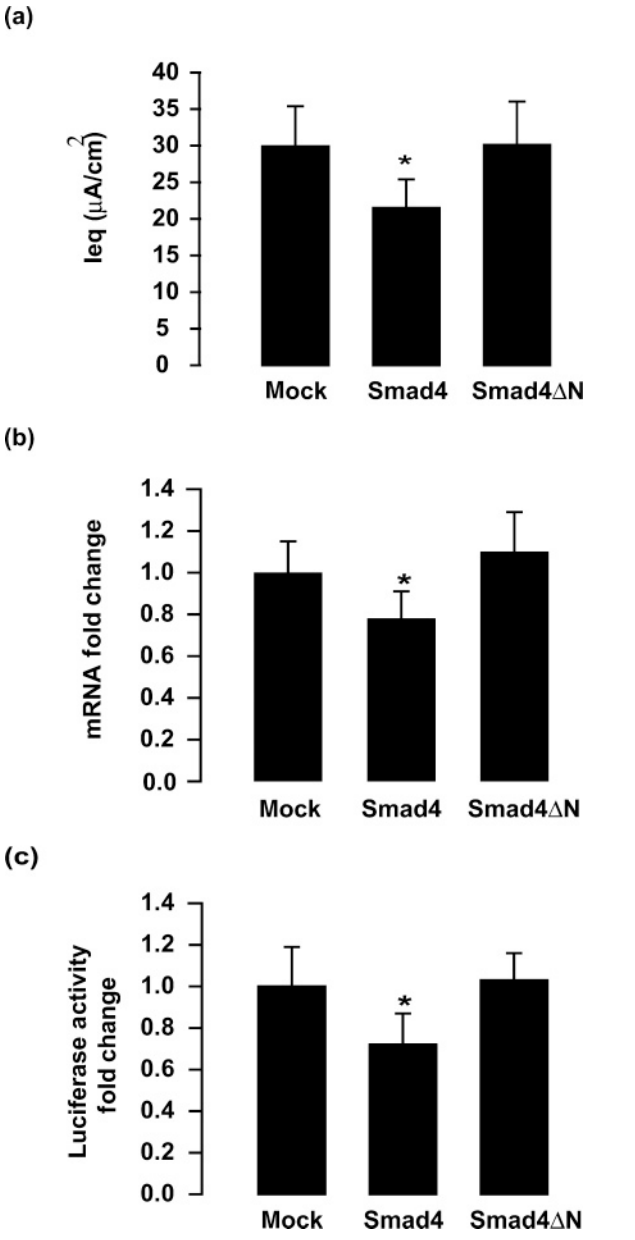

Fig. 5. N-terminus of Smad4 was required in the Smad4-mediated alteration in I_{eq}, α-ENaC mRNA expression and α-ENaC promoter activity. The (**a**) I_{eq}, (**b**) α-ENaC mRNA expression and (**c**) α-ENaC promoter activity in stable cell lines overexpressing wild-type Smad4 (Smad4) were lower than those of stable cell lines overexpressing N-terminus truncated Samd4 (Smad4ΔN). These parameters were studied in mock-transfection cell lines used as negative control ($n = 6$, $^*P < 0.05$).

AmsIsc. The TGF-β1-mediated inhibitory effect on AmsIsc was polarized. Application of TGF-β1 to the apical side of cells did not cause alteration of AmsIsc, whereas TGF-β1 administered to the basal side led to a significant decrease of the current, about the same as when TGF-β1 was applied to both sides of the cells. As TGF-β receptors are distributed on apical and basolateral aspects of mpkCCDcl$_4$ cells (data not shown), we suspect that the different effects of TGF-β1 on AmsIsc might be explained by the existence of a subpopulation of TGF-β receptors localized only at the basolateral aspect of the cells, which can only be approached by basolateral administration of TGF-β1.

The most important amiloride-sensitive channel is ENaC. Our data also demonstrated that the TGF-β1-induced reduction of the amiloride-sensitive short circuit current was correlated with a decrease of the α-ENaC mRNA expression, and this was further confirmed by studying the α-ENaC promoter reporter system. On the other hand, TGF-β1 did not affect the expression of β- or γ-ENaC. These three ENaCs are not always simultaneously increased following stimulation with cytokines or hormones. For instance, Masilamani *et al*. have shown that elevation of aldosterone level following sodium restriction selectively increases the α-ENaC protein but not β or γ subunits [17]. Wakida *et al*. have recently shown that administration of TGF-β1 leads to a decrease in all of α-, β- and γ-ENaC mRNA expressions in cortical collecting duct cells [9]. The discrepancy between their and our results might be attributable to the doses of TGF-β1 used (20 ng/ml by them versus 1–5 ng/ml by us). We speculate that α-ENaC might be more sensitive to a lower concentration

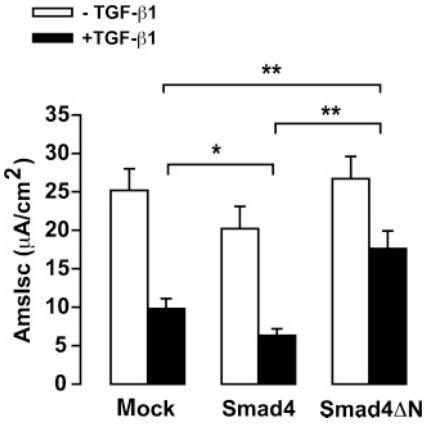

Fig. 6. The functional Smad4 was required in the TGF-β1-induced reduction of AmsIsc. TGF-β1 (1 ng/ml) administered in the basal side of the filters for 24 h reduced a larger degree of AmsIsc in stable cell lines overexpressing wild-type Smad4 (Smad4) than in stable cell lines overexpressing N-terminus truncated Smad4 (Smad4ΔN) ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.001$).

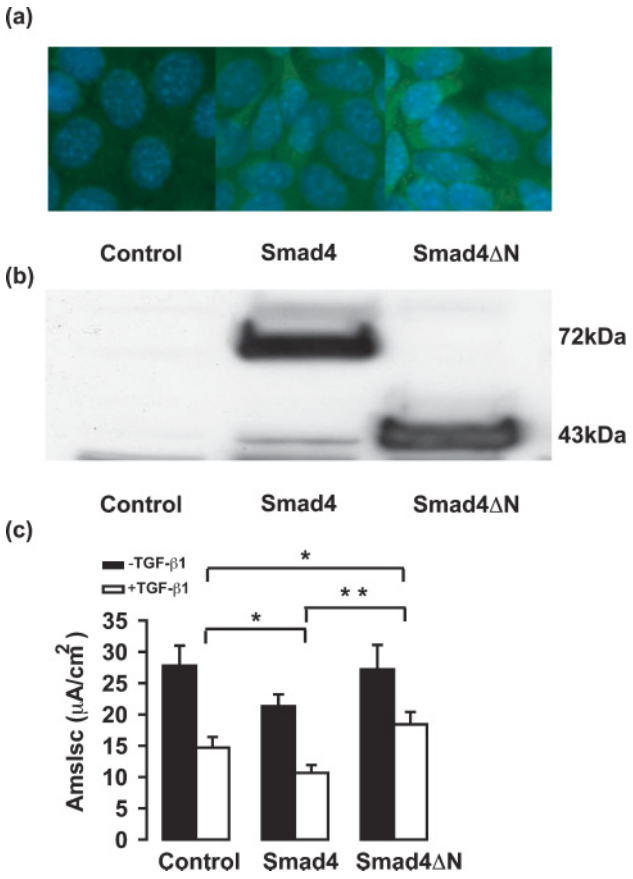

Fig. 7. Confirmation of the requirement of functional Smad4 in the TGF-β1-induced reduction of AmsIsc by the adenovirus-mediated gene transfer. The mpkCCDc1$_4$ cells grown in filters were infected with adenoviruses containing Flag-tagged wild-type (Smad4, viral titer 10^9 Infectious Unit/ml) or N-terminus truncated Smad4 (Smad4ΔN, viral titer 3.7×10^8 Infectious Unit/ml) genes for 48 h. The success of virus-mediated gene transfer was confirmed by immunofluorescent staining (**a**) and western blot analysis (**b**) using anti-Flag antibody. Cells infected with a virus carrying a shuttle vector alone were used as control (**c**) AmsIsc measured in virus infected cells treated with TGF-β1 (1 ng/ml) for 24 h was lower in that of Smad4 infected cells than that in control or Smad4ΔN infected cells ($n = 7$, $^{*}P < 0.05$, $^{**}P < 0.001$).

of TGF-β1 whereas altering of β- or γ-ENaC might require stimulation by a higher concentration of TGF-β1. In line with this theory, the β- and γ-ENaC mRNA expression was decreased in mpkCCDcl$_4$ cells after exposure to 20 ng/ml of TGF-β1 (data not shown).

Supported by the evidence that aldosterone is the main regulator enhancing the ENaC expression and activity and by our result that TGF-β1 inhibits ENaC functionality, it would behoove us to determine if TGF-β1 can prevail over the aldosterone-driven increase of the ENaC activity. To address this issue, we treated mpkCCDcl$_4$ cells treated with TGF-β1 and aldosterone 5×10^{-7} M for 24 h following which we measured the equivalent current and α-ENaC mRNA expression. The result of this processing demonstrated that TGF-β1 suppressed the aldosterone-induced increase of the equivalent current in a dose-dependent manner (data not shown). This was in accord with the findings of Stokes *et al*., who demonstrated that TGF-β1 blocked the stimulatory effect of aldosterone on the ENaC activity and sodium transport [18]. The administration of aldosterone also enhanced the α-ENaC gene expression detected by real-time PCR, but addition of up to 5 ng/ml of TGF-β1 did not alter the aldosterone-induced increase of the α-ENaC gene expression (data not shown). We speculate that the inhibitory effect of TGF-β1 on the aldosterone-induced increase of the transepithelial equivalent current requires post-transcriptional modification of α-ENaC. As TGF-β1 and aldosterone have been shown to influence serum and glucocortieoid-inducible kinase 1 (Sgk 1) [2], it would be interesting in the future to determine if Sgk1 plays a crucial role in the TGF-β1-mediated inhibition of the aldosterone-induced ENaC functionality.

The MAPK signalling pathways including p38, JNK or ERK1/2 signal cascades are important downstream signalling pathways of TGF-β1. In rat alveolar cells, the inhibitory effect of TGF-β1 on the activity of the α-ENaC promoter and on transepithelial sodium transport is mediated through an ERK1/2-dependent pathway rather than through the p38 or JNK signalling pathways [7]. In the current study, TGF-β1, at a concentration of 5 ng/ml, did not activate the ERK1/2 signal and the inhibition of the signal by a specific ERK inhibitor did not prevent the TGF-β1-induced decrease of the transepithelial electric current in mpkCCDcl$_4$ cells which therefore indicate that TGF-β1 decreased α-ENaC functionality independent of the ERK signalling pathway. Similarly, TGF-β1 did not activate the p38 or JNK signal and the inhibition of these two signalling pathways by using their specific inhibitors did not block the TGF-β1-mediated decrease of I_{eq}. These results suggested that the transduction of the p38 or JNK signal likely was not involved in the TGF-β1-mediated decrease of I_{eq}.

Among the various cell functions, the most crucial downstream TGF-β1 signal pathway is the Smad signalling pathway. Smad4, co-Smad, is considered an obligatory mediator for the transduction of all TGF-β1 superfamily signals and it has the capability of binding to DNA to activate the transcription process. In addition, the N-terminus of Smad4 plays an important part in the functionality of Smad4, for the N-terminal depleted Smad4

has an inhibitory effect on the TGF-β1-induced increase of p3TPLux [19] as well as on the TGF-β1-mediated increase of the type I collagen gene expression [20]. Although TGF-β1 has been shown able to regulate transepithelial sodium transport in cortical collecting ducts, the mechanism of this effect remains unclear. We have searched for the α-ENaC promoter sequence in the Genome Bioinformatics database and found one potential Smad-binding site located at position $-208 \sim -216$ upstream of mouse 5′ untranslated sequences of the α-ENaC promoter region (data not shown), implying that Smads might have a role in the regulation of the α-ENaC expression. Our current study demonstrated that the overexpression of Smad4 reduced the AmsIsc, α-ENaC mRNA expression and α-ENaC promoter activity, indicating that Smad4 can function as a suppressor in the α-ENaC activity and expression. The N-terminus of Smad4 was essential for Smad4 functionality in these responses, as the deletion of the N-terminus of Smad4 led to a loss of the Smad4-induced reduction in the AmsIsc, α-ENaC mRNA expression and α-ENaC promoter activity. It has been shown that the DNA binding domain of Smad4 lies in its N-terminus area. Whether the N-terminus of Smad4 directly binds to the α-ENaC promoter requires further investigation. Nevertheless, the addition of TGF-β1 to the wild-type or N-terminus truncated Smad4-overexpressing cells caused a still further reduction in AmsIsc, implying that TGF-β1 activated an endogenous Smad4 complex or another α-ENaC mediator such as prostasin to further inhibit the ENaC activity [8]. More importantly, the TGF-β1-induced reduction of AmsIsc was alleviated in the N-terminus truncated Smad4-ovexpressing cell when compared with the mock-transfected cells or with the wild-type Smad4-overexpressing cells. The identical results were obtained from both stable cell lines and from the cells overexpressing wild-type or N-terminus truncated Smad4 by the adenovirus-mediated gene transfer. This suggests that the TGF-β1-mediated inhibition of transepithelial sodium transport requires the intact function of Smad4, especially its N-terminus region.

In conclusion, we have demonstrated that in mpkCCDcl$_4$ cells TGF-β1 can inhibit the transepithelial electrical current, which is mainly amiloride sensitive and relies on the α-ENaC expression. We provide the first evidence that, in mouse cortical collecting duct cells, the TGF-β1-mediated reduction of transepithelial sodium transport is regulated by the downstream Smad signalling pathway.

Acknowledgements. We thank Dr Alain Vandewalle (INSERM Unit 773, Paris) for kindly providing the mpkCCDcl$_4$ cell line and Dr André Dagenais (University of Montreal) and Dr Liliana Attisano (University of Toronto) for the their generous gifts of the α-ENaC promoter and p3TPLux reporter plasmids.

Conflict of interest statement. None declared.

References

1. Warnock DG, Rossier BC. Renal sodium handling: the role of the epithelial sodium channel. *J Am Soc Nephrol* 2005; 16: 3151–3153
2. Verrey F. Sodium reabsorption in aldosterone-sensitive distal nephron: news and contributions from genetically engineered animals. *Curr Opin Nephrol Hypertens* 2001; 10: 39–47
3. Lingueglia E, Voilley N, Waldmann R *et al*. Expression cloning of an epithelial amiloride-sensitive Na^+ channel. A new channel type with homologies to Caenorhabditis elegans degenerins. *FEBS Lett* 1993; 318: 95–99
4. Hummler E, Barker P, Talbot C *et al*. A mouse model for the renal salt-wasting syndrome pseudohypoaldosteronism. *Proc Natl Acad Sci U S A* 1997; 94: 11710–11715
5. Shen JP, Cotton CU. Epidermal growth factor inhibits amiloride-sensitive sodium absorption in renal collecting duct cells. *Am J Physiol Renal Physiol* 2003; 284: F57–F64
6. Sakharova OV, Taal MW, Brenner BM. Pathogenesis of diabetic nephropathy: focus on transforming growth factor-beta and connective tissue growth factor. *Curr Opin Nephrol Hypertens* 2001; 10: 727–738
7. Frank J, Roux J, Kawakatsu H *et al*. Transforming growth factor-beta1 decreases expression of the epithelial sodium channel alphaENaC and alveolar epithelial vectorial sodium and fluid transport via an ERK1/2-dependent mechanism. *J Biol Chem* 2003; 278: 43939–43950. Epub 42003 Aug 43920
8. Tuyen do G, Kitamura K, Adachi M *et al*. Inhibition of prostasin expression by TGF-beta1 in renal epithelial cells. *Kidney Int* 2005; 67: 193–200
9. Wakida N, Kitamura K, Tuyen DG *et al*. Inhibition of prostasin-induced ENaC activities by PN-1 and regulation of PN-1 expression by TGF-beta1 and aldosterone. *Kidney Int* 2006; 70: 1432–1438
10. Tian YC, Phillips AO. Interaction between the transforming growth factor-beta type II receptor/Smad pathway and beta-catenin during transforming growth factor-beta1-mediated adherens junction disassembly. *Am J Pathol* 2002; 160: 1619–1628
11. Bens M, Vallet V, Cluzeaud F *et al*. Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. *J Am Soc Nephrol* 1999; 10: 923–934
12. Goel M, Sinkins WG, Schilling WP. Selective association of TRPC channel subunits in rat brain synaptosomes. *J Biol Chem* 2002; 277: 48303–48310
13. Chang CT, Bens M, Hummler E *et al*. Vasopressin-stimulated CFTR Cl^- currents are increased in the renal collecting duct cells of a mouse model of Liddle's syndrome. *J Physiol* 2005; 562: 271–284
14. Tian YC, Chen YC, Hung CC *et al*. Leptospiral outer membrane protein induces extracellular matrix accumulation through a TGF-beta1/Smad-dependent pathway. *J Am Soc Nephrol* 2006; 17: 2792–2798
15. Xu J, Attisano L. Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor beta signaling by targeting Smads to the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* 2000; 97: 4820–4825
16. Dennler S, Itoh S, Vivien D *et al*. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J* 1998; 17: 3091–3100
17. Masilamani S, Kim GH, Mitchell C *et al*. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 1999; 104: R19–R23
18. Husted RF, Sigmund RD, Stokes JB. Mechanisms of inactivation of the action of aldosterone on collecting duct by TGF-beta. *Am J Physiol Renal Physiol* 2000; 278: F425–F433
19. de Caestecker MP, Hemmati P, Larisch-Bloch S *et al*. Characterization of functional domains within Smad4/DPC4. *J Biol Chem* 1997; 272: 13690–13696
20. Tsuchida K, Zhu Y, Siva S *et al*. Role of Smad4 on TGF-beta-induced extracellular matrix stimulation in mesangial cells. *Kidney Int* 2003; 63: 2000–2009

Received for publication: 31.10.06
Accepted in revised form: 8.10.07