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Transforming growth factor β1 antagonizes the transcription, expression and vascular signaling of guanylyl cyclase/natriuretic peptide receptor A – role of δEF1

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The objective of this study was to determine the role of transforming growth factor β1 (TGF-β1) in transcriptional regulation and function of the guanylyl cyclase A/natriuretic peptide receptor A gene (*Npr1*) and whether cross-talk exists between these two hormonal systems in target cells. After treatment of primary cultured rat thoracic aortic vascular smooth muscle cells and mouse mesangial cells with TGF-β1, the *Npr1* promoter construct containing a δ-crystallin enhancer binding factor 1 (δEF1) site showed 85% reduction in luciferase activity in a time- and dose-dependent manner. TGF-β1 also significantly attenuated luciferase activity of the *Npr1* promoter by 62%, and decreased atrial natriuretic peptide-mediated relaxation of mouse denuded aortic rings *ex vivo*. Treatment of cells with TGF-β1 increased the protein levels of δEF1 by 2.4–2.8-fold, and also significantly enhanced the phosphorylation of Smad 2/3, but markedly reduced *Npr1* mRNA and receptor protein levels. Over-expression of δEF1 showed a reduction in *Npr1* promoter activity by 75%, while deletion or site-directed mutagenesis of δEF1 sites in the *Npr1* promoter eliminated the TGF-β1-mediated repression of *Npr1* transcription. TGF-β1 significantly increased the expression of α-smooth muscle actin and collagen type I α2 in rat thoracic aortic vascular smooth muscle cells, which was markedly attenuated by atrial natriuretic peptide in cells over-expressing natriuretic peptide receptor A. Together, the present results suggest that an antagonistic cascade exists between the TGF-β1/Smad/δEF1 pathways and *Npr1* expression and receptor signaling that is relevant to renal and vascular remodeling, and may be critical in the regulation of blood pressure and cardiovascular homeostasis.

Introduction

The cardiac hormones atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) play critical roles in reducing the of blood pressure and cardiac disorders, in relevance to renal, cardiovascular, endocrine, skeletal and neural homeostasis [1–5]. ANP and BNP bind and activate guanylyl cyclase A/natriuretic

Abbreviations
ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; COL1A2, collagen type I α2; GC-A/NPRA, guanylyl cyclase A/natriuretic peptide receptor A; MMCs, mouse mesangial cells; RTASMCs, rat thoracic aortic vascular smooth muscle cells; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; δEF1, δ-crystallin enhancer binding factor 1.

peptide receptor A (GC-A/NPRA), which catalyze formation of the intracellular second messenger cGMP [6–8]. NPRA is a major biological natriuretic peptide receptor with a wide range of physiological actions; however, the molecular mechanism of its functional expression and regulation is not well understood. The 5′ flanking region (~ 500 bp upstream of the start codon) of the *Npr1* gene (encoding GC-A/NPRA) contains binding sites for several known transcription factors, and appears to play a critical role in functional regulation and expression of this gene [9–12]. Previous studies from our laboratory and by others have focused on the regulation of *Npr1* gene expression and function, however, the complete molecular machinery regulating its expression and function is yet to be established [9,13–16].

Transforming growth factor β1 (TGF-β1) belongs to the TGF-β family of peptides, which regulate various cellular processes such as proliferation, differentiation, apoptosis, and specification of cell type during embryonic development [17,18]. Hypertension, nephropathy and cardiac hypertrophy are associated with significantly elevated levels of TGF-β1 and collagen in *Npr1* gene knockout mice [19–25]. Previous findings indicated that TGF-β1 decreased *Npr1* mRNA levels in cultured aortic smooth muscle cells; however, the underlying molecular mechanisms were not determined [26]. It has been shown that BNP inhibited TGF-β1-induced proliferation in cardiac fibroblasts and opposed almost 88% of TGF-β1-stimulated gene expression [27]. Furthermore, TGF-β1 has been shown to stimulate collagen production in fibroblasts and to modulate the extracellular matrix by induction of fibronectin, collagen and related proteins [28–31]. Genes involved in positive regulation of the cell cycle, fibrosis, inflammation, myofibroblast transformation and extracellular matrix production have been shown to be up-regulated by TGF-β1, while the same genes appear to be down-regulated by BNP [32].

Interestingly, the E2 box repressor, δ-crystallin enhancer binding factor 1 (δEF1), was identified as a nuclear protein that binds to a lens-specific enhancer, and has been suggested to be regulated by TGF-β1 in vascular smooth muscle cells [33–36]. Several studies have shown that δEF1 acts as a mediator of TGF-β1 signaling in transcriptional repression of genes involved in cell differentiation and tissue-specific cellular responses [37–40]. In the present study, we examined the effect of TGF-β1 on *Npr1* gene transcription in rat thoracic aortic smooth muscle cells (RTASMCs) and mouse mesangial cells (MMCs), which represent attractive systems to study functional aspects of ANP/NPRA signaling [41,42]. Glomerular mesangial cells are the target of diverse pathophysiological processes, particularly in hypertension and immune inflammatory diseases. Both RTASMCs and MMCs express functional GC-A/NPRA, providing novel model systems for elucidating the regulatory mechanisms involved in *Npr1* gene transcription and expression [43]. The findings reported here demonstrate that TGF-β1 represses *Npr1* gene transcription and functional expression via activation of δEF1 and its recruitment to the *Npr1* promoter.

Results

In the presence of TGF-β1, the *Npr1* proximal promoter region −356/+55 from the transcription start site exhibited reductions in promoter activity by 81% in RTASMCs and by 85% in MMCs, in a time-dependent manner (Fig. 1A,B). Treatment of cells with increasing concentrations of TGF-β1 resulted in a marked repression of *Npr1* promoter activity in RTASMCs and MMCs compared with untreated controls (Fig. 1C,D). A real-time RT-PCR assay showed approximately 62% and 66% attenuation in *Npr1* mRNA levels in RTASMCs and MMCs, respectively, treated with TGF-β1 compared with untreated controls (Fig. 1E,F). In addition, there was a 55% reduction in NPRA protein expression in RTASMCs and a 59% reduction in MMCs treated with increasing concentrations of TGF-β1 compared with control cells (Fig. 1G,H). There was also significant decrease in ANP-stimulated intracellular accumulation of cGMP by 59% in RTASMCs and 52% in MMCs in TGF-β1-treated cells compared with untreated control cells (Fig. 2A).

A schematic map of deletion constructs of the *Npr1* promoter region (−356 to +359) containing δEF1 binding sites is shown in Fig. 2B. Treatment with TGF-β1 significantly decreased the luciferase activity of *Npr1* promoter constructs −356/+55, −356/+96 and −356/+359 with δEF1 binding sites compared with their untreated controls, suggesting that δEF1 binding sites are required for TGF-β1-mediated *Npr1* transcriptional repression (Fig. 2C). To examine the role of δEF1 in *Npr1* basal promoter activity, RTASMCs were co-transfected with *Npr1* promoter deletion constructs and δEF1 expression plasmids. Over-expression of δEF1 significantly reduced luciferase activity of *Npr1* promoter constructs −356/+55, −356/+96 and −356/+359 containing δEF1 binding sites (Fig. 2D).

A schematic map of *Npr1* promoter constructs containing combinations of wild-type and mutant δEF1A or δEF1B binding sites is presented in Fig. 2E. Over-expression of δEF1 in RTASMCs transfected with the *Npr1* promoter constructs −356/+55 and −356/+359

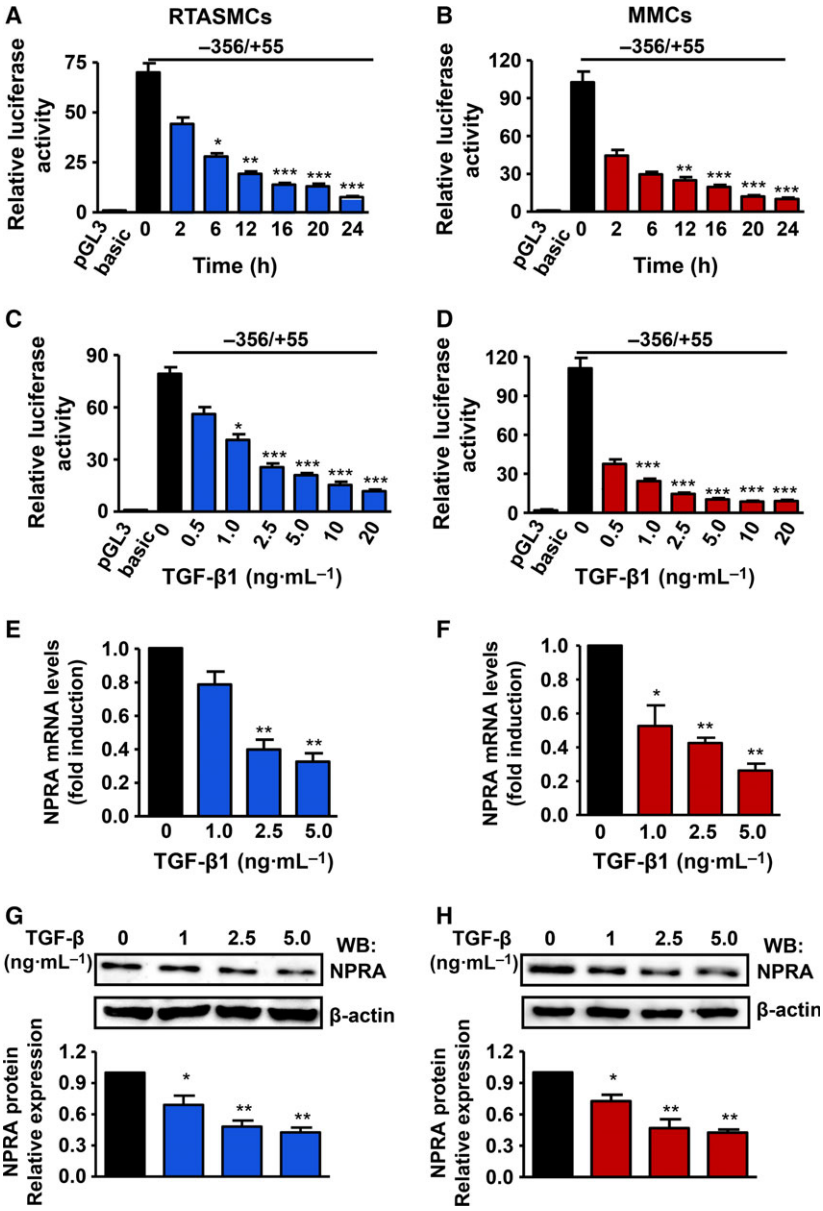

Fig. 1. Effect of TGF-β1 on *Npr1* gene transcription and expression in a time- and dose-dependent manner. (A,B) Luciferase activity of the *Npr1* proximal promoter construct −356/+55 in RTASMCs (A) and MMCs (B) treated with TGF-β1 (5 ng·mL^{-1}). (C,D) Effect of increasing concentrations of TGF-β1 on *Npr1* promoter activity in transfected RTASMCs (C) and MMCs (D) as measured by the luciferase assay. (E,F) Dose-dependent effect of TGF-β1 on *Npr1* mRNA levels in RTASMCs (E) and MMCs (F) as determined by real-time RT-PCR with β-actin as an internal control. (G,H) NPRA protein expression by densitometry analysis in treated RTASMCs (G) and MMCs (H). β-actin was used as a loading control. WB, Western blot. Values are means ± SE of eight independent experiments in triplicate. Asterisks indicate statistically significant differences compared with the controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

with wild-type δEF1 sites repressed promoter activity by 60% and 80%, respectively (Fig. 2F). Mutation of δEF1 site A in construct −356/+55 markedly increased luciferase activity. To examine the effect of δEF1 site B and the combination of sites A and B, we utilized the construct −356/+359. The mutation of site A in the construct −356/+55 resulted in reversal of δEF1-mediated repression of *Npr1* promoter activity. Mutation of site B in construct −356/+359 increased the promoter activity 3.2-fold compared with the wild-type control construct. However, mutation of both δEF1 sites in construct −356/+359 showed 6.6-fold enhanced promoter activity compared with the wild-type construct in transfected RTASMCs. However, co-transfection of δEF1 expression plasmids with *Npr1* promoter constructs bearing mutant δEF1 binding sites did not show any effect on the promoter activity, further confirming the role of δEF1 binding sites in mediating the repressive effects on *Npr1* gene expression.

Knockdown of δEF1 by siRNA abolished the δEF1-mediated repression of *Npr1* promoter activity (Fig. 3A). There was a 68% reduction in luciferase activity of *Npr1* promoter construct −356/+359 in MMCs over-expressing δEF1, whereas knockdown of δEF1 by siRNA significantly increased the luciferase activity (Fig. 3B). An ~ 80% reduction in endogenous δEF1 protein expression was observed in δEF1

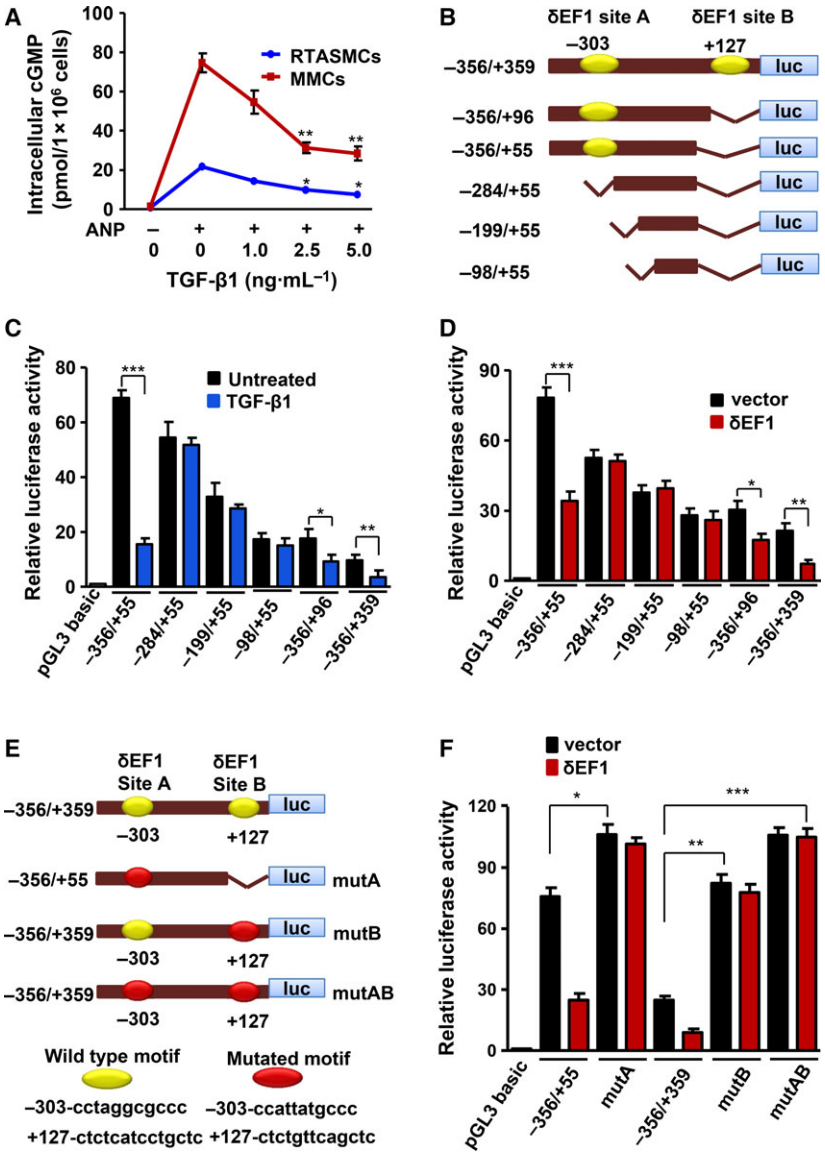

Fig. 2. Luciferase activity of *Npr1* promoter constructs containing deletions and mutations of δEF1 binding sites in RTASMCs. (A) Intracellular accumulation of cGMP in TGF-β1-treated RTASMCs and MMCs induced using ANP. (B) Schematic map of *Npr1* promoter deletion constructs from which δEF1 binding sites A and B were deleted either alone or in combination. (C) Luciferase activity of *Npr1* promoter deletion constructs transiently transfected into cells that were treated with TGF-β1 (2.5 ng·mL^{-1}) for another 24 h. (D) Luciferase activity of *Npr1* promoter deletion constructs in RTASMCs co-transfected with δEF1 expression plasmid or an empty vector. (E) Diagrammatic representation of *Npr1* promoter constructs harboring wild-type or mutated δEF1 binding sites. (F) Luciferase activity of *Npr1* promoter mutation constructs in RTASMCs co-transfected with δEF1 expression plasmid or an empty vector. Values are means ± SE of six independent experiments in triplicate. Asterisks indicate statistically significant differences as indicated or compared with controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

siRNA-transfected RTASMCs compared with untransfected cells (Fig. 3C). Over-expression of δEF1 protein was observed in RTASMCs and MMCs transfected with the δEF1 expression plasmid (Fig. 3C,D).

In order to confirm whether endogenous δEF1 protein binds to its consensus sequence in the *Npr1* promoter, EMSA and a ChIP assay were performed. In a gel shift assay, incubation of untreated RTASMC nuclear extract with δEF1 site A and δEF1 site B oligonucleotides showed formation of specific nucleoprotein complexes (Fig. 4A,B, lane 2), and the binding was markedly enhanced in the presence of TGF-β1-treated nuclear extract (Fig. 4A,B, lane 3). DNA–protein binding was inhibited in the presence of a 100-fold excess molar concentration of competitor DNA (Fig. 4A,B, lane 4). The specificity of the protein–DNA complex was confirmed by a δEF1 antibody supershift assay (Fig. 4C,D, lane 3). Figure 5A shows the position of δEF1 sites in the *Npr1* promoter used for ChIP assay. Treatment with TGF-β1 greatly enhanced occupancy of δEF1 and pSmad2/3 at the *Npr1* promoter compared with untreated cells (Fig. 5B). *In vivo* binding of δEF1 to sites A and B was also observed in untreated MMCs (Fig. 5C). We further examined the effect of TGF-β1 on δEF1 protein expression by treating the cells with increasing concentrations of TGF-β1 and performing Western blot analysis with the cell lysates. The Western blot analysis demonstrated that TGF-β1 increased the expression of endogenous δEF1 by 2.4- and 2.8-fold in

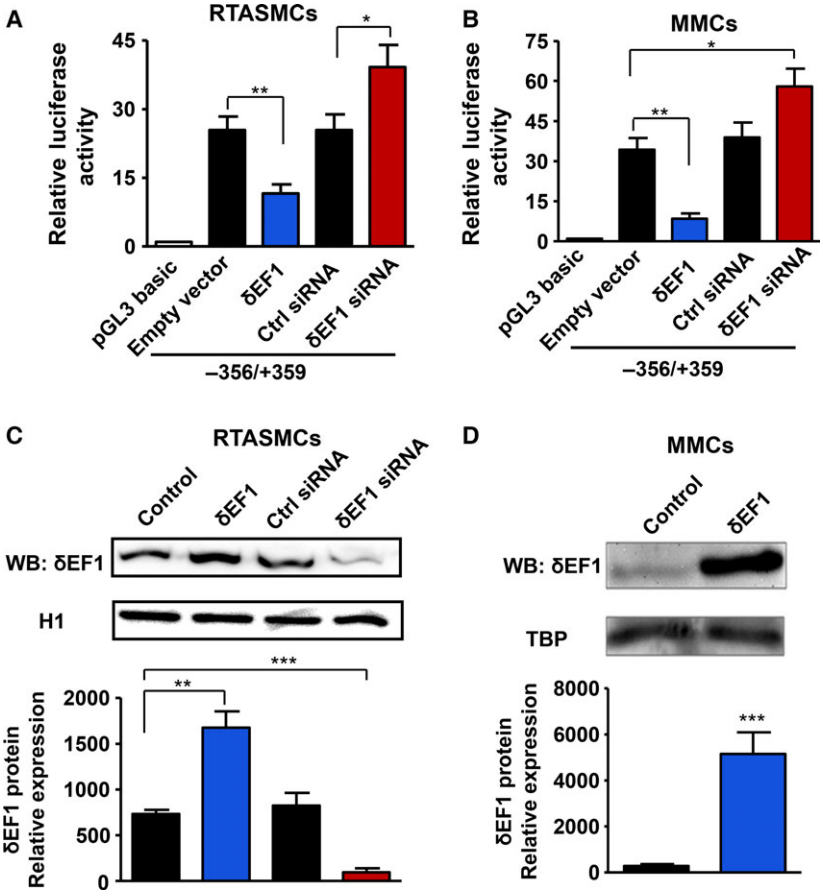

Fig. 3. Effect of over-expression and knockdown of δEF1 on *Npr1* gene transcription in RTASMCs and MMCs. (A,B) Luciferase activity of *Npr1* promoter construct − 356/+ 359 co-transfected with δEF1 expression plasmid, empty vector, δEF1 siRNA or control siRNA in RTASMCs (A) and MMCs (B) as measured by the luciferase assay. (C) Western blot and densitometry analysis of δEF1 protein in RTASMCs transfected with δEF1 expression plasmid or δEF1 siRNA. H1 expression is shown as a loading control. (D) Western blot analysis of over-expression of δEF1 protein in MMCs. Expression of TATA box-binding protein (TBP) is shown as a loading control. WB, Western blot. Values are means ± SE of six independent experiments in triplicate. Asterisks indicate statistically significant differences as indicated or compared with the control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

RTASMCs and MMCs, respectively, in a dose-dependent manner, compared with untreated cells (Fig. 5D, E). Treatment of cells with TGF-β1 significantly increased phosphorylation of Smad2/3 proteins in RTASMCs and MMCs compared with their untreated controls (Fig. 5F,G). However, there was no change in the expression level of Smad2/3 protein under TGF-β1 treatment.

As ANP/NPRA and TGF-β1 signaling are known to antagonize each other, we further tested this response under our experimental conditions. Western blot analysis of TGF-β1-treated RTASMCs showed a significant increase in protein expression for α-smooth muscle actin (α-SMA) and collagen type I α2 (COL1A2), which was markedly attenuated by ANP treatment in NPRA over-expressing cells pre-treated with TGF-β1 (Fig. 6A). Treatment of cells with ANP significantly attenuated TGF-β1-induced nuclear translocation of pSmad2/3 (Fig. 6B). To further confirm the functional effects of TGF-β1 on *Npr1* expression, we performed *ex vivo* experiments using denuded aortic rings from C57/BL6 male mice. There was a 65% reduction in luciferase activity of *Npr1* promoter construct −356/+359 in transiently transfected aortic rings treated with TGF-β1 compared with untreated control aortic rings (Fig. 7A). Treatment of aortic rings with TGF-β1 resulted in a 62% reduction in *Npr1* mRNA levels (Fig. 7B). Incubation of denuded aortic rings with TGF-β1 resulted in a 70% reduction in NPRA protein expression and significantly increased expression of TGF-β1-responsive proteins, namely α-SMA and COL1A2 (Fig. 7C). Treatment with increasing concentrations of ANP (IC_{50} = 6×10^{-9} m) relaxed denuded aortic rings that had been contracted using prostaglandin F2α; however, pre-treatment of aortic rings with TGF-β1 significantly attenuated ANP-mediated relaxation (Fig. 7D). Interestingly, endothelium-intact vessels were not affected by TGF-β1 incubation.

Discussion

The findings of the present study suggest that transcriptional repression of *Npr1* is modulated by the TGF-β1/Smad/δEF1 pathway. Our results demonstrate that TGF-β1 inhibits *Npr1* promoter activity by 80–90% in a time- and dose-dependent manner, and significantly reduced *Npr1* mRNA expression and protein

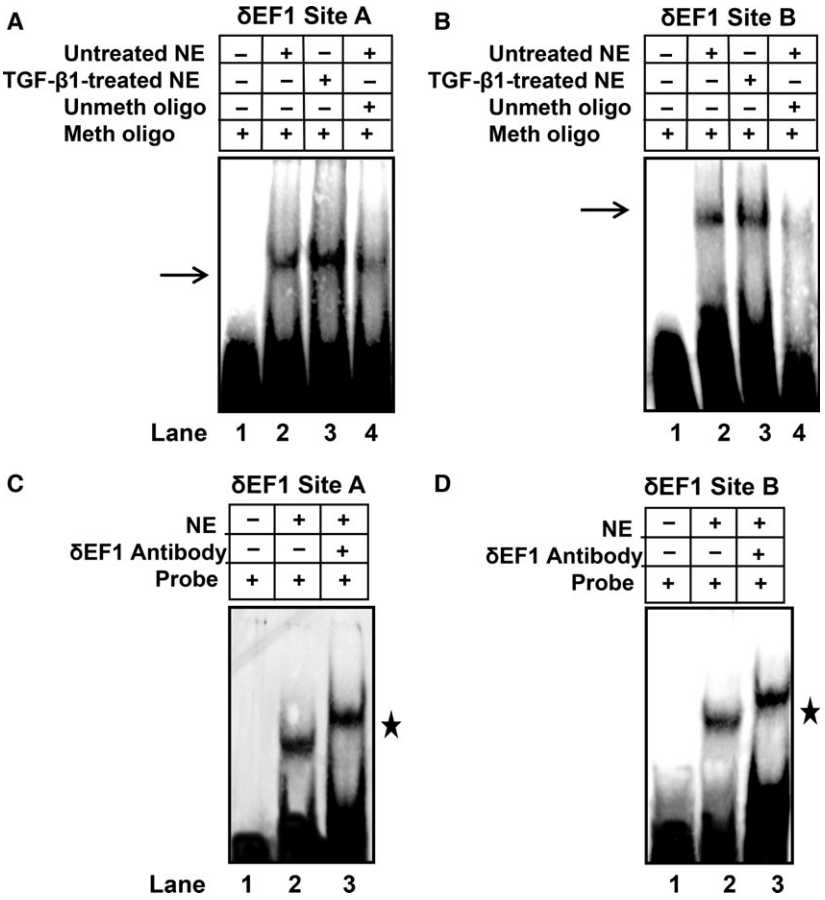

Fig. 4. Electrophoretic mobility shift assay showing *in vitro* binding of δEF1 to the consensus binding sites in the *Npr1* promoter. (A,B) RTASMC nuclear extract was incubated with oligonucleotides for δEF1 site A (A) and δEF1 site B (B). Arrows indicate specific DNA–protein binding complexes in untreated nuclear extract (lane 2) and TGF-β1-treated nuclear extract (lane 3); the binding was inhibited in presence of a 100-fold molar excess of unlabeled competitor DNA in lane 4. (C,D) Incubation of nuclear extract with oligonucleotides for δEF1 site A (C) and δEF1 site B (D) in the presence of anti-δEF1 antibody shows a supershift of the DNA–δEF1 protein complex in lane 3. Asterisks indicate the supershifted complex. Values are means ± SE of four independent experiments. NE, nuclear extract; meth/unmeth, methylated/unmethylated.

levels in cultured primary RTASMCs, MMCs and denuded aortic rings. Two δEF1 binding sites have been predicted in the *Npr1* promoter (−356/+359) using the TRANSFAC 3.2 database (www.biobase-international.com/product/transcription-factor-sites, Qiagen, Waltham, MA, USA), namely δEF1 site A (−303 to −293) and δEF1 site B (+127 to +139) (positions relative to the transcription start site) [10,13]. *Npr1* promoter deletion analysis showed that repression of *Npr1* gene transcription due to δEF1 was eliminated for constructs that did not have δEF1 binding sites. Over-expression of δEF1 showed significant repression of *Npr1* promoter activity for constructs with δEF1 binding sites. However, over-expression of δEF1 did not result in any change in activity of constructs lacking δEF1 binding sites, suggesting that the absence of δEF1 site de-represses *Npr1* promoter activity. Site-directed mutagenesis of δEF1 binding sites and endogenous δEF1gene silencing by siRNA transfection confirmed that the repression of *Npr1* promoter was due to δEF1. Previously, a TGF-β1-mediated decrease in *Npr1* mRNA levels in cultured SMCs had been demonstrated, but the underlying molecular mechanisms were not known [26]. Our data provides the evidence for the involvement of δEF1 in mediating TGF-β1 effects on *Npr1* gene transcription. It has been shown that δEF1 promotes breast cancer cell proliferation through down-regulation of p21 expression [44]. Over-expression of δEF1 family proteins has been shown to repress E-cadherin promoter activity [45,46]. Ectopic expression of δEF1 represses transcription of the estrogen receptor-α by binding to the E2 box on its promoter [38]. Our *in vivo* ChIP binding assay data showed that δEF1 formed nucleoprotein complexes with the endogenous *Npr1* gene promoter, which were absent in the negative controls, providing evidence that the mechanism of *Npr1* promoter repression by δEF1 is due to direct binding to the *Npr1* promoter DNA.

Interaction between δEF1 and TGF-β1 signaling has been observed in several cellular processes [35,47,48]. It has been shown that TGF-β1 activates genes such as vimentin and represses E-cadherin by δEF1-mediated assembly of Smad proteins and other transcription factors at the promoter regions of the respective genes [47,48]. Our results from Western blot analysis showed a significant increase in phosphorylation of Smad2/3 proteins, confirming their involvement in the TGF-β1/δEF1 signaling cascade. Moreover, the

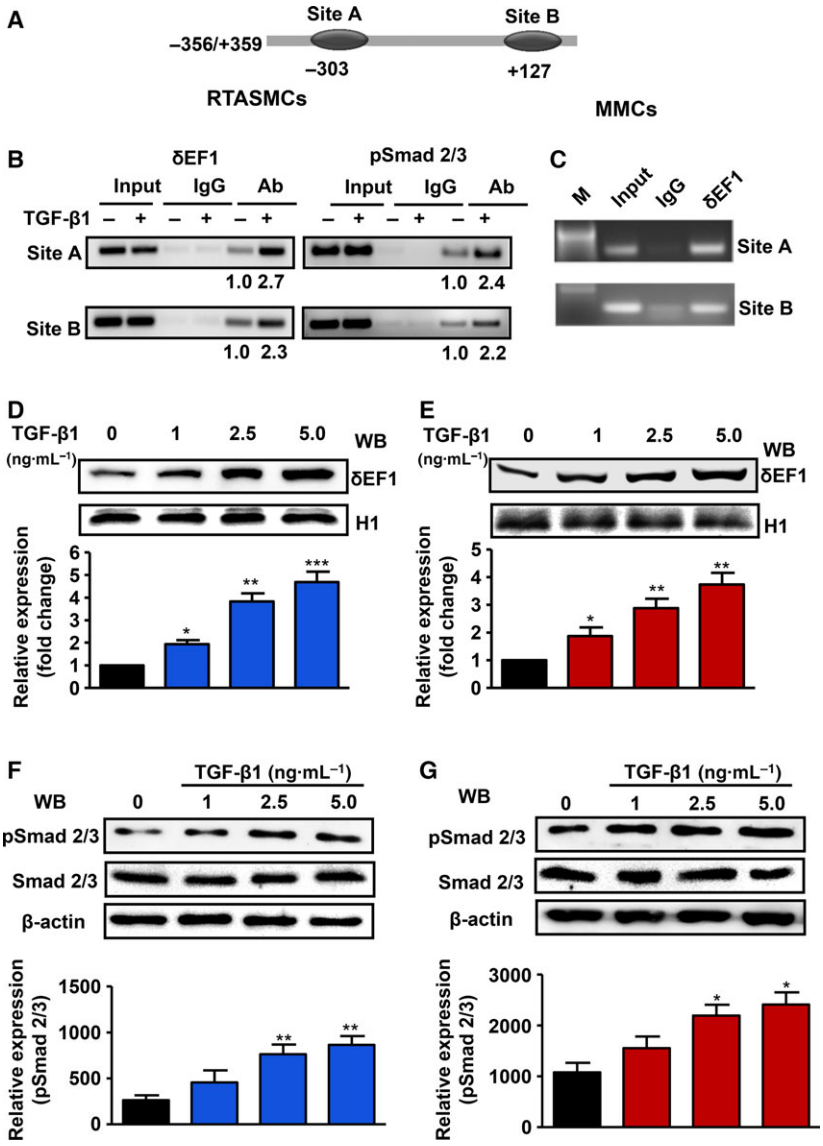

Fig. 5. TGF-β1-dependent δEF1 protein expression, and binding of δEF1 to the *Npr1* promoter. (A) Schematic map showing δEF1 binding sites on the *Npr1* promoter. (B) ChIP analysis demonstrating *in vivo* recruitment of δEF1 and pSmad2/3 to the *Npr1* promoter in TGF-β1-treated and untreated RTASMCs. (C) Expression of δEF1 protein in untreated MMCs. The intensity of DNA bands was quantified using ALPHAVIEW software (ProteinSimple, San Jose, CA, USA). Representative gels from three independent experiments are shown. (D,E) Western blot and densitometry analyses of δEF1 protein expression in RTASMCs (D) and MMCs (E) treated with increasing concentrations of TGF-β1. H1 expression is shown as a loading control. (F,G) Western blot and densitometry analysis of phosphorylated and unphosphorylated Smad2/3 protein expression in TGF-β1-induced RTASMCs (F) and MMCs (G). β-actin was used as a loading control. WB, Western blot. Values are means ± SE of six independent experiments in triplicate. Asterisks indicate statistically significant differences compared with controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

results of the present study showed that TGF-β1 repressed *Npr1* gene transcription and expression by inducing direct binding of δEF1 and pSmad2/3 to the *Npr1* promoter (Fig. 8). Recent studies have shown that TGF-β1 transcriptionally regulates the expression of many *trans*-acting factors, including the zinc-finger factors Snail and Slug and the two-handed zinc-finger factors of δEF1 family proteins δEF1 and Smad interacting protein 1(SIP1), which are involved in the induction of the epithelial to mesenchymal transition, particularly through transcriptional repression of E-cadherin and epithelial splicing regulatory proteins [37,49–51]. Our results showed that TGF-β1 treatment increased δEF1 protein levels compared with untreated controls. Targeted deletion of δEF1 in mice results in skeletal defects, which are similar to those in mice with gene knockout of TGF-β1 family proteins [47,52]. Down-regulation of FXYD3, a member of the FXYD family of proteins that have a single transmembrane segment and share a signature sequence of four amino acids (Phe-x-Tyr-Asp), is induced by TGF-β1 signaling via δEF1 in human mammary epithelial cells [53].

Our results demonstrate that TGF-β1 exerts negative repressive effects on transcription and expression of *Npr1* and receptor signaling in ANP target cells, including MMCs and RTASMCs, as well as denuded aortic segments. Interestingly, treatment withTGF-β1 significantly attenuated ANP-mediated dose-dependent relaxation of denuded intact aortic rings. Conversely, ANP/NPRA signaling markedly attenuated the TGF-β1-induced nuclear translocation of pSmad2/3 and expression of COL1A2 and α-SMA in these target

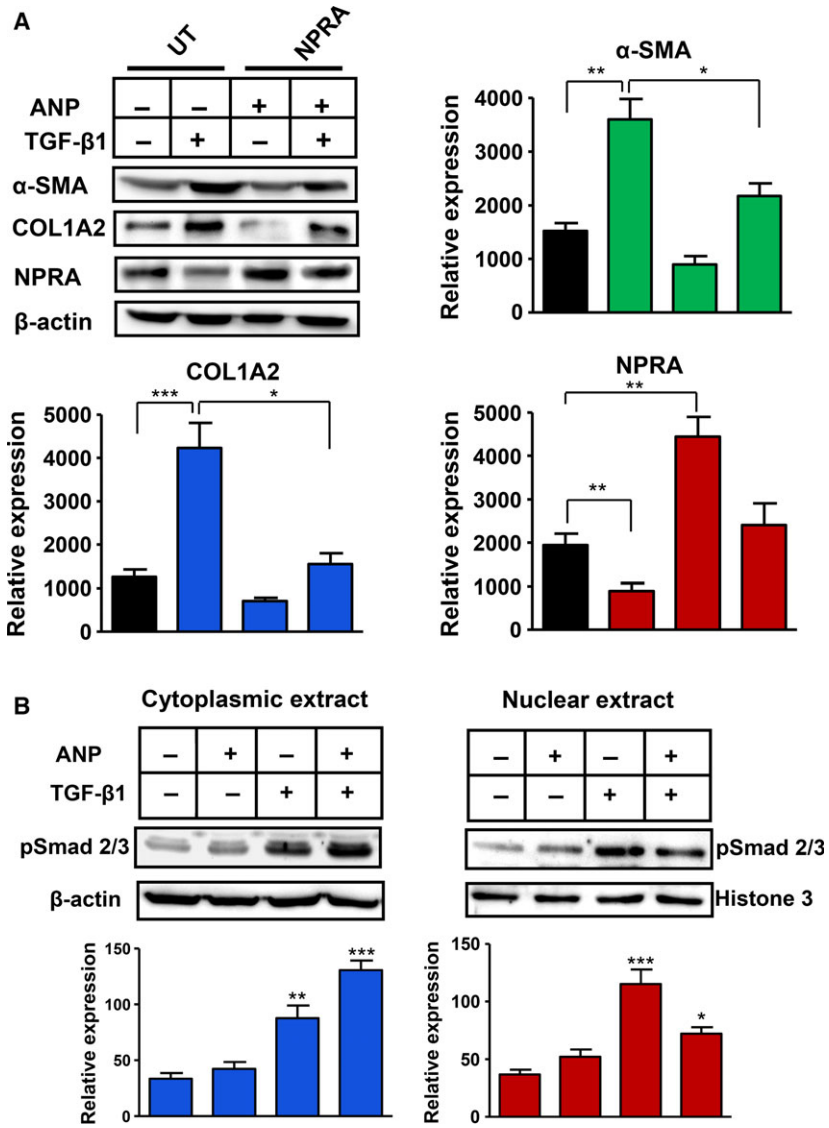

Fig. 6. Effect of ANP treatment on TGF-β1 signaling in RTASMCs. (A) Western blot and densitometry analysis of NPRA, α-SMA and COL1A2 protein expression in cells transfected with NPRA expression plasmid and treated with and without ANP and TGF-β1. (B) Western blot and densitometry analysis of nuclear translocation of pSmad2/3 (Ser423/425) in cytoplasmic and nuclear extracts of RTASMCs treated with and without ANP and TGF-β1. β-actin and H1 expression are shown as loading controls. Values are means ± SE of six independent experiments in triplicate. Asterisks indicate statistically significant differences as indicated or compared with controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

cells, indicating the antagonistic actions between TGF-β1 and ANP/NPRA systems. Interestingly, ANP/NPRA signaling has been shown to exert its antifibrogenic effect by blocking TGF-β1-induced nuclear translocation of Smad2/3 and extracellular matrix expression in pulmonary aortic smooth muscle cells [40,54,55]. Mechanical stretch has been shown to increase BNP and NPRA expression in human cardiac fibroblasts, which in turn attenuates TGF-β1-induced myocardial fibrosis by inhibiting expression of α-SMA and COL1A2 [56]. Studies using targeted disruption of the *Npr1* gene in mice have shown enhanced activation of pro-inflammatory cytokines, including TGF-β1, in the heart and kidneys [19,20,24,57,58]. In contrast, activated TGF-β1 has been shown to participate in the pathogenesis of cardiac hypertrophy, renal fibrosis and vascular remodeling via its downstream signaling pathway [25,28,59–61]. The findings of the present study demonstrate that TGF-β1 induces the expression of δEF1 and its binding to the *Npr1* promoter, thus repressing *Npr1* gene transcription, expression and function in the physiological context. Our results identify novel molecular mechanisms of TGF-β1 action on *Npr1* gene repression, which will enhance our understanding of the counter-regulatory mechanisms of TGF-β1, Smad 2/3, *trans*-acting factor δEF1 and ANP/NPRA/cGMP signaling that are relevant to renal and vascular remodeling in cardiovascular disease states.

In conclusion, the present results demonstrate that TGF-β1 mediates its effect via inducing Smad2/3 protein phosphorylation and δEF1 expression, and their binding to the *Npr1* promoter. The results in primary cultured RTASMCs, MMCs and denuded aortic rings

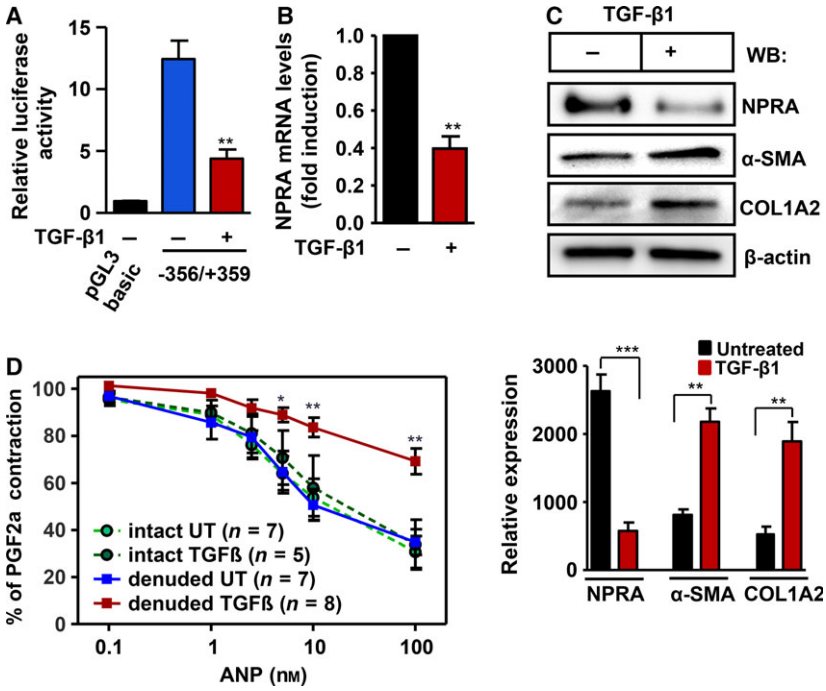

Fig. 7. Effect of TGF-β1 treatment on *Npr1* gene transcription and expression and ANP-induced vaso-relaxation in aortic rings. (A) Luciferase activity of denuded aortic rings transfected with the *Npr1* proximal promoter construct −356/+55 and treated with TGF-β1. (B,C) *Npr1* mRNA levels (B) and Western blot analysis of NPRA, α-SMA and COL1A2 expression (C) in TGF-β1-induced aortic rings. β-actin was used as a loading control. (D) Vaso-relaxation of aortic rings in the presence of ANP with or without TGF-β1 treatments. WB, Western blot. Values are means ± SE of 5–8 independent experiments in triplicate. Asterisks indicate statistically significant differences as indicated or compared with controls (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

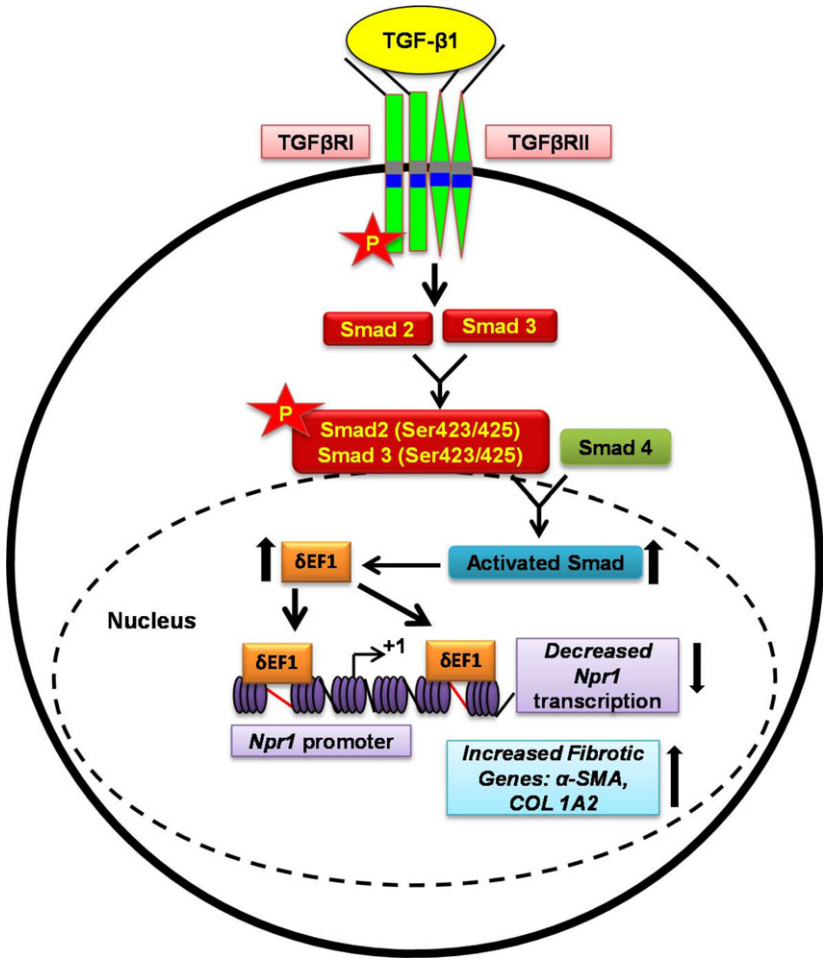

Fig. 8. Regulation of *Npr1* gene transcription by TGF-β1 signaling. Activation of TGF-β1 signaling results in increased levels of δEF1, which causes repression of *Npr1* gene transcription. δEF1 directly binds to the *Npr1* gene promoter in response to TGF-β1 induction, and represses its activity. It is possible that δEF1 interacts with Smad proteins in the nucleus. The bold upward arrows indicate increases in δEF1 protein expression; the bold downward arrows indicate decreases in *Npr1* gene transcription and expression.

showed that the inhibitory effect of TGF-β1 on NPRA/cGMP signaling is transduced by direct repressive effects of *Npr1* transcription, expression and physiological function. On the other hand, the antagonistic action of ANP/NPRA on TGF-β1 signaling is evident from the repressive effects on TGF-β1-induced expression of COL1A2 and α-SMA in RTASMCs and aortic rings. Identification of TGF-β1/Smad/δEF1 signaling as a suppressor of functional expression of NPRA provides new molecular targets for developing therapeutic strategies for treatment of hypertension and related cardiovascular disorders.

Experimental procedures

Plasmids and promoter constructs

The *Npr1* promoter–luciferase reporter constructs were generated by cloning various lengths of the *Npr1* promoter into the pGL3 basic vector as previously described [13,15]. The sequences of primers used in generation of constructs −284/+55, −98/+55, −356/+96 and −356/+359 are provided in Table 1. The expression plasmid δEF1 was obtained from Michel M. Sanders (University of Minnesota, Minneapolis, MN, USA).

Cell transfection and luciferase assay

RTASMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal bovine serum, and MMCs were grown in DMEM enriched with 10% fetal bovine serum and insulin/transferrin/sodium selenite as described previously [43]. The cultures were maintained at 37 °C in a 5% CO_2/95% O_2 humidified atmosphere. Cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Grand Island, NY, USA) with 1 μg promoter–reporter construct and 0.3 μg pRL-TK plasmid (Promega, Madison, WI, USA), which was used as an internal transfection control, and luciferase activity was measured as previously described [12,13]. For calculation of the luciferase activity of various *Npr1* promoter constructs, the pGL3 basic plasmid (Promega) was used as the control, and the results are expressed as relative luciferase activity compared with the pGL3 basic plasmid. In co-transfection experiments, 0.5 μg of the δEF1 expression plasmid was used, and total DNA content was equalized by inclusion of empty vector. In ectopic over-expression experiments, cells were transfected with expression plasmids for δEF1 or NPRA, and total DNA content was equalized by inclusion of empty vector. For treatment with TGF-β1 at 24 h after transfection, cells were serum-starved for 12 h in DMEM containing 0.1% BSA, and further stimulated with increasing concentrations of TGF-β1 (EMD Millipore, Billerica, MA, USA) for 24 h.

Whole-cell lysate and nuclear extract preparation

Cells were harvested by scraping 24 h after TGF-β1 treatment or δEF1 transfections. Cells were washed with PBS and centrifuged at 250 ***g*** for 10 min at 4 °C. The pellet was lysed in buffer containing 25 mM HEPES, pH 7.4, 0.05% 2-mercaptoethanol, 1% Triton X-100, 1 mM sodium vanadate, 10 mm sodium fluoride, 0.2 mm phenylmethanesulfonyl flu-

Table 1. List of primers used in cloning, EMSA and the ChIP assay.

Experiment	Primer sequence (5′→3′)	Orientation
Cloning		
Construct − 284/+ 55	tacggaacgcgtcgggtgctgccaagggagggaaacc	Forward
	tacggaagatctgcgggtgcgccagcgaggaaagg	Reverse
Construct − 98/+ 55	tacggaacgcgtctggctcgccttgtggtcccgtcc	Forward
	tacggaagatctgcgggtgcgccagcgaggaaagg	Reverse
Construct − 356/+ 96	tacggaacgcgtgagggggggcagcttcctcac	Forward
	tacggaagatctgagcgagagaac gagagggcg	Reverse
Construct 356/+ 359	tacggaacgcgtgagggggggcagcttcctcac	Forward
	tacggaagatctcagcgagcgcagcgacggagc	Reverse
EMSA		
δEF1 site A − 303	cccccgcggcctaggcgccc	Forward
	gggcgcctaggccgcggggg	Reverse
δEF1 site B + 127	tgcgctcgctctcacctgctctaaagcac	Forward
	gtgctttagagcaggtgagagcgagcgca	Reverse
ChIP		
δEF1 site A at − 303	ttcctcacacccttcctcagtcct	Forward
	cgccagttattgctgaccctctt	Reverse
δEF1 site B + 127	ctcttcttagatcgccctctcgtt	Forward
	agggtgcttagagcaggtgaga	Reverse

oride, 10 μg·mL^{-1}M aprotinin and 10 μg·mL^{-1} leupeptin. The cell extract was passed 15–20 times through a 1 mL syringe with a 21-gauge needle, and centrifuged at 14 000 ***g*** for 10 min at 4 °C. The clear cell lysate was collected and stored at −80 °C until use. Nuclear extract was prepared from cells as previously described [62]. Cells were harvested and centrifuged at 250 ***g*** for 10 min. The cell pellet was washed with PBS and centrifuged again at 250 ***g*** for 10 min. The resulting pellet was resuspended in five volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride), incubated on ice for 10 min and centrifuged at 250 ***g*** for 10 min at 4 °C. The pellet was again resuspended in three volumes of buffer A to which 0.05% v/v Nonidet P-40 (Sigma-Aldrich, St. Louis, MO, USA) had been added. The suspension was homogenized using 20–25 strokes of a tight-fitting Dounce homogenizer to release the nuclei, and centrifuged at 250 ***g*** for 10 min to pellet the nuclei. The pellet thus obtained was resuspended in buffer C (5 mM HEPES, pH 7.9, 26% v/v glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride), and NaCl was added to a final concentration of 300 mM. The suspension was incubated on ice for 30 min, and centrifuged at 24 000 ***g*** for 20 min. The centrifugation steps described above were performed at 4 °C. Aliquots of the supernatant were stored at −80 °C. The protein concentration was estimated by the Bradford method using a Bio-Rad protein assay kit (Hercules, CA, USA).

Ex vivo mouse aortic ring assays

C57BL/6 male mice were killed by deep anesthesia comprising isoflurane inhalation. Aortic segments were prepared as described previously [63]. Immediately after thoracotomy, the thoracic aorta was removed and placed in cold Dulbecco's PBS (Sigma-Aldrich, St Louis, MO, USA) containing 136.8 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_2, pH 7.4. Then the aorta was cleaned by removing the surrounding fat and connective tissues. A small segment of aorta with intact endothelium was saved for control studies, and the endothelium was removed mechanically from the remaining segment of the aorta. Denudation of the endothelium was achieved by scraping the lumen of the aorta using a 26-gauge monofilament surgical steel wire (Ethicon, Somerville, NJ, USA). Blood and denuded endothelial cells were removed by gently flushing Dulbecco's PBS through the lumen of the aorta. Finally, the aorta was cut into 3–4 mm rings for experiments. After 4–5 h incubation in DMEM enriched with 10% fetal bovine serum and 5 mL.L^{-1} penicillin/streptomycin, the aortic rings were serum-starved overnight and treated with TGF-β1 for 12 h. Aortic rings were homogenized by sonication in lysis buffer, centrifuged at 14 000 ***g*** for 10 min at 4 °C, and the supernatants were stored at −80 °C for later use in Western blot experiments. For RNA extraction, the aortae were crushed using a 1.5 mL tube and pestle, and RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Denuded aortic rings were transfected using aortic smooth muscle cell transfection reagent (Altogen Biosystems, Las Vegas, NV, USA) with 3 μg promoter–reporter construct. After 24 h transfection, aortic rings were serum-starved for 12 h in DMEM containing 0.1% BSA, and further stimulated with TGF-β1 for 24 h. Aortic rings were homogenized by sonication in passive lysis buffer, and luciferase activity was measured as previously described [9].

Aortic rings relaxation assay

The aorta was excised as described above and cut into 2 mm rings. Some rings were denuded and some were left with the endothelium intact. Rings were placed into a 24-well culture dish in DMEM containing vehicle (PBS containing 0.1% BSA) or 2.5 ng·mL^{-1} TGF-β1. After 24 h incubation, rings were mounted onto a Danish Myotechnology multi-chamber myograph system (model 620M; Ann Arbor, MI, USA) and set to an initial tension of 10 mN as previously described [64]. After an initial incubation period followed by contraction using 80 mM KCl and washout, endothelial function was tested by contracting vessels using 5 μM prostaglandin F2α followed by 1 μM acetylcholine. Vessels with more than 50% relaxation were considered endothelium-intact. After washing with physiological salt solution at 37 °C (3 × 5 min each wash), vessels were then contracted again using 5 μM prostaglandin F2α, and exposed to increasing concentrations of ANP (10^{-10}–10^{-7} M). The results are expressed as percentage relaxation from prostaglandin F2α contraction.

Real-time RT-PCR

A total RNA isolation kit from Promega was used to isolate total RNA, and first-strand cDNA was reverse-transcribed using Smartscribe reverse transcriptase (Clontech, Mountain View, CA, USA). Cells were treated with increasing concentrations of TGF-β1 for 24 h, lysed, and total RNA was extracted. Real-time RT-PCR was performed using a Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA), and the data were analyzed using MXPRO QPCR software (Agilent Technologies). Primers for amplification of *Npr1* and β-actin were purchased from Qiagen. PCR amplifications (in triplicates) were performed in a 25 μL reaction volume using RT2 real-time™ SYBR Green/ROX PCR Master Mix (Roche, New York, NY, USA). The reaction conditions were 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min, followed by one cycle of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s for the dissociation curve. Standard curves were generated

for *Npr1* and β-actin separately. Relative expression of the *Npr1* gene was determined by the comparative C_T method using MXPRO QPCR software (Agilent Technologies). The size of the PCR product for *Npr1* was 70 bp and that for β-actin was 200 bp.

Western blot analysis

Whole-cell lysate (40–50 μg proteins) from each sample was mixed with sample loading buffer, and separated by 10% SDS/PAGE. Proteins were electrotransferred onto a polyvinylidene fluoride membrane, blocked using 1 × Tris-buffered saline/Tween 20 containing 5% fat-free milk for 1 h at room temperature, and then incubated overnight at 4 °C in 1 × Tris-buffered saline/Tween 20 containing 3% fat-free milk with primary antibodies (1 : 250 dilution). The membrane was treated with corresponding secondary anti-mouse or anti-chicken horseradish peroxidase-conjugated antibodies (1 : 5000 dilutions). Protein bands were developed using a SuperSignal West Femto Chemiluminescent kit (Thermo Fisher Scientific), and visualized using an Alpha Innotech detection system (Proteinsimple, Santa Clara, CA, USA). The intensity of protein bands was quantified using ALPHAVIEW software. Primary antibodies against δEF1 (catalog number sc-10573), pSmad2/3 (Ser423/425; catalog number sc-11769), H1 (catalog number sc-10806), α-SMA (catalog number Sc-56499), COL1A2 (catalog number sc-8788) and β-actin (catalog number Sc-47778 HRP) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Primary antibody for NPRA was produced as previously described [16,65].

cGMP assay

Twenty-four hours after plating, cells were made serum-free for 12 h, and treated with TGF-β1 for 24 h. Cells were stimulated with ANP at 37 °C for 15 min in the presence of 0.2 mM 3-isobutyl-1-methylxanthine, washed three times with PBS, and scraped into 0.5 M HCl. The cell suspension was subjected to five freeze/thaw cycles, then centrifuged at 10 000 ***g*** for 10 min. The supernatant collected was used for cGMP assay using a direct ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions.

siRNA transfection

Cells were cultured to 80–90% confluence and transfected with δEF1 siRNA comprising a pool of three target-specific 20–25 nt siRNAs (Santa Cruz Biotechnology) using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). A non-targeting 20–25 nt siRNA was used as a negative control. Twenty-four after transfection, cells were lysed to measure firefly and Renilla luciferase activity by using the Dual-luciferase reporter assay system (promega).

Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was performed in nuclear extract prepared from RTASMCs as described above. EMSA was performed utilizing biotin-labeled probes and a Lightshift chemiluminescent kit (Thermo Scientific Pierce, Rockford, IL, USA), according to the manufacturer's instructions. Approximately 5–10 μg of nuclear extract was incubated with 20 fmol of biotin-labeled probe in the presence of 1 × binding buffer in a final reaction volume of 20 μL. The EMSA reaction was incubated for an additional 25 min at room temperature, and the nucleo-protein complexes were resolved by 5% non-denaturing PAGE and visualized by the chemiluminescent method. For supershift assays, δEF1 polyclonal antibody was added to the protein–DNA complexes, and the reaction was incubated for an additional 30 min. The sequences of the oligonucleotides used for δEF1 site A at −303 and δEF1 site B at +127 are provided in Table 1.

Chromatin immunoprecipitation assay

The ChIP assay was performed using a ChIP-IT Express enzymatic kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were treated with 1% formaldehyde for 10 min to crosslink protein–DNA complexes, and the reaction was quenched using 0.1 $mol \cdot L^{-1}$ glycine. Cells were scraped, resuspended in 1 mL of lysis buffer on ice, homogenized with a Dounce homogenizer, and centrifuged at 2400 ***g*** for 10 min at 4 °C. The chromatin extracted from the cells was enzymatically sheared by incubation at 37 °C for 10 min, and immunoprecipitated using Protein G magnetic beads and δEF1 antibody or control IgG at 4 °C overnight. After washing the magnetic beads, bound protein was eluted by gentle rotation for 15 min in elution buffer at 22 °C. In the eluted protein–DNA complex, cross-linking was reversed at 65 °C overnight to release DNA. Immunoprecipitated DNA was sequentially treated with RNase A and proteinase K and then purified. The DNA was PCR-amplified. The sequences of primers used for PCR amplification of δEF1 site A and δEF1 site B are provided in Table 1.

In vitro site-directed mutagenesis

Npr1 promoter constructs with mutated δEF1 sites were custom-synthesized by Eurofins Genomics (Huntsville, AL, USA). The mutant construct was transfected into the cells using Lipofectamine 2000 as previously described [8,66].

Statistical analysis

Statistical analyses were performed by one-way analysis of variance, followed by Dunnett's multiple comparison tests using PRISM software (GraphPad Software, San Diego, CA,

USA). A P-value < 0.05 was considered significant. Values are means ± SE.

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Author contributions

AS, PK and KNP planned the experiments. AS, PK, SHL, PVGK and KNP performed the experiments and analyzed the data. AS, PK, RG, PVGK, SHL, MB and KNP contributed reagents or other essential material. AS, PK and KNP wrote the paper.

References

1 Brenner BM, Ballermann BJ, Gunning ME & Zeidel ML (1990) Diverse biological actions of atrial natriuretic peptide. *Physiol Rev* **70**, 665–699.
2 de Bold AJ, Borenstein HB, Veress AT & Sonnenberg H (1981) A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci* **28**, 89–94.
3 Pandey KN (2005) Biology of natriuretic peptides and their receptors. *Peptides* **26**, 901–932.
4 Maack T (2006) The broad homeostatic role of natriuretic peptides. *Arq Bras Endocrinol Metabol* **50**, 198–207.
5 Venugopal J (2003) Pharmacological modulation of the natriuretic peptide system. *Expert Opin Ther Pat* **13**, 1386–1409.
6 Garbers DL (1992) Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. *Cell* **71**, 1–4.
7 Koller KJ, de Sauvage FJ, Lowe DG & Goeddel DV (1992) Conservation of the kinase-like regulatory domain is essential for activation of the natriuretic peptide receptor guanylyl cyclases. *Mol Cell Biol* **12**, 2581–2590.
8 Pandey KN & Singh S (1990) Molecular cloning and expression of murine guanylate cyclase/atrial natriuretic factor receptor cDNA. *J Biol Chem* **265**, 12342–12348.
9 Kumar P, Garg R, Bolden G & Pandey KN (2010) Interactive roles of Ets-1, Sp1, and acetylated histones in the retinoic acid-dependent activation of guanylyl cyclase/atrial natriuretic peptide receptor-A gene transcription. *J Biol Chem* **285**, 37521–37530.
10 Garg R, Oliver PM, Maeda N & Pandey KN (2002) Genomic structure, organization, and promoter region analysis of murine guanylyl cyclase/atrial natriuretic peptide receptor-A gene. *Gene* **291**, 123–133.
11 Kumar P & Pandey KN (2009) Cooperative activation of Npr1 gene transcription and expression by interaction of Ets-1 and p300. *Hypertension* **54**, 172–178.
12 Arise KK & Pandey KN (2006) Inhibition and down-regulation of gene transcription and guanylyl cyclase activity of NPRA by angiotensin II involving protein kinase C. *Biochem Biophys Res Commun* **349**, 131–135.
13 Kumar P, Arise KK & Pandey KN (2006) Transcriptional regulation of guanylyl cyclase/natriuretic peptide receptor-A gene. *Peptides* **27**, 1762–1769.
14 Chen S, Olsen K, Grigsby C & Gardner DG (2007) Vitamin D activates type A natriuretic peptide receptor gene transcription in inner medullary collecting duct cells. *Kidney Int* **72**, 300–306.
15 Garg R & Pandey KN (2003) Angiotensin II-mediated negative regulation of Npr1 promoter activity and gene transcription. *Hypertension* **41**, 730–736.
16 Kumar P, Tripathi S & Pandey KN (2014) Histone deacetylase inhibitors modulate the transcriptional regulation of guanylyl cyclase/natriuretic peptide receptor-A gene: interactive roles of modified histones, histone acetyltransferase, p300, and Sp1. *J Biol Chem* **289**, 6991–7002.
17 Heldin CH, Miyazono K & ten Dijke P (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465–471.
18 Shi Y & Massague J (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* **113**, 685–700.
19 Vellaichamy E, Kaur K & Pandey KN (2007) Enhanced activation of pro-inflammatory cytokines in mice lacking natriuretic peptide receptor-A. *Peptides* **28**, 893–899.
20 Vellaichamy E, Das S, Subramanian U, Maeda N & Pandey KN (2014) Genetically altered mutant mouse models of guanylyl cyclase/natriuretic peptide receptor-A exhibit the cardiac expression of proinflammatory mediators in a gene dose-dependent manner. *Endocrinology* **155**, 1045–1056.
21 Vellaichamy E, Khurana ML, Fink J & Pandey KN (2005) Involvement of the NF-κB/matrix metalloproteinase pathway in cardiac fibrosis of mice lacking guanylyl cyclase/natriuretic peptide receptor A. *J Biol Chem* **280**, 19230–19242.
22 Kishimoto I, Tokudome T, Nakao K & Kangawa K (2011) Natriuretic peptide system: an overview of studies using genetically engineered animal models. *FEBS J* **278**, 1830–1841.

23 Pandey KN (2011) The functional genomics of guanylyl cyclase/natriuretic peptide receptor-A: perspectives and paradigms. *FEBS J* **278**, 1792–1807.
24 Das S, Au E, Krazit ST & Pandey KN (2010) Targeted disruption of guanylyl cyclase-A/natriuretic peptide receptor-A gene provokes renal fibrosis and remodeling in null mutant mice: role of proinflammatory cytokines. *Endocrinology* **151**, 5841–5850.
25 Hathaway CK, Gasim AM, Grant R, Chang AS, Kim HS, Madden VJ, Bagnell CR Jr, Jennette JC, Smithies O & Kakoki M (2015) Low TGFβ1 expression prevents and high expression exacerbates diabetic nephropathy in mice. *Proc Natl Acad Sci USA* **112**, 5815–5820.
26 Fujio N, Gossard F, Bayard F & Tremblay J (1994) Regulation of natriuretic peptide receptor A and B expression by transforming growth factor-β1 in cultured aortic smooth muscle cells. *Hypertension* **23**, 908–913.
27 Kapoun AM, Liang F, O'Young G, Damm DL, Quon D, White RT, Munson K, Lam A, Schreiner GF & Protter AA (2004) B-type natriuretic peptide exerts broad functional opposition to transforming growth factor-β in primary human cardiac fibroblasts: fibrosis, myofibroblast conversion, proliferation, and inflammation. *Circ Res* **94**, 453–461.
28 Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH *et al.* (1986) Transforming growth factor type β: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc Natl Acad Sci USA* **83**, 4167–4171.
29 Ignotz RA & Massague J (1986) Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem* **261**, 4337–4345.
30 Zimmerman KA, Graham LV, Pallero MA & Murphy-Ullrich JE (2013) Calreticulin regulates transforming growth factor-β-stimulated extracellular matrix production. *J Biol Chem* **288**, 14584–14598.
31 Pan X, Chen Z, Huang R, Yao Y & Ma G (2013) Transforming growth factor β1 induces the expression of collagen type I by DNA methylation in cardiac fibroblasts. *PLoS ONE* **8**, e60335.
32 He J, Chen Y, Huang Y, Yao F, Wu Z, Chen S, Wang L, Xiao P, Dai G, Meng R *et al.* (2009) Effect of long-term B-type natriuretic peptide treatment on left ventricular remodeling and function after myocardial infarction in rats. *Eur J Pharmacol* **602**, 132–137.
33 Funahashi J, Kamachi Y, Goto K & Kondoh H (1991) Identification of nuclear factor δEF1 and its binding site essential for lens-specific activity of the δ1-crystallin enhancer. *Nucleic Acids Res* **19**, 3543–3547.
34 Funahashi J, Sekido R, Murai K, Kamachi Y & Kondoh H (1993) δ-crystallin enhancer binding protein δEF1 is a zinc finger-homeodomain protein implicated in postgastrulation embryogenesis. *Development* **119**, 433–446.
35 Nishimura G, Manabe I, Tsushima K, Fujiu K, Oishi Y, Imai Y, Maemura K, Miyagishi M, Higashi Y, Kondoh H *et al.* (2006) δEF1 mediates TGF-β signaling in vascular smooth muscle cell differentiation. *Dev Cell* **11**, 93–104.
36 Fontemaggi G, Gurtner A, Damalas A, Costanzo A, Higashi Y, Sacchi A, Strano S, Piaggio G & Blandino G (2005) δEF1 repressor controls selectively p53 family members during differentiation. *Oncogene* **24**, 7273–7280.
37 Horiguchi K, Sakamoto K, Koinuma D, Semba K, Inoue A, Inoue S, Fujii H, Yamaguchi A, Miyazawa K, Miyazono K *et al.* (2012) TGF-β drives epithelial-mesenchymal transition through δEF1-mediated downregulation of ESRP. *Oncogene* **31**, 3190–3201.
38 Guo S, Li Y, Tong Q, Gu F, Zhu T, Fu L & Yang S (2012) δEF1 down-regulates ER-α expression and confers tamoxifen resistance in breast cancer. *PLoS ONE* **7**, e52380.
39 Castro NE, Kato M, Park JT & Natarajan R (2014) Transforming growth factor β1 (TGF-β1) enhances expression of profibrotic genes through a novel signaling cascade and microRNAs in renal mesangial cells. *J Biol Chem* **289**, 29001–29013.
40 Li P, Oparil S, Novak L, Cao X, Shi W, Lucas J & Chen YF (2007) ANP signaling inhibits TGF-β-induced Smad2 and Smad3 nuclear translocation and extracellular matrix expression in rat pulmonary arterial smooth muscle cells. *J Appl Physiol* **102**, 390–398.
41 Pandey KN (1996) Vascular action: natriuretic peptide receptor. In Contemporary Endocrinology: Endocrinology of the Vasculature (Sowers JR, ed), pp. 255–267. Humana Press, Totowa, NJ.
42 Pandey KN (1992) Kinetic analysis of internalization, recycling and redistribution of atrial natriuretic factor–receptor complex in cultured vascular smooth-muscle cells. Ligand-dependent receptor down-regulation. *Biochem J* **288**, 55–61.
43 Pandey KN, Nguyen HT, Li M & Boyle JW (2000) Natriuretic peptide receptor-A negatively regulates mitogen-activated protein kinase and proliferation of mesangial cells: role of cGMP-dependent protein kinase. *Biochem Biophys Res Commun* **271**, 374–379.
44 Hu F, Wang C, Du J, Sun W, Yan J, Mi D, Zhang J, Qiao Y, Zhu T & Yang S (2010) δEF1 promotes breast cancer cell proliferation through down-regulating p21 expression. *Biochim Biophys Acta* **1802**, 301–312.
45 Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, Berx G, Cano A, Beug H & Foisner R (2005) δEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* **24**, 2375–2385.

46 Shirakihara T, Saitoh M & Miyazono K (2007) Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. *Mol Biol Cell* **18**, 3533–3544.
47 Postigo AA (2003) Opposing functions of ZEB proteins in the regulation of the TGFβ/BMP signaling pathway. *EMBO J* **22**, 2443–2452.
48 Liu Y, El-Naggar S, Darling DS, Higashi Y & Dean DC (2008) Zeb1 links epithelial–mesenchymal transition and cellular senescence. *Development* **135**, 579–588.
49 Perrot CY, Gilbert C, Marsaud V, Postigo A, Javelaud D & Mauviel A (2013) GLI2 cooperates with ZEB1 for transcriptional repression of CDH1 expression in human melanoma cells. *Pigment Cell Melanoma Res* **26**, 861–873.
50 Naber HP, Drabsch Y, Snaar-Jagalska BE, ten Dijke P & van Laar T (2013) Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion. *Biochem Biophys Res Commun* **435**, 58–63.
51 Dhasarathy A, Phadke D, Mav D, Shah RR & Wade PA (2011) The transcription factors Snail and Slug activate the transforming growth factor-β signaling pathway in breast cancer. *PLoS ONE* **6**, e26514.
52 Takagi T, Moribe H, Kondoh H & Higashi Y (1998) δEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. *Development* **125**, 21–31.
53 Yamamoto H, Mukaisho K, Sugihara H, Hattori T & Asano S (2011) Down-regulation of FXYD3 is induced by transforming growth factor-β signaling via ZEB1/δEF1 in human mammary epithelial cells. *Biol Pharm Bull* **34**, 324–329.
54 Gong K, Xing D, Li P, Hilgers RH, Hage FG, Oparil S & Chen YF (2011) cGMP inhibits TGF-β signaling by sequestering Smad3 with cytosolic β2-tubulin in pulmonary artery smooth muscle cells. *Mol Endocrinol* **25**, 1794–1803.
55 Li P, Wang D, Lucas J, Oparil S, Xing D, Cao X, Novak L, Renfrow MB & Chen YF (2008) Atrial natriuretic peptide inhibits transforming growth factor β-induced Smad signaling and myofibroblast transformation in mouse cardiac fibroblasts. *Circ Res* **102**, 185–192.
56 Watson CJ, Phelan D, Xu M, Collier P, Neary R, Smolenski A, Ledwidge M, McDonald K & Baugh J (2012) Mechanical stretch up-regulates the B-type natriuretic peptide system in human cardiac fibroblasts: a possible defense against transforming growth factor-β mediated fibrosis. *Fibrogenesis Tissue Repair* **5**, 9.
57 Nishikimi T, Inaba-Iemura C, Ishimura K, Tadokoro K, Koshikawa S, Ishikawa K, Akimoto K, Hattori Y, Kasai K, Minamino N *et al.* (2009) Natriuretic peptide/natriuretic peptide receptor-A (NPR-A) system has inhibitory effects in renal fibrosis in mice. *Regul Pept* **154**, 44–53.
58 Das S, Periyasamy R & Pandey KN (2012) Activation of IKK/NF-κB provokes renal inflammatory responses in guanylyl cyclase/natriuretic peptide receptor-A gene-knockout mice. *Physiol Genomics* **44**, 430–442.
59 Rosenkranz S (2004) TGF-β1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res* **63**, 423–432.
60 Loeffler I & Wolf G (2014) Transforming growth factor-β and the progression of renal disease. *Nephrol Dial Transplant* **29** (Suppl 1), i37–i45.
61 Xie WB, Li Z, Miano JM, Long X & Chen SY (2011) Smad3-mediated myocardin silencing: a novel mechanism governing the initiation of smooth muscle differentiation. *J Biol Chem* **286**, 15050–15057.
62 Dignam JD (1990) Preparation of extracts from higher eukaryotes. *Methods Enzymol* **182**, 194–203.
63 Ponnoth DS, Sanjani MS, Ledent C, Roush K, Krahn T & Mustafa SJ (2009) Absence of adenosine-mediated aortic relaxation in A_{2A} adenosine receptor knockout mice. *Am J Physiol Heart Circ Physiol* **297**, H1655–H1660.
64 Lindsey SH, Carver KA, Prossnitz ER & Chappell MC (2011) Vasodilation in response to the GPR30 agonist G-1 is not different from estradiol in the mRen2.Lewis female rat. *J Cardiovasc Pharmacol* **57**, 598–603.
65 Mani I, Garg R, Tripathi S & Pandey KN (2015) Subcellular trafficking of guanylyl cyclase/natriuretic peptide receptor-A with concurrent generation of intracellular cGMP. *Biosci Rep* **35**, e00260.
66 Pandey KN, Nguyen HT, Sharma GD, Shi SJ & Kriegel AM (2002) Ligand-regulated internalization, trafficking, and down-regulation of guanylyl cyclase/atrial natriuretic peptide receptor-A in human embryonic kidney 293 cells. *J Biol Chem* **277**, 4618–4627.