HHS Public Access

Author manuscript

FEBS J. Author manuscript; available in PMC 2016 June 03.

Published in final edited form as:

FEBS J. 2016 May ; 283(9): 1767–1781. doi:10.1111/febs.13701.

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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Abstract

The objective of this study was to determine the role of transforming growth factor-beta 1 (TGF-β1) in transcriptional regulation and function of guanylyl cyclase-A/natriuretic peptide receptor-A (GC-A/NPRA) gene (*Npr1*) and whether a cross-talk exists between these two hormonal systems in target cells. After treatments of primary cultured rat thoracic aortic vascular smooth muscle cells (RTASMCs) and mouse mesangial cells (MMCs) with TGF-β1, the *Npr1* promoter construct embodying delta-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 *Npr1* promoter by 62% and decreased the ANP-mediated relaxation of mouse denuded aortic rings *ex vivo*. Treatment of cells with TGF-β1, stimulated the protein levels of δEF1 by 2.4- to 2.8-fold and also significantly enhanced the phosphorylation of Smad 2/3; however, markedly reduced *Npr1* mRNA and receptor protein levels. Overexpression of δEF1 showed a reduction in *Npr1* promoter activity by 75% while the deletion or site-directed mutagenesis of δEF1 sites in *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 1 alpha 2 in RTASMCs, which were markedly attenuated by ANP in NPRA overexpressing cells. Together, the present results suggest that an antagonistic cascade exists between TGF-β1/Smad/δEF1 pathways and *Npr1* expression and receptor signaling relevant to renal and vascular remodeling, which might be critical in the regulation of blood pressure and cardiovascular homeostasis.

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Wrote the paper: Anagha Sen, Prerna Kumar, and Kailash N. Pandey

Disclosures No conflicts of interest, financial or otherwise, are declared by the author(s).

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Keywords

Atrial natriuretic peptide; particulate guanylyl cyclase-A; chromatin immunoprecipitation; Smad; gene expression

Introduction

Cardiac hormones, atrial and brain natriuretic peptides (ANP and BNP) play critical roles in the reduction of blood pressure and cardiac disorders with relevance to renal, cardiovascular, endocrine, skeletal, and neural homeostasis [1–5]. ANP and BNP bind and activate guanylyl cyclase-A/natriuretic peptide receptor-A (GC-A/NPRA), which catalyzes the formation of intracellular second messenger cGMP [6–8]. NPRA is considered 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 upstream of the start codon (~500 base pairs, bp), 5′ flanking region of the *Npr1* (coding for GC-A/NPRA) promoter contains the binding sites for several known transcription factors and seems to play a critical role in the functional regulation and expression of this gene [9–12]. The 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-beta1 (TGF-β1) belongs to a group of peptides known as TGF-β family, which regulate different cellular processes such as proliferation, differentiation, apoptosis, and specification of cell type during embryonic development [17, 18]. Hypertension, nephropathy, and cardiac hypertrophy are known to be associated with significantly elevated levels of TGF-β1 and collagen in *Npr1* gene-knockout mice [19–25]. Earlier findings indicated that TGF-β1 decreased *Npr1* mRNA levels in cultured aortic smooth muscle cells (SMCs); however, the underlying molecular mechanisms were not determined [26]. Previously, it has been shown that BNP inhibited the TGF-β1-induced proliferation in cardiac fibroblasts as well as opposed nearly 88% of the TGF-β1-stimulated gene expression events [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 feedback of cell cycle, fibrosis, inflammation, myofibroblast transformation, and extracellular matrix production have been shown to be upregulated by TGF-β1 while the same genes seems to be downregulated by BNP [32].

Interestingly, E2 box repressor, delta-crystallin enhancer binding factor 1 (δEF1) was identified as a nuclear protein that binds to lens specific enhancer and has been suggested to be regulated by TGF-β1 in vascular SMCs [33–36]. Several studies have shown that δEF1 acts as a mediator of TGF-β1 signaling in the 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 the functional aspects of ANP/NPRA signaling [41, 42]. Glomerular

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mesangial cells are the frequent target of diverse pathophysiological processes, particularly, in hypertension and immune inflammatory diseases. Both RTASMCs and MMCs express functional GC-A/NPRA, which provide a 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 *Npr1* promoter.

Results

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

A schematic map of deletion constructs of *Npr1* promoter region −356 to +359 containing δEF1 binding sites is shown in Figure 2B. Treatment with TGF-β1 significantly decreased luciferase activity of *Npr1* promoter constructs −356/+55, −356/+96, and −356/+359 having δEF1 binding sites as 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 on *Npr1* basal promoter activity, RTASMCs were cotransfected with *Npr1* promoter deletion constructs and δEF1 expression plasmids. Overexpression of δEF1 significantly reduced luciferase activity of *Npr1* promoter constructs −356/+55, −356/+96, and −356/+359 having δEF1 binding sites (Fig. 2D). Schematic map of *Npr1* promoter constructs having wild-type or mutant δEF1A or δEF1B binding sites individually or together is presented in Fig. 2E. Overexpression of δEF1 in RTASMCs, transfected with the *Npr1* promoter constructs −356/+55 and −356/+359 having wild-type δEF1 sites, repressed promoter activity by 60% and 80%, respectively (Fig. 2F). Mutation of δEF1 site A in the construct −356/+55 markedly augmented luciferase activity. To examine the effect of δEF1 site B and both sites A and B, we utilized the construct −356/+359 as mutation of site A in the construct −356/+55 exhibited reversal of δEF1-mediated repression on *Npr1* promoter activity. Mutation of site B in the construct −356/+359 increased the promoter activity by 3.2-fold compared with the wild-type control construct. However, mutation of both δEF1 sites in the construct −356/+359 showed 6.6-fold enhanced promoter activity compared with wild-type construct in transfected RTASMCs. On the other hand, cotransfection of δEF1 expression plasmid with *Npr1* promoter constructs having mutant δEF1 binding sites did not show any effect on the promoter activity, further confirming the role of δEF1 binding sites in mediating its repressive effects on *Npr1* gene expression. On the other hand, knockdown of δEF1 by siRNA abolished the δEF1-mediated repression of

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Npr1 promoter activity (Fig. 3A). There was 68% reduction in luciferase activity of the *Npr1* promoter construct −356/+359 in MMCs overexpressing δEF1; whereas knockdown of δEF1 by siRNA significantly increased the *Npr1* promoter activity (Fig. 3B). Approximately, 80% reduction in endogenous δEF1 protein expression occurred in δEF1 siRNA-transfected RTASMCs compared with untransfected cells (Fig. 3C). Overexpression of δEF1 protein was observed in RTASMCs and MMCs, transfected with δEF1 expression plasmid (Fig. 3C and D).

In order to confirm whether endogenous δEF1 protein binds to its consensus sequence present in the *Npr1* promoter, EMSA and ChIP assay were performed. In gel shift assay, the incubation of untreated RTASMCs nuclear extract with δEF1 site A and δEF1 site B oligonucleotides showed the formation of specific nucleoprotein complexes (Fig. 4A and B, *lane 2*) and the binding was markedly enhanced with TGF-β1-treated nuclear extract (Fig. 4A and B, *lane 3*). DNA-protein binding was inhibited in the presence of 100-fold excess molar concentrations of competitor DNA (Fig. 4A and B, *lane 4*). The specificity of the protein-DNA complex was confirmed by δEF1 antibody supershift assay (Fig. 4C and 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 δEF1 and phosphorylated mothers against decapentaplegic homolog 2/3 (pSmad 2/3) occupancy at both the sites on the *Npr1* promoter compared with untreated cells (Fig. 5B). The *in vivo* binding of δEF1 to site 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 performed Western blot analysis in the cell lysates. The Western blot analysis demonstrated that TGF-β1 increased the expression of endogenous δEF1 by almost 2.4- to 2.8-fold in RTASMCs and MMCs, respectively, in a dose-dependent manner, compared with untreated cells (Fig. 5D and E). Treatment of cells with TGF-β1 significantly increased phosphorylation of Smad 2/3 proteins in RTASMCs and MMCs compared with their untreated controls (Fig. 5F and G). However, there was no change in the expression level of Smad 2/3 protein with TGF-β1 treatment.

Since ANP/NPRA and TGF-β1 signaling are known to antagonize each other we further tested this response in our experimental conditions. Western blot analysis of TGF-β1-treated RTASMCs showed significant increase in α-smooth muscle actin (α-SMA) and collagen type 1 alpha 2 (COL1A2) protein expression which was markedly attenuated by ANP treatment in NPRA overexpressing cells pretreated with TGF-β1 (Figure 6A). As shown by Western blot analysis, treatment of cells with ANP significantly attenuated TGF-β1-induced nuclear translocation of phosphorylated Smad 2/3 (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 65% reduction in luciferase activity of the *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 showed 62% reduction in *Npr1* mRNA levels (Fig. 7B). Incubation of denuded aortic rings with TGF-β1 exhibited 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 contracted with prostaglandin F2α (PGF2α); however,

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pretreatment 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 the transcriptional repression of *Npr1* gene is modulated by TGF-β1-Smad-δEF1 pathway. Our results demonstrate that TGF-β1 inhibited the *Npr1* promoter activity by 80–90% in a time-and dose-dependent manner and significantly reduced the *Npr1* mRNA expression and protein 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, namely δEF1 site A (−303 to −293) and δEF1 site B (+127 to +139) relative to TSS [10, 13]. *Npr1* promoter deletional analysis exhibited that repression of *Npr1* gene transcription due to δEF1, was eliminated in the constructs, which did not have δEF1 binding sites. Overexpression of δEF1 demonstrated significant repression of *Npr1* promoter activity in the constructs having δEF1 binding sites. On the other hand, overexpression of δEF1 did not produce any change in the activity of the constructs deficient in δEF1 binding sites, which suggests that the absence of δEF1 derepresses the *Npr1* promoter activity. Site-directed mutagenesis of δEF1 binding sites and endogenous δEF1 gene silencing by siRNA transfection confirmed that the repression of *Npr1* promoter was due to δEF1. Previously, TGF-β1-mediated decrease in *Npr1* mRNA levels in cultured SMCs has been shown but the underlying molecular mechanisms were not known [26]. Our data provides the evidence of 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 the down-regulation of p21 expression [44]. Overexpression of δEF1 family of proteins has been shown to repress the E-cadherin promoter activity [45, 46]. Ectopic expression of δEF1 represses estrogen receptor-α transcription by binding to E2-box on its promoter [38]. Our *in vivo* ChIP binding assay data showed that δEF1 formed the nucleoprotein complexes with the endogenous *Npr1* gene promoter, which were absent in the negative controls and provided the 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 repress E-cadherin by δEF1-mediated assembly of Smads 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 Smad 2/3 proteins confirming their involvement in TGF-β1-δEF1 signaling cascade. Moreover, the results from the present study showed that TGF-β1 repressed *Npr1* gene transcription and expression by inducing direct binding of δEF1 and pSmad 2/3 to *Npr1* promoter (Fig. 8). Recent studies have shown that TGF-β1 transcriptionally regulates the expression of many transacting factors, including the zinc-finger factors Snail and Slug and the two-handed zinc-finger factors of δEF1 family proteins δEF1 and SIP1, which are involved in the induction of epithelial to mesenchymal transition (EMT) particularly through the transcriptional repression of E-cadherin and epithelial splicing regulatory proteins [37, 49–51]. Our results showed that TGF-β1 treatment induced δEF1 protein levels as compared to untreated controls. Targeted deletion

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of δEF1 in mice has skeletal defects, which are similar to those in mice with gene knock-out of TGF-β1 family of proteins [47, 52]. Downregulation of FXYD3 a member of the FXYD family proteins, which have a single transmembrane segment, and share a signature sequence of four amino acids "FXYD" (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, the treatments with TGF-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 pSmad 2/3 and expression of COL1A2 and α-SMA in these target 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 Smad 2/3 and extracellular matrix expression in pulmonary aortic SMCs [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 α-SMA and collagen 1 expression [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 by 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 *Npr1* promoter, henceforth, represses the *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 and transacting factor δEF1 and ANP/NPRA/cGMP signaling relevant to renal and vascular remodeling in the cardiovascular disease states.

In conclusion, the present results demonstrate that TGF-β1 mediates its effect via inducing the Smad 2/3 protein phosphorylation, δEF1 expression, and their binding to *Npr1* promoter. The results in primary cultured RTASMCs, MMCs, and denuded aortic rings 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 by the repressive effects on TGF-β1-induced expression of COL1A2 and α-SMA in RTASMCs and aortic rings. Identification of TGF-β1-Smad-δEF1signaling as a suppressor of functional expression of NPRA should provide new molecular targets for developing the therapeutic strategies for the treatment of hypertension and related cardiovascular disorders.

Materials and Methods

Plasmids and Promoter Constructs

The *Npr1* promoter-luciferase reporter constructs were generated by cloning different lengths of *Npr1* promoter in pGL3 basic vector as previously described [13, 15]. Primers

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used in the generation of constructs −284/+55, −98/+55, −356/+96, and −356/+359 are provided in Table 1. The expression plasmid δEF1 was obtained from Dr. 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 calf serum (FCS) and mouse mesangial cells (MMCs) were grown in DMEM enriched with 10% FCS 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 reagent (Thermo Fisher scientific, Grand Island, NY, USA) with 1 μg of promoter reporter construct and 0.3 μg of pRL-TK plasmid, which was used as internal transfection control and luciferase activity was measured as previously described [12, 13]. For calculation of luciferase activity of various *Npr1* promoter constructs pGL3-basic plasmid was taken as control and the results are expressed as relative luciferase activity compared with the pGL3-basic plasmid. In co-transfection experiments, 0.5 μg of δEF1 expression plasmid was used and total DNA content was equalized by inclusion of empty vector. In ectopic overexpression 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, 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 24 h after TGF-β1-treatment or δEF1 transfections. Cells were washed with phosphate-buffered saline (PBS) and lysed in buffer containing: 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 0.05% 2-mercaptoethanol, 1 % Triton X-100, 1 mM sodium vanadate, 10 mm sodium fluoride, 0.2 mm phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cell extract was passed 15–20 times through a 1 cc syringe with a 21-guage needle and centrifuged at 14,000 rpm for 10 min. The clear cell lysate was collected and stored at −80°C until used. 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 PMSF) and incubated on ice for 10 min and centrifuged as above. The pellet was again resuspended in three volumes of buffer A to which Nonidet P-40 (0.05%, v/v) was added. The suspension was homogenized with 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% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM PMSF) 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. Centrifugation steps described above were carried out at 4°C. Aliquots of the supernatant were stored at −80°C. Protein concentration was estimated by Bradford method using Bio-Rad (Hercules, CA, USA) protein assay kit.

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Ex vivo mouse aortic ring assays

C57BL/6 male mice were euthanized by deep anesthesia with isoflurane inhalation. Aortic segments were prepared by using the previously described method with a minor modification [63]. Immediately, thoracotomy was performed, thoracic aorta was removed and placed in cold Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich Co., St. Luis, Missouri, USA; D8537) 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. Later, 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 in the remaining segment of the aorta. Denudation of endothelium was achieved by scraping the lumen of the aorta with a 26 gauge monofilament surgical steel wire (Ethicon, Somerville, NJ, USA). Subsequently, blood and denuded endothelial cells were removed by gently flushing DPBS through the lumen of the aorta. Finally, aorta was cut into 3 to 4 mm rings for experiments. After 4–5 h of incubation in DMEM enriched with 10% FCS and 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, and supernatant were stored at −80°C to be later used in Western blot experiments. For RNA extraction RNeasy mini kit was used to crush the aortae with a 1.5-ml tube pestle and followed the protocol provided by the manufacturers (Qiagen, Valencia, CA, USA). Denuded aortic rings were transfected using aortic smooth muscle cells (ASMC) Transfection Reagent (Altogen Biosystems, Las Vegas, NV, USA) with 3 µg of promoter reporter construct. After 24 h of 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

Aorta was excised as described above and cut into 2 mm rings. Some rings were denuded and some were left endothelium-intact. Rings were placed into a 24-well culture dish in DMEM containing vehicle or 2.5ng/ml TGFß1. After 24 h incubation, rings were mounted onto a Danish Myotechnology (DMT) Multi-Chamber Myograph System (Model 620M) and set to an initial tension of 10 mN as previously described [64]. After an initial incubation period followed by contraction to 80 mM KCl and washout, endothelial function was tested by contracting vessels to 5 µM PGF2α followed by 1 µM acetylcholine. Vessels with more than 50% relaxation were considered endothelium-intact. After washing, vessels were then contracted again with 5 µM PGF2α and exposed to increasing concentrations of ANP (10^{-10} to 10^{-7} M). Data is expressed as percent relaxation from PGF2α contraction.

Real-time Reverse Transcription-Polymerase Chain Reaction Assay

Total RNA isolation kit from Promega (Madison, WI, USA) was used to isolate total RNA and first-strand cDNA was reverse transcribed using Smartscribe reverse transcriptase from Clontech Laboratories, Inc. (Mountain View, CA, USA). Cells were treated with increasing concentrations of TGF-β1 for 24 h, lysed, and total RNA was extracted. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Mx3000P real-time PCR system and data were analyzed with MxPro software (Agilent Technologies, Santa

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Clara, CA, USA). Primers for amplification of *Npr1* and β-actin were purchased from Qiagen. PCR amplifications (in triplicates) were carried out in a 25 μl reaction volume using RT2 real-time™ SYBR Green/ROX PCR Master Mix from Roche (New York, NY). 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 1 cycle at 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 Ct value using MxPro QPCR software. 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% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred onto a polyvinylidene fluoride membrane, and blocked with 1x Tris-buffered saline-Tween 20 (TBST) containing 5% fat-free milk for 1 h at room temperature and then incubated overnight at 4°C in TBST 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 and visualized using an Alpha Innotech detection system from Proteinsimple (Santa Clara, CA, USA). The intensity of protein bands was quantified by Alphaview software. The primary antibodies; δEF1 (catalog # sc-10573), pSmad2/3 (Ser423/425; catalog # sc-11769), H1 (catalog # sc-10806), α-SMA (catalog # A-7607), COL1A2 (catalog # sc-8788), and β-actin (catalog # A5316) 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 phosphate-buffered saline (PBS), and scraped into 0.5 N HCl. Cell suspension was subjected to five cycles of freeze and thaw, then centrifuged at 10,000 rpm for 10 min. The supernatant thus collected was used for the cGMP assay using a direct enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocol.

Small Inhibitory RNA Transfection

Cells were cultured to 80%–90% confluence and transfected with δEF1 small interfering RNA (siRNA; a pool of 3 target-specific 20- to 25-nucleotide sequence siRNAs) purchased from Santa Cruz Biotechnology using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). A nontargeting 20-25-nucleotide sequence siRNA was used as a negative control. Twenty-four after transfection, cells were lysed to measure firefly and Renilla luciferase activity.

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Electrophoretic mobility shift assay

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

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express Enzymatic Kit (Active Motif, Carlsbad, CA, USA) following manufacturer's protocol. Briefly, cells were treated with 1% formaldehyde for 10 min to crosslink protein-DNA complexes and the reaction was quenched with 0.1 mol/L of glycine. Cells were scraped, resuspended in 1 ml of lysis buffer on ice, and homogenized with a Dounce homogenizer and centrifuged. The chromatin extracted from the cells was enzymatically sheared by incubating 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 minutes 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. Primers used for PCR amplification of δEF1 site A and site B are listed in Table 1.

In Vitro Site-Directed Mutagenesis

Npr1 promoter constructs with mutated δEF1 site was custom-synthesized from Eurofins Genomics (Huntsville, Alabama). The mutant construct was transfected in 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 the PRISM software (GraphPad software, San Diego, CA, USA). A value of <0.05 was considered significant. Results are expressed as mean ± S.E. of 7–8 independent experiments done in the triplicates.

Acknowledgements

Authors wish to thank Ms. Gevoni Bolden and Ms. Vicki Nguyen for technical assistance and Mrs. Kamala Pandey for assistance in the preparation of this manuscript. We sincerely thank Dr. Michel M. Sanders (University of Minnesota, Minneapolis, MN) for the kind gift of expression vectors. This work was supported by NIH grants R01HL057531 and R01HL062147.

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Abbreviations

ANP and BNP	atrial and brain natriuretic peptides
α-SMA	α-smooth muscle actin
δEF1	delta-crystallin enhancer binding factor 1
COL1A2	collagen type 1 alpha 2
ChIP	chromatin immunoprecipitation
DMT	Danish Myo Technology
EMSA	Electrophoretic mobility shift assay
ELISA	enzyme-linked immunosorbent assay
GC-A/NPRA	guanylyl cyclase-A/natriuretic peptide receptor-A
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MMCs	mouse mesangial cells
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
RTASMCs	rat thoracic aortic vascular smooth muscle cells
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
pSmad 2/3	phosphorylated mothers against decapentaplegic homolog 2/3
SMCs	smooth muscle cells
TBST	tris-buffered saline-Tween 20
TGF-β1	transforming growth factor-beta 1.

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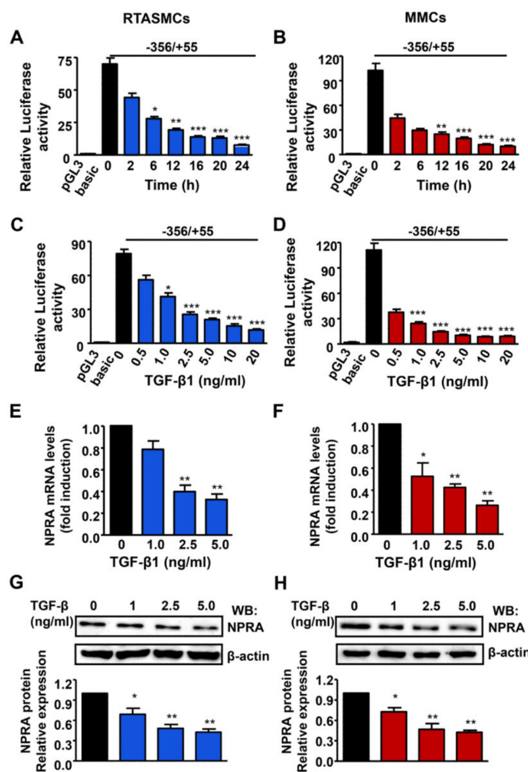

Figure 1.

Effect of TGF-β1 on *Npr1* gene transcription and expression in a time- and dose-dependent manner. (A) Luciferase activity of *Npr1* proximal promoter construct −356/+55 in RTASMCs and (B) MMCs treated with TGF-β1 (5 ng/ml) in a time-dependent manner. (C) Effect of increasing concentrations of TGF-β1 on *Npr1* promoter activity in transfected RTASMCs and (D) MMCs as measured by luciferase assay. (E) Dose-dependent effect of TGF-β1 on *Npr1* mRNA levels in RTASMCs and (F) MMCs as determined by real-time RT-PCR with β-actin as an internal control and (G) NPRA protein expression with densitometry analysis and β-actin expression is shown as loading controls in treated RTASMCs and (H) MMCs. Bar represents the mean ± SE of 8 independent experiments in triplicates. WB, Western blot; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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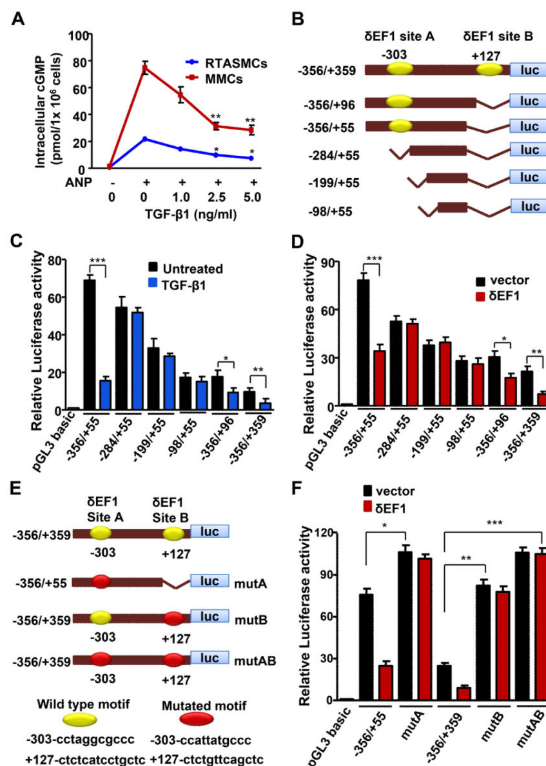

Figure 2.

Luciferase activity of *Npr1* promoter containing deletion and mutation of δEF1 binding sites in RTASMCs. (A) Intracellular accumulation of cGMP in TGF-β1-treated RTASMCs and MMCs and induced with ANP. (B) Schematic map of the *Npr1* promoter deletion constructs having δEF1 binding sites A and B deleted either alone or in combination. (C) Luciferase activity of *Npr1* promoter deletion constructs transiently transfected in cells and treated with TGF-β1 (2.5 ng/ml) for another 24 h. (D) Luciferase activity of *Npr1* promoter deletion constructs in RTASMCs cotransfected with δEF1 expression plasmid or an empty vector. (E) Diagrammatic representation of *Npr1* promoter constructs harboring wild-type or mutant δEF1 binding sites and (F) luciferase activity in RTASMCs cotransfected with δEF1 expression plasmid or an empty vector. Bar represents the mean ± SE of 6 independent experiments in triplicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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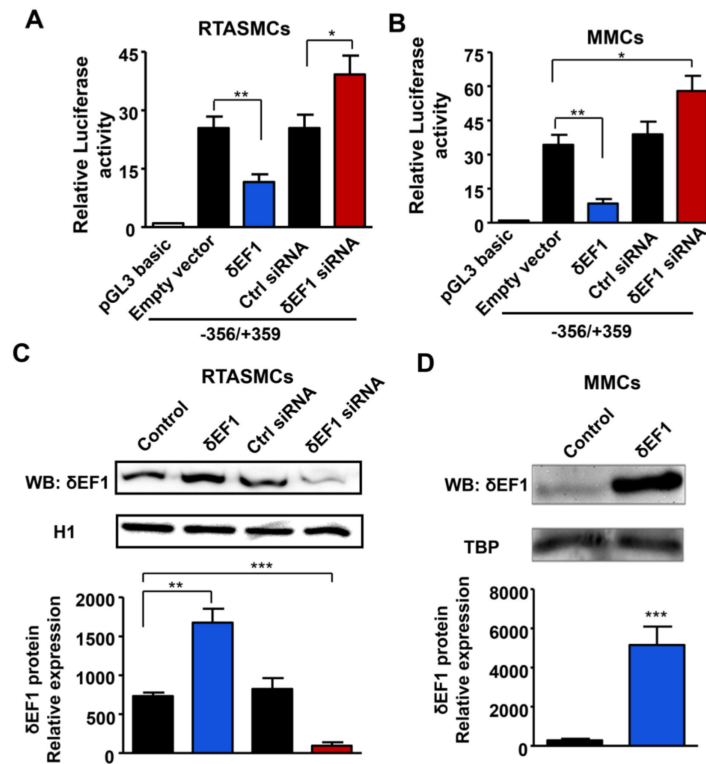

Figure 3.

Effect of overexpression and knockdown of δEF1 on *Npr1* gene transcription in RTASMCs and MMCs. (A) Luciferase activity of *Npr1* promoter construct −356/+359 cotransfected with δEF1 expression plasmid, empty vector, δEF1 siRNA, and control siRNA in RTASMCs and (B) MMCs as measured by luciferase assay. (C) Western blot and densitometry analysis of δEF1 protein in δEF1expression plasmid or δEF1 siRNA transfected RTASMCs and H1 expression is shown as loading controls. (D) Western blot analysis of over expression of δEF1 protein in MMCs and TBP expression is shown as loading controls. Data shown mean ± SE of 6 independent experiments in triplicates. WB, Western blot; *, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

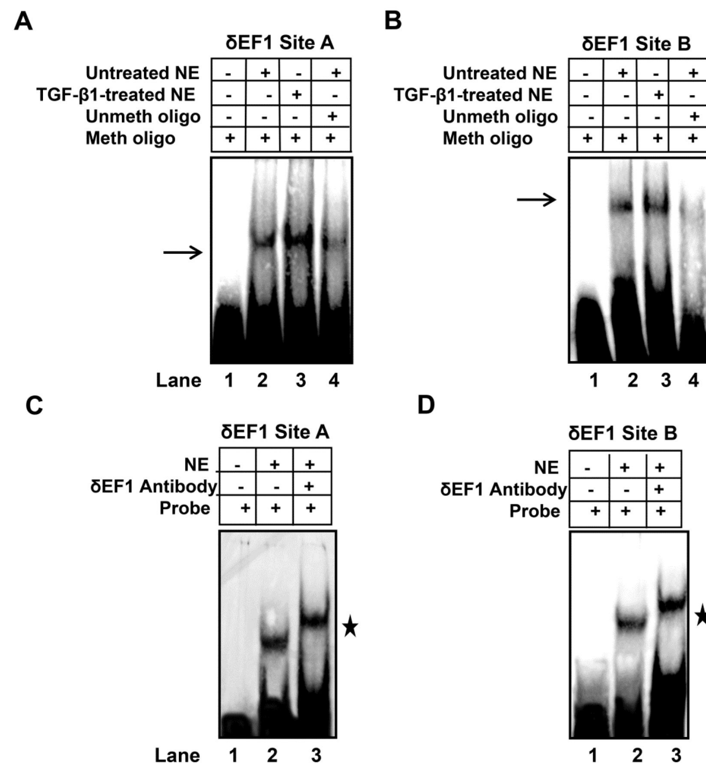

Figure 4.

Electrophoretic mobility shift assay showing in vitro binding of δEF1 to the consensus binding sites in the *Npr1* promoter. (A) RTASMCs nuclear extract was incubated with δEF1 Site A and (B) δEF1 Site B oligonucleotides. Arrows indicate a specific DNA-protein binding complex in untreated nuclear extract (lane 2) and TGF-β1-treated nuclear extract (lane 3); the binding was inhibited in presence of 100-fold molar excess of unlabeled competitor DNA in lane 4. (C) Incubation of nuclear extract with δEF1 Site A and (D) δEF1 Site B oligonucleotides in presence of anti-δEF1 antibody shows supershift of the DNA-δEF1 protein complex in lane 3. Asterisk indicates supershifted complex. Data shown mean ± SE of 4 independent experiments. NE, nuclear extract; meth, methylated.

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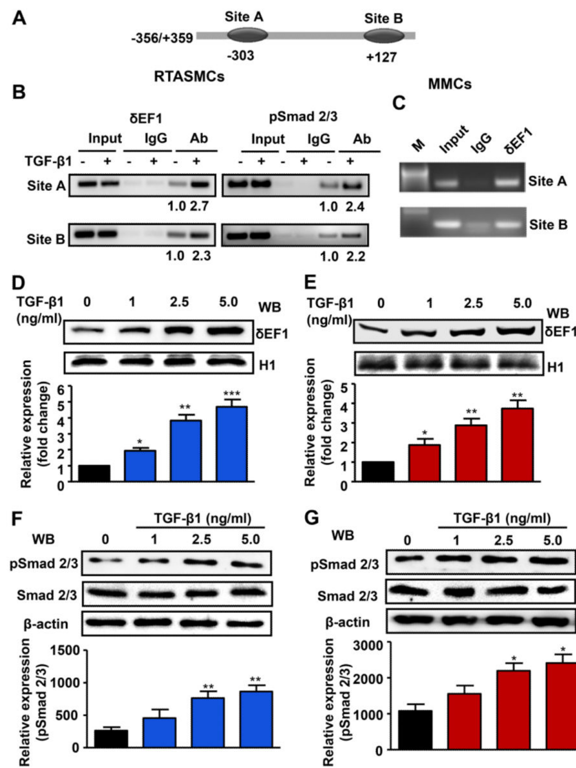

Figure 5.

TGF-β1-dependent δEF1 protein expression and its binding 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 pSmad 2/3 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 by Alpha Innotech analysis software. Representative gels from three independent experiments are shown. (D) Western blot and densitometry analyses of δEF1 protein expression in RTASMCs and (E) MMCs treated with increasing concentrations of TGF-β1 and H1 expression is shown as loading control. (F) Western blot and densitometry analysis of phosphorylated and unphosphorylated Smad 2/3 protein expression in TGF-β1-induced RTASMCs and (G) MMCs and β-actin expression is shown as loading controls. Bar represents the mean ± SE of 6 independent experiments in triplicates. WB, Western blot; *, $p< 0.05$; **, $p < 0.01$.

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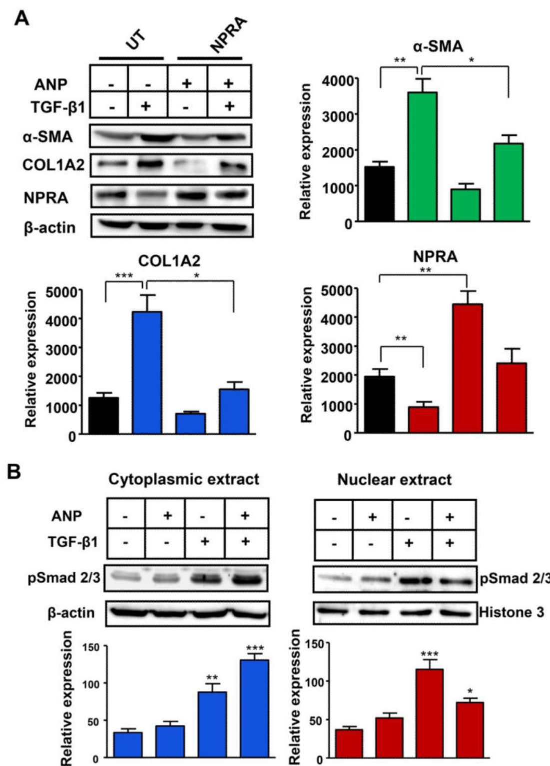

Figure 6.

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 NPRA expression plasmid transfected cells treated with and without ANP and TGF-β1. (B) Western blot and densitometry analysis of nuclear translocation of phosphorylated Smad 2/3 (Ser 423/425) in cytoplasmic and nuclear extract of RTASMCs treated with and without ANP and TGF-β1. β-actin and H1 expression is shown as loading control. Bar represents the mean ± SE of 6 independent experiments in triplicates.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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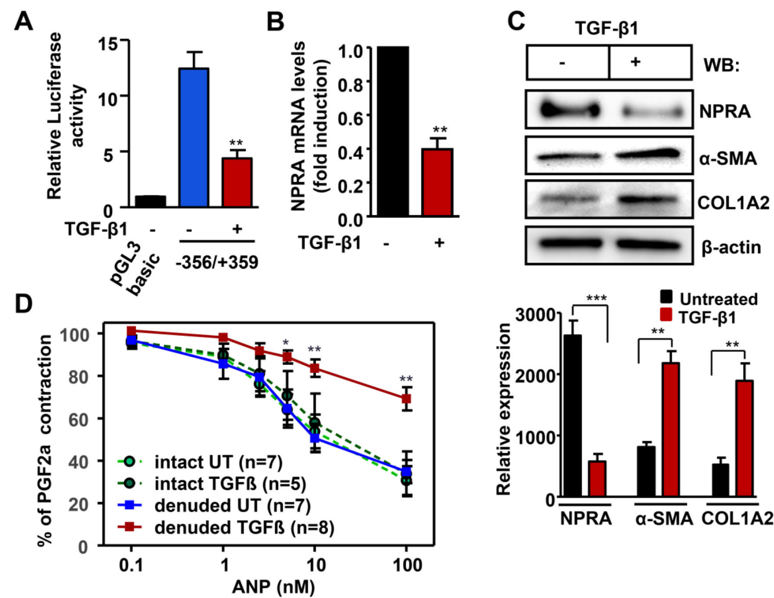

Figure 7.

Effect of TGF-β1 treatment on *Npr1* gene transcription and expression and ANP-induced vasorelaxation in aortic rings. (A) Luciferase activity of denuded-aortic rings transfected with *Npr1* proximal promoter construct −356/+55 and treated with TGF-β1. (B) *Npr1* mRNA levels and (C) Western blot analysis of NPRA, α-SMA, COL1A2 expression in TGF-β1-induced aortic rings and β-actin expression as loading controls. (D) Vasorelaxation of aortic rings in the presence of ANP with or without TGF-β1 treatments. Bars represent the mean ± SE of 5–8 independent experiments in triplicates. WB, Western blot; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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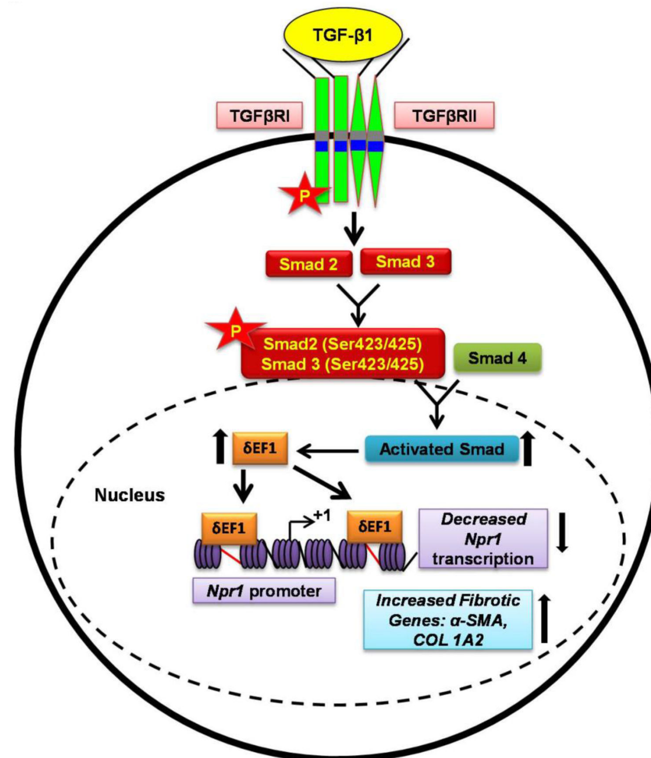

Figure 8.

Schematic of 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 *Npr1* gene promoter in response to TGF-β1 and represses its activity. There is a possibility of its interaction with Smads in the nucleus. The bold upward arrows indicate increase in δEF1 protein expression; whereas the bold downward arrows indicate decrease in *Npr1* gene transcription and expression.

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Table 1

List of primers used in cloning, electrophoretic mobility shift assay, and chromatin immunoprecipitation (ChIP) assay.

Experiment	Primer (Sequence 5' to 3')	Orientation
Cloning		
Construct −284/+55	tacggaacgcgtcgggtgctgccaagggagggaaacc	Forward
	tacggaagatctgcgggtgcgccagcgaggaaagg	Reverse
Construct −98/+55	tacggaacgcgtctggctcgccttgtggtcccgtcc	Forward
	tacggaagatctgcgggtgcgccagcgaggaaagg	Reverse
Construct −356/+96	tacggaacgcgtgaggggggggcagcttcctcac	Forward
	tacggaagatctgagcgagagaac gagagggcg	Reverse
Construct 356/+359	tacggaacgcgtgaggggggggcagcttcctcac	Forward
	tacggaagatctcagcgagcgcagcgacggagc	Reverse
EMSA		
δ*EF1 site A −303*	cccccgcggcctaggcgccc	Forward
	gggcgcctaggccgcggggg	Reverse
δ *EF1 site B +127*	tgcgctcgctctcacctgctctaaagcac	Forward
	gtgctttagagcaggtgagagcgagcgca	Reverse
ChIP		
δ*EF1 site A at −303*	ttcctcacacccttcctcagtcct	Forward
	cgccagttattgctgaccctctt	Reverse
δ *EF1 site B +127*	ctcttcttagatcgccctctcgtt	Forward
	agggtgcttagagcaggtgaga	Reverse