TGF-β1 Downregulates AT1 Receptor Expression via PKC-δ-Mediated Sp1 Dissociation From KLF4 and Smad-Mediated PPAR-γ Association With KLF4

Xin-hua Zhang, Bin Zheng, Chun Gu, Jian-ran Fu, Jin-kun Wen

Objective—Cardiovascular effects of angiotensin II are primarily mediated via the angiotensin II type 1 receptor (AT1R). Krüppel-like factor 4 (KLF4), a transcription factor that binds to the transforming growth factor (TGF)-β control element (TCE), regulates a variety of receptor expression in vascular smooth muscle cells. In the present study, we investigated the mechanisms of TGF-β-mediated KLF4 regulation of AT1R expression.

Methods and Results—Coimmunoprecipitation, chromatin immunoprecipitation, and luciferase assays were performed, with the results suggesting that Sp1 forms a complex with KLF4 bound to the TCE of the AT1R promoter and cooperatively activates AT1R transcription in vascular smooth muscle cells under basal conditions. On activation of TGF-β1 signaling, Sp1 is dissociated from the KLF4-Sp1 complex through PKC-δ-mediated KLF4 phosphorylation at Thr401, downregulating AT1R expression. Simultaneously, TGF-β1 facilitates KLF4-PPAR-γ complex formation and its binding to the TCE of the AT1R promoter through Smad-mediated KLF4 phosphorylation at Ser470, subsequently leading to inhibition of AT1R transcription.

Conclusion—KLF4 functions as a protein platform that is able to bind to the TCE of the AT1R promoter. On activation of TGF-β signaling, KLF4 mediates Sp1 dissociation from, and PPAR-γ association with, the AT1R promoter, leading to downregulation of AT1R expression in VSMCs. (Arterioscler Thromb Vasc Biol. 2012;32:1015-1023.)

Key Words: Vascular biology • KLF4 • PPAR-γ • Sp1 • vascular smooth muscle cells

Genes that control cell proliferation and differentiation by transforming growth factor (TGF)-β are regulated by Krüppel-like factor 4 (KLF4), a zinc-finger containing transcription factor, which binds to TGF-β control elements (TCE) on gene promoters.2 In turn, KLF4, a TGF-β-inducible transcription factor,3 can regulate genes involved in cell proliferation, cell cycle, survival, and differentiation.4,5 KLF4 activity can also be regulated not only by de novo synthesis but also through posttranslational modifications including acetylation,6,7 phosphorylation,8 ubiquitinoylation,8,9 and methylation,10 as well as protein–protein interactions.1,3,11 Thus, KLF4 can act as an activator or repressor of gene transcription depending on the interaction partners and the promoter contexts. Consistent with these findings, a recent study by our group indicated that all-trans retinoic acid activates and platelet-derived growth factor-BB represses smooth muscle cell differentiation marker gene SM22α expression through KLF4 binding to, or dissociating from, its various cis-elements in an acetylation/deacetylation-dependent manner in vascular smooth muscle cells (VSMCs).6 Furthermore, TGF-β1 induces KLF4 phosphorylation via Smad and p38 MAPK signaling pathways, and phosphorylated KLF4 interacts with Smad2 to cooperatively activate the TGF-β type I receptor (TβRI) promoter in VSMCs.3 Given the importance of KLF4 in the regulation of VSMC proliferation and differentiation, identification of its binding partners, effectors, and downstream targets may allow a better understanding of its various promoter contexts.

Angiotensin II (Ang II), a bioactive peptide promoting VSMC proliferation, plays a key role in the pathogenesis of cardiovascular diseases.12 Most of the known physiological and pathophysiological effects of Ang II are mediated via the Ang II type 1 receptor (AT1R).12,13 This receptor subtype is expressed in VSMCs,13 and its expression level defines the biological efficacy of Ang II. Although a variety of physiological and pathophysiological stimuli regulate AT1R expression in VSMCs,14 the exact mechanisms of transcriptional control remain poorly understood. Although there exists 4 putative KLF4-binding sequences within the −1423/−33 bp region of the rat AT1R promoter, it remains unclear whether and how KLF4 modulates AT1R gene expression.

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In this study, we aimed to investigate whether and how TGF-β-mediated KLF4 expression regulates AT1R expression in VSMCs. We determined that Sp1 forms a complex with KLF4 that is bound to the TCE of the AT1R promoter in the absence of TGF-β to maintain basal level expression of the AT1R gene in VSMCs. On TGF-β activation, AT1R expression is suppressed through 2 coordinate pathways: TGF-β1 promotes dissociation of the KLF4-Sp1 complex from the TCE of the AT1R promoter, through PKC-δ-mediated KLF4 phosphorylation at Ser470, subsequently leading to the TCE of the AT1R promoter through Smad-mediated signaling. Moreover, TGF-β1 also decreases AT1R mRNA expression and increased KLF4 mRNA expression in a time- and dose-dependent manner (Figure 1C and 1D). Similar results were obtained in rat pulmonary artery smooth muscle cells (Figure 1 in the online-only Data Supplement). Unlike in VSMCs, in human aortic smooth muscle cells, the protein and mRNA expression of both KLF4 and AT1R were reduced by TGF-β1 treatment (Figure II in the online-only Data Supplement). TGF-β1 inhibited AT1R expression in VSMCs infected with pAd empty vector. Without TGF-β signaling, KLF4 overexpression markedly increased AT1R expression; however, AT1R expression was dramatically inhibited by both KLF4 overexpression and TGF-β signaling (Figure 1E).

In contrast, when VSMCs were transfected with KLF4-specific siRNA (siKLF4) to block endogenous KLF4 expression, suppression of AT1R expression by TGF-β and KLF4 was abrogated (Figure 1F). We then constructed an AT1R promoter-luciferase reporter construct including the region spanning from −1423 to +33 of the rat AT1R promoter and performed a luciferase assay in 293A cells. Our results indicated that KLF4 overexpression markedly increased AT1R promoter activity in the absence of TGF-β activation. When cells were transfected with a constitutively active mutant of TβRI (TβRI-CA), AT1R promoter activity was significantly reduced and this inhibiting effect was increased by KLF4 overexpression (Figure 1G). In cells where KLF4 was knocked down by transfecting siKLF4, AT1R promoter activity was reduced and no longer affected by transfecting TβRI-CA (Figure 1H). These results indicate that on activation of TGF-β signaling, the function of KLF4 in regulating AT1R expression is reversed from activation to inhibition and
that KLF4 mediates TGF-β1-induced suppression of the AT1R gene.

TGF-β1 Inhibits the Interaction of KLF4 With Sp1 and Increases the Interaction of KLF4 With PPAR-γ

Sp1, PPAR-γ, and NF-κB can regulate AT1R expression in VSMCs. A coimmunoprecipitation assay showed that KLF4 forms a complex with Sp1 prior to TGF-β1 treatment, and that TGF-β1 decreased this association in a time-dependent manner. Meanwhile, TGF-β1 time-dependently increased the levels of PPAR-γ present in anti-KLF4 immunoprecipitates. Likewise, reciprocal immunoprecipitation experiments confirmed these findings (Figure 2A). Although interactions between Sp1 and PPAR-γ, as well as between KLF4 and NF-κB were also observed, TGF-β1 addition had no effect on these interactions (Figure 2A and 2B). Collectively, these results indicate that KLF4 forms a protein complex with Sp1 under basal conditions, with the complex severely disrupted by TGF-β1 treatment. In contrast, TGF-β1 addition facilitated KLF4-PPAR-γ complex formation in VSMCs.

TGF-β1-Inducible Dissociation of KLF4 From Sp1 Requires KLF4 Phosphorylation at the Thr Residue Mediated by PKC-δ

As shown in Figure 3A, TGF-β1 rapidly stimulated KLF4 phosphorylation on Ser and Thr residues, but not on Tyr residues within 5 minutes, and this response was time-dependent for up to 40 minutes. Total KLF4 levels remained unchanged during the time course of TGF-β1 stimulation. The existence of several putative PKC phosphorylation sites (Thr401, Thr410, Thr412, Thr448, Thr455) in the KLF4 ZNF domain (predicted by NetPhosK1.0 program; www.cbs.dtu.dk/services/NetPhosK/) suggests that PKC may directly phosphorylate KLF4 at Thr residues. Among the multiple PKCs, PKC-δ is a member of the novel PKC subfamily (nPKC) of serine-threonine kinases. It can be activated by TGF-β1 and has been shown to inhibit the proliferation of rat VSMCs. Consistently, Western blot analyses revealed that TGF-β1 significantly induced PKC-δ phosphorylation in VSMCs within 5 minutes, whereas PKC-ζ phosphorylation was undetectable in TGF-β1–treated cells. The levels of total PKC-δ and total PKC-ζ were not significantly changed (Figure 3B). Coimmunoprecipitation assays showed that TGF-β1 time-dependently increased the levels of KLF4 present in anti-PKC-δ immunoprecipitates, which is consistent with the time course of KLF4 phosphorylation changes. Likewise, reciprocal immunoprecipitation experiments paralleled these findings. As a negative control, the interaction between KLF4 and PKC-ζ was not observed, as demonstrated by immunoprecipitation of cell lysates with anti-KLF4 followed by anti-PKC-ζ immunoblotting (Figure 3C).

Rottlerin, a pharmacological inhibitor of PKC-δ, was used to block the activity of PKC-δ. Figure 3D shows that Rottlerin completely blocked TGF-β1–induced PKC-δ phosphorylation without affecting the expression of total PKC-δ, as detected by Western blot analyses. Correspondingly, Rottlerin completely abrogated TGF-β1–induced KLF4 phosphorylation at Thr residues and the interaction of KLF4 with PKC-δ. Additionally, Rottlerin completely prevented TGF-β1–induced dissociation of Sp1 from KLF4 without influencing the interaction between KLF4 and PPAR-γ induced by TGF-β1 (Figure 3E). Knockdown of PKC-δ by transfection of VSMCs with PKC-δ-specific siRNA (siPKC-δ) abrogated TGF-β1–induced KLF4 phosphorylation at Thr residues and dissociation of Sp1 from KLF4. Expression and phosphorylation of PKC-δ were markedly reduced in siPKC-δ–transfected cells when compared with siControl-transfected cells (Figure 3F). Interestingly, either Rottlerin treatment or siPKC-δ transfection completely abolished inhibition of AT1R expression by TGF-β1 (Figure IIIA and IIIB in the online-only Data Supplement).

Moreover, point mutation experiments demonstrated that the phosphorylation-deficient KLF4 mutants, KLF4-T448A and KLF4-T455A, were phosphorylated by TGF-β1 at levels comparable to those of wild-type KLF4. In contrast, KLF4-T401A and KLF4-T410/412A mutants were phosphorylated by TGF-β1 to a lesser extent than that of wild-type KLF4. Interestingly, mutation of Thr401 prevented the dissociation of Sp1 from KLF4 in response to TGF-β1 (Figure 3G and Figure IV in the online-only Data Supplement). Together, these results suggest that KLF4 is phosphorylated by PKC-δ at multiple Thr residues (Thr401, Thr410, Thr412), and phosphorylation of Thr401 leads to the dissociation of Sp1 from KLF4 in response to TGF-β1.
TGF-β1-Induced Interactions Between PPAR-γ and KLF4 Requires KLF4 Phosphorylation at Ser Residue Mediated by Smad Signaling

Treatment of VSMCs with SB431542, a specific TβRI inhibitor that inhibits Smad phosphorylation by TGF-β1, markedly blocked KLF4 phosphorylation at Ser residues and its interaction with PPAR-γ induced by TGF-β1. In contrast, the dissociation of Sp1 from KLF4 induced by TGF-β1 was not affected through SB431542 treatment (Figure 3H). In addition, we knocked down Smad2 and Smad3 both individually or simultaneously using their corresponding siRNA and demonstrated that knockdown of Smad2 or and Smad3 largely blocked TGF-β1–induced recruitment of PPAR-γ to KLF4. There was a significant reduction in Smad2 and Smad3 protein expression in siSmad2- and/or siSmad3-transfected cells when compared with untransfected control cells or siControl-transfected cells (Figure 3I). Moreover, SB431542 treatment or siSmad2/siSmad3 transfection largely abolished inhibition of AT1R expression by TGF-β1 (Figure VA and VB in the online-only Data Supplement).

Because our laboratory has demonstrated that KLF4 is phosphorylated at Ser470 by Smad signaling in response to TGF-β1,3 point mutation experiments were next conducted to verify the requirement of this site for interaction between KLF4 and PPAR-γ in response to TGF-β1. As expected, the S470A substitution markedly reduced TGF-β1–induced KLF4 phosphorylation and association of PPAR-γ with KLF4 (Figure 3J). These data provide strong evidence that KLF4 phosphorylation at Ser470 by TGF-β1 is necessary for its interaction with PPAR-γ in response to TGF-β1.

KLF4-Mediated Activation of the AT1R Promoter Is Enhanced by Sp1 and Inhibited by PPAR-γ

Luciferase reporter gene assays were performed using rat AT1R promoter reporter plasmids containing TCE- or GC-site deletion. Various deletion mutants of KLF4 and Sp1 sites are named Wt, TCE1/TCE2 deletion, TCE3/TCE4 deletion, and GC deletion, respectively (Figure 4A). Results from these experiments showed that KLF4 or Sp1 alone activated wild-type AT1R promoter and TCE1/TCE2 deletion mutant by 3-fold, whereas coexpression of KLF4 and Sp1 increased the reporter activity of these 2 constructs by 5-fold over that seen with the reporter alone (Wt). Cotransfection of TβRI-CA, KLF4, and Sp1 markedly reduced these 2 reporter activities to almost basal level (Figure 4B and 4C). In contrast, KLF4

40-minute incubation with or without TGF-β1 (2 ng/mL). Total protein lysates were immunoprecipitated with antiphosphoserine antibody and analyzed using Western immunoblotting for KLF4 (top). Interactions of Sp1 or PPAR-γ with KLF4 were examined with a communoprecipitation assay. I, VSMCs were transfected with siSmad2 or and siSmad3 or siControl for 24 hours. Cells were then treated with TGF-β1 (2 ng/mL) for 40 minutes, after which the interaction between KLF4 and PPAR-γ was determined by a communoprecipitation assay. Total forms of Smad2 and Smad3 were also examined using Western immunoblotting. J, VSMCs were transfected for 48 hours with the indicated vectors and then stimulated with or without TGF-β1 (2 ng/mL) for 40 minutes, after which the phosphorylated (at Ser residue) KLF4 and its interaction with PPAR-γ were determined as described above. IgG was used as a negative control for immunoprecipitation.
Overexpression of the TCE3/TCE4 deletion reporter, although KLF4 and Sp1 failed to activate this construct synergistically (Figure 4D). Furthermore, deletion of the GC box sequence did not affect activation of the AT1R promoter by either KLF4 alone or combination of KLF4 and Sp1 (Figure 4E).

Overexpression of PPAR-γ alone had little effect on AT1R promoter activity when compared with the reporter alone (Wt, TCE1/TCE2 deletion, TCE3/TCE4 deletion, and GC deletion), whereas coexpression of both KLF4 and PPAR-γ led to a marked repression of the AT1R promoter activation by KLF4 (Figure 4F). Furthermore, reduction in AT1R promoter activity was observed when cells were cotransfected with TβRI-CA, KLF4, and PPAR-γ (Figure 4F, 4G, and 4I). However, cotransfection of KLF4 or/and PPAR-γ showed no obvious effects on the TCE3/TCE4-deleted AT1R promoter (Figure 4H). Taken together, these results indicate that Sp1 increased and PPAR-γ decreased the ability of KLF4 to transactivate the AT1R promoter through TCE3/TCE4.

**TGF-β1 Alters the Assembly of KLF4, Sp1, and PPAR-γ on TCE4 of the AT1R Promoter**

ChIP assays were performed to determine the effect of TGF-β1 on the assembly of KLF4, Sp1, and PPAR-γ on the AT1R promoter. As a result, the binding of KLF4 to TCE3/4 was increased in response to TGF-β1, peaking at 20 minutes and remaining constant for at least 40 minutes (Figure 5A). PPAR-γ was also bound to TCE3/4 following stimulation with TGF-β1 for 20 and 40 minutes (Figure 5B). In contrast, Sp1 dissociated from TCE3/4 in the presence of TGF-β1, with binding decreasing at 10 minutes following TGF-β1 treatment and decreasing to undetectable levels at 20 minutes (Figure 5C). The dissociation of Sp1 from TCE3/4 induced by TGF-β1 was blocked with Rottlerin treatment (Figure 5D).

Furthermore, we performed sequential ChIP analysis in which VSMC chromatin was immunoprecipitated first with anti-KLF4 and second with anti-Sp1 or anti-PPAR–γ. As shown in Figure 5E, without TGF-β1 signaling, the TCE3/4-containing region could be amplified in the immunoprecipitates pulled down with anti-Sp1. On TGF-β1 signaling activation, this region was amplified in the immunoprecipitates pulled down with anti-PPAR–γ, suggesting that TGF-β1 treatment disrupted the co-occupancy of TCE3/4 by KLF4 and Sp1. In contrast, TGF-β1 enhanced the recruitment of KLF4-PPAR–γ complex to TCE3/4 compared with TGF-β1–untreated cells. In addition, we also repeated the sequential ChIP assays using immunoprecipitation first with anti-Sp1 or anti-PPAR–γ and second with anti-KLF4 and obtained the same results (Figure 5F and G). We used nonimmune IgG as a negative control for the second ChIP assays and demonstrated the specificity of immunoprecipitation (Figure 5E–5G).

Oligo pull-down experiments were next performed to distinguish between TCE3 and TCE4. As shown in Figure 5H, TGF-β1 markedly increased the binding of KLF4 to TCE4, but not TCE3, and disrupted the binding of Sp1 to TCE4. PPAR-γ was only bound to TCE4, but not TCE3 in response to TGF-β1. These data support a model in which TGF-β promotes the dissociation of Sp1 from the KLF4-Sp1 complex bound to TCE4 and stimulates recruitment of PPAR-γ to KLF4 prebound to TCE4 of the AT1R promoter to cooperatively inhibit AT1R gene transcription.

**TGF-β1 Inhibits VSMC Proliferation via Downregulation of AT1R Expression**

Treatment with TGF-β1 increased the expression of the VSMC differentiation markers SM22α and SMα-actin and decreased AT1R and proliferating cell nuclear antigen levels in a time- and dose-dependent manner (Figure 6A and 6B). In
addition, BrdU incorporation showed that TGF-β1 inhibited VSMC proliferation in a time- and dose-dependent manner (Figure 6C and 6D). In contrast, Ang II significantly decreased SM22α and SMα-actin expression and increased proliferating cell nuclear antigen expression. However, these effects were completely abrogated by TGF-β1 preincubation (Figure 6E). Moreover, preincubation of the cells with TGF-β1 largely blocked Ang II-induced proliferation (Figure 6F). We then evaluated the role of KLF4/Sp1/PPAR-γ complex in TGF-β1-mediated inhibition of cell proliferation. As shown in Figure 6G, knockdown of KLF4 or/and PPAR-γ largely abolished inhibition of cell proliferation by TGF-β1. In contrast, knockdown of Sp1 further enhanced TGF-β1-mediated growth inhibition. These results demonstrate that Sp1 dissociation from the KLF4-Sp1 complex and PPAR-γ association with KLF4 play an essential role in the inhibition of VSMC proliferation by TGF-β1.

Discussion

The present study demonstrates that on activation of TGF-β signaling, KLF4-mediated regulation of AT1R expression is altered from activation to inhibition. KLF4 and Sp1 physically interact, occupy the AT1R promoter and induce AT1R transcription in VSMCs in a co-operative manner under basal conditions. TGF-β1 addition causes the dissociation of Sp1 from KLF4 that is bound to the TCE of the AT1R promoter, leading to the suppression of AT1R transcription. In addition, TGF-β1 addition facilitates KLF4-PPAR-γ complex formation and its binding to the AT1R promoter, resulting in the inhibition of AT1R transcription (Figure 6H). For the first
time, our data provide insight into molecular mechanism by which KLF4 regulates TGF-β-mediated AT1R expression in VSMCs.

KLF4 and Sp1 are 2 members of the Sp/XKLF family of transcription factors. They physically interact and synergistically activate target gene promoters that have multiple binding sites for these factors, such as the rat laminin γ1 chain promoter.11 We examined the rat AT1R promoter region and found that the promoter has several KLF4- and Sp1-putative binding sites (CACCC sequence and GC-rich element), suggesting that AT1R promoter may simultaneously recruit KLF4 and Sp1, and thus be synergistically activated. As expected, KLF4 and Sp1 form a complex and cooperatively trigger the AT1R promoter without TGF-β activation (Figures 2 and 4B). This synergistic effect occurs through the TCE3/4, because KLF4 and Sp1 failed to synergistically activate the TCE3/4 deleted AT1R promoter (Figure 4D).

Figure 6. Transforming growth factor (TGF-β) inhibits vascular smooth muscle cell (VSMC) proliferation via downregulating angiotensin II type 1 receptor (AT1R). A–D, VSMCs were incubated in serum-free medium for 24 hours, followed by treatment with TGF-β for the indicated times and doses. A and B, Expression of AT1R, SM22α, SMα-actin, and proliferating cell nuclear antigen (PCNA) was detected using Western immunoblotting. C and D, VSMC proliferation was tested using the bromodeoxyuridine (BrdU) incorporation assay. *$P<0.05$ vs untreated group. E, VSMCs were pretreated for 6 hours with or without TGF-β (2 ng/ml) and then exposed to 0 mol/L or 10⁻⁷ M Ang II for 24 hours. Expression of AT1R, SM22α, SMα-actin, and PCNA was detected using Western immunoblotting. F, VSMCs were pretreated for 6 hours with or without TGF-β (2 ng/ml) and then exposed to 0 mol/L, 10⁻⁷ M or 10⁻⁸ M Ang II for 24 hours. VSMC proliferation was tested using the BrdU incorporation assay. *$P<0.05$ vs treated group; #$P<0.05$ vs untreated group. G, VSMCs transfected with Krüppel-like factor 4 (KLF4) or/and PPAR-γ siRNA or Sp1 siRNA were exposed to TGF-β( 2 ng/ml) for 24 hours and cell proliferation was analyzed using the BrdU incorporation assay. *$P<0.05$ vs untreated group; #$P<0.05$ vs group treated with TGF-β alone. H, Model of KLF4-mediated suppression of the AT1R gene by TGF-β1.
TCE3/4 it acts as a coactivator of KLF4 to transactivate the AT1R promoter. When TGF-β1 signaling is present, Sp1 dissociates from KLF4 bound to the AT1R promoter, leading to the deactivation of the AT1R promoter (Figures 2, 4B–4E, and 5).

KLF4 phosphorylation plays a key role in the recruitment/derecruitment of other transcription factors. In this study, we demonstrated that treatment with TGF-β1 stimulates KLF4 phosphorylation at Ser and Thr residues, but not at Tyr residues (Figure 3A), and that the PKC-δ signaling pathway mediates KLF4 phosphorylation at Thr residues. The PKC-δ signaling pathway is an important non-Smad–mediated pathway in TGF-β signaling in VSMCs. Consistently, our studies showed that TGF-β1 rapidly induces PKC-δ expression within 3 hours (data not shown) in VSMCs. Importantly, we also demonstrated that PKC-δ can be phosphorylated rapidly by TGF-β1 within 5 minutes (Figure 3B) and that activated PKC-δ binds to and phosphorylates KLF4 at Thr residues, leading to the dissociation of Sp1 from KLF4 (Figure 3C–3F). In addition, point mutation experiments demonstrated that KLF4 is phosphorylated by PKC-δ at multiple Thr residues (Thr401, Thr410/412), and that phosphorylation of Thr401 leads to the dissociation of Sp1 from KLF4 in response to TGF-β1 (Figure 3G and Figure IV in the online-only Data Supplement). We hypothesized that phosphorylation at Thr401 by TGF-β1 causes a conformational change in KLF4, leading to the release of Sp1 from KLF4. These data suggest that Sp1 interacts with KLF4 and functions as a coactivator of KLF4 at the AT1R promoter and that TGF-β1 disrupts the KLF4–Sp1 complex through TGF-β1–induced KLF4 phosphorylation at Thr401 mediated by PKC-δ signaling.

Based on our findings that Sp1 functions as a coactivator of KLF4 in regulating AT1R expression under basal conditions, and because of its dissociation from KLF4 on TGF-β1 signaling activation, we speculate that KLF4 may function as a protein platform that binds to the TCE of the AT1R promoter to derecruit its coactivator and recruit its coinhibitor when TGF-β1 signaling is activated. Therefore, we attempted to determine the coinhibitor of KLF4 in regulating AT1R transcription. As a candidate, PPAR-γ is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Activators of PPAR-γ (thiazolidinediones or glitazones) antagonize Ang II effects in vivo and in vitro, in part by decreasing the expression levels of AT1R. Mechanistically, Sugawara et al demonstrated that ligand-activated PPAR-γ suppressed AT1R gene expression at the transcriptional level by inhibiting the binding of Sp1 to GC-box–related sequence via protein–protein interactions. Recently, Liu et al demonstrated that unliganded and ligand-activated PPAR-γ may show an opposite effect in regulating target gene transcription. In this study, we demonstrated that KLF4 recruits unliganded PPAR-γ to bind to the AT1R promoter on TGF-β1 signaling activation in a time-dependent manner and that KLF4 phosphorylation at Ser470 via Smad signaling is necessary for this interaction (Figure 3H–3I). Canonically, phosphorylated Smad2/3 by TGF-β translocate into the nucleus with Smad4 and regulate the expression of TGF-β target genes in cooperation with other activators and repressors. Here, we speculated that Smad2/3 may act as a scaffold protein of other protein kinase to mediate the phosphorylation of KLF4 at Ser470. Functionally, PPAR-γ decreased the ability of KLF4 to transactivate the AT1R promoter through its interaction with KLF4 bound to the TCE of the AT1R promoter, as demonstrated by promoter reporter assays (Figure 4F–4I). These data demonstrated that TGF-β1 triggers the phosphorylation of KLF4 at Ser470 and that phosphorylated KLF4 recruits unliganded PPAR-γ to bind to the TCE of the AT1R promoter, subsequently leading to inhibition of the AT1R transcription. Transcription factor may be phosphorylated by various kinases at different sites, such as Ser/Thr/Tyr residues, within the protein. In this study, it is possible that KLF4 be dually phosphorylated at T401 and S470 on TGF-β1 stimulation.

In summary, we have presently shown that Sp1 forms a complex with KLF4 bound to the TCE of the AT1R promoter and cooperatively activates AT1R transcription in VSMCs under basal conditions. On TGF-β1 activation, Sp1 is dissociated from the KLF4–Sp1 complex through PKC-δ–mediated KLF4 phosphorylation at Thr401, downregulating AT1R expression. Simultaneously, TGF-β1 facilitates the formation of the KLF4–PPAR-γ complex and its binding to the TCE of the AT1R promoter through Smad-mediated KLF4 phosphorylation at Ser470, subsequently leading to inhibition of AT1R transcription (Figure 6H). For the first time, our results propose a molecular mechanism by which TGF-β1 downregulates AT1R expression and further expand the interaction between the renin-angiotensin system and TGF-β1 signaling. These studies also indicate that KLF4 functions as a protein platform that binds to the TCE of the AT1R promoter to derecruit or recruit other transcription factors to serve as a “molecular switch” to regulate AT1R expression in VSMCs.

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Disclosures
None.

References


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Supplemental Methods

Cell Culture and Treatment

Human aortic smooth muscle cells (HASMCs) (ScienCell, no. 6110) and rat pulmonary artery smooth muscle cells (PASMCs) (Cell Biologics, no. R1154) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Male Sprague-Dawley rats were sacrificed, aortas were removed, and VSMCs were isolated as described previously. VSMCs were cultured in DMEM supplemented with 10% FBS and maintained in 5% CO₂ at 37°C within a humidified atmosphere. All studies utilized cells from passages three to six. Prior to stimulation with TGF-β1 (R&D Systems, Minneapolis, MN), cells were incubated in serum-free medium for 24 h. For inhibitor studies, cells were pretreated for 2 h with the PKC-δ inhibitor Rottlerin (5 μmol/l) (Calbiochem, San Diego, CA) or the TβRI inhibitor SB431542 (10 μmol/l) (Promega, Madison, WI) prior to application of TGF-β1 (2 ng/ml). Human embryonic kidney 293A cells were obtained from ATCC (Manassas, VA) and maintained in high glucose DMEM supplemented with 10% FBS.

Adenovirus Expression Vector and Plasmid Constructs

pEGFP-KLF4 and adenovirus pAd-KLF4 were prepared as previously described. Expression plasmid for Sp1 (pPac-Sp1) was a generous gift from Dr. Tijan (University of California, Berkeley). The PPAR-γ expression plasmid Flag-PPAR-γ was created by the placement of PPAR-γ cDNA into the pCMV-FLAG-MAT-Tag-1 vector (Promega).
Flag-tagged wild type TβRI (Flag-TβRI) was created by placing rat TβRI cDNA into the pCMV-FLAG-MAT-Tag-1 vector. The 5' regulatory region of AT1R (−1423 to +33 bp) was amplified by PCR using the primer pairs 5'-CGGGGTACCTCGCTACTGGAGGTCTGCTGCTA-3' (sense) and 5'-CGAAGATCTGGGACAGCATCATCCAGTCCCTC-3' (antisense) for the promoter assay. The PCR product was cloned into the pGL3-Basic vector (Promega) in order to generate the AT1R promoter-reporter pGL3-AT1R-luc.

**RNA Preparation and Quantitative Real-time PCR**

Total RNA was extracted by TRIzol (Invitrogen) and 1 μg of RNA was subjected to reverse transcription using first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. Real-time PCR analysis was done with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the DyNAmo™ ColorFlash® SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland). PCR thermocycling parameters were 95°C for 7 min, 35 repeats of the denaturation step at 95°C for 10 sec, at 60°C for 20 sec, at 72°C for 20 sec, a final elongation step at 72°C for 7 min, and establishment of a melting curve from 65 to 90°C. As an internal control, GAPDH primers were used for RNA template normalization. All PCRs were performed in triplicate. The relative expression level was calculated using the following equation: relative gene expression = 2^((-ΔΔCt)) where ΔCt = Ct(sample) - Ct(control). Primers were: rat GAPDH, 5'-GGTGTGAACCACGAGAAATATGAC-3' and 5'-CTCCAGGCAGCATGTCCAGATCCAC-3'; rat AT1R, 5'-TGGCCCTTAACTTTCTTGCTGA-3' and 5'-CATAAGTCAGCAAGGGCAT-3'; rat
KLF4, 5'-CGGAAGGGAGAAGACACTGC-3' and 5'-GCTAGCTGGGGAAGACGAGGA-3'; human GAPDH, 5'-AAGGTGAAGGTCGGAGTC-3' and 5'-GATTTTGGAGGGATCTCG-3'; human AT1R, 5'-CACCATGTTTTGAGGTTGAGTGAC-3' and 5'-CAGGCTAGGGAGATTGCATTTCTG-3'; human KLF4, 5'-CCCAATTACCCATCCTTCCT-3' and 5'-CGTCCCAGTCACAGTGGTAA-3'.

**Immunoblotting**

Proteins were isolated from SMCs as previously described,\(^3\) electrophoresed on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 5% dry milk in TTBS for 2 h at 37°C and incubated overnight at 4°C with the following primary antibodies: 1:200 rabbit anti-AT1R (Santa Cruz Biotechnology, Inc., catalog no. sc-1173), 1:400 rabbit anti-KLF4 (Santa Cruz Biotechnology, Inc., catalog no. sc-20691), 1:200 rabbit anti-Sp1 (Santa Cruz Biotechnology, Inc., catalog no. sc-59), 1:400 rabbit anti-PPAR-\(\gamma\) (Santa Cruz Biotechnology, Inc., catalog no. sc-7196), 1:500 rabbit anti-NF-\(\kappa\)B p65 (Santa Cruz Biotechnology, Inc., catalog no. sc-109), 1:1000 rabbit anti-phospho-PKC-\(\delta\) (Cell Signaling, catalog no. 2055, Tyr311), 1:1000 rabbit anti-PKC-\(\delta\) (Cell Signaling, catalog no. 2058), 1:1000 rabbit anti-phospho-PKC-\(\zeta\) (Abcam, catalog no. ab62372, Thr560), 1:1000 rabbit anti-PKC-\(\zeta\) (Abcam, catalog no. ab4137), 1:1000 rabbit anti-Smad2 (Cell Signaling, catalog no. 3122), 1:1000 rabbit anti-Smad3 (Cell Signaling, catalog no. 9523), 1:1000 rabbit anti-SM22\(\alpha\) (Abcam, catalog no. ab14106), 1:500 mouse anti-SM\(\alpha\)-actin (Abcam, catalog no. ab5694), 1:400 mouse anti-PCNA (Santa Cruz Biotechnology, Inc., catalog no. sc-7907) and
1:1000 mouse anti-β-actin (Santa Cruz Biotechnology, Inc., catalog no. sc-47778). Following incubation with the appropriate secondary antibody, antibody-antigen complexes were imaged using the Chemiluminescence Plus Western immunoblot analysis kit (Santa Cruz Biotechnology, Inc.).

**Co-immunoprecipitation Assay**

Co-immunoprecipitation was performed as previously described. In brief, cell extracts were precleared with 25 μl of protein A-agarose (50% v/v). The supernatants were immunoprecipitated with 2 μg of anti-phosphoserine (Santa Cruz Biotechnology, Inc., catalog no. sc-81514), anti-phosphothreonine (Santa Cruz Biotechnology, Inc., catalog no. sc-5267), anti-phosphotyrosine (Santa Cruz Biotechnology, Inc., catalog no. sc-7020), anti-KLF4, anti-Sp1, anti-PPAR-γ, or anti-PKC-δ antibodies for 1 h at 4°C, followed by incubation with protein A-agarose overnight at 4°C. Protein A-agarose-antigen-antibody complexes were collected using centrifugation at 12,000 rpm for 60 s at 4°C. The pellets were washed five times with 1 ml of IPH buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride) for 20 min at 4°C. Bound proteins were resolved using SDS-PAGE, followed by Western immunoblotting with anti-KLF4, anti-Sp1, anti-PPAR-γ, anti-NF-κB p65 or anti-PKC-δ antibodies.

**Site-directed Mutagenesis**

Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Agilent Technologies-Stratagene, La Jolla, CA) according to the
manufacturer’s instructions. A constitutively active mutant of rat TβRI [Flag-TβRI-CA (T198D)] was obtained with the mutagenic primer pairs 5'-GATATCGTGCTACAAGAAAGC-3' (sense) and 5'-GCTTTCTTGTAGCAGATATC-3' (antisense). Various deletion mutants of the AT1R promoter-reporter plasmid (named TCE1/TCE2 deletion, TCE3/TCE4 deletion, and GC deletion, respectively) were generated from pGL3-AT1R-luc. Serine-to-alanine mutations of GFP-KLF4 at Ser470 (S470A) were described previously.\(^5\) Threonine-to-alanine mutations of GFP-KLF4 at Thr401 (T401A), Thr410/412 (T410/412A), Thr448 (T448A), and Thr455 (T455A) were generated from pEGFP-KLF4. All constructs were verified using DNA sequence analysis.

**Plasmid Transfection**

Early passage cultures (P1-P3) of VSMCs were plated in 6-well culture dishes for 18 h, at which time they had reached about 70% confluence in DMEM without antibiotics. Cells were washed twice with serum-free DMEM, and then 1 ml of serum-free Opti-MEM 1 medium was added to each well. The DNA-lipofectamine reagent complex was prepared according to the instructions of the manufacturer (Invitrogen). The amount of transfected plasmid and lipofectamine reagent added to each well was 6 μg and 30 μl, respectively. The DNA-lipofectamine reagent complex (0.5 ml per well) was added to each well, followed by incubation at 37°C for 4 h. After transfection, the cells were maintained in DMEM containing 1% FBS for 48 h and then treated with TGF-β1 (2 ng/ml) for 40 min. Cells were then collected and used in the experiments.
Small Interfering RNA Transfection

Small interfering RNA (siRNA) targeting rat PKC-δ were designed according to the methods as described by Naoaki Saito.\textsuperscript{6} siRNAs against the rat sequences for Smad2 (accession number NM_019191) and Smad3 (accession number NM_013095) were designed and synthesized by Sigma. siRNAs specific for rat PKC-δ and KLF4 were synthesized by Sigma. The siRNA sequences utilized in these studies were as follows: PKC-δ siRNA, 5’-UGACAAAGAUUAUGGCCGCAU-3’ and 5’-CGGACCAUAUCUUGUCATT-3’; Smad2 siRNA, 5’-CGGACCAUAUGGACGACAAAGATT-3’ and 5’-UCUUAUGGUGGCACGATT-3’; Smad3 siRNA, 5’-GGAAUUUCUGCCUUCGATT-3’ and 5’-UAGGAGGGCAGCAAUVCCT-3’; KLF4 siRNA, 5’-CAGAUAUCAAGAGCUAUGCCACCAG-3’ and 5’-CCGUGGCAUGACUUGAUAUGG-3’. Non-specific siRNA (siControl) and siRNAs specific for rat Sp1 and PPAR-γ were purchased from Santa Cruz Biotechnology. Transfection was performed using Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions. Twenty-hours following transfection, VSMCs were treated with TGF-β1 (2 ng/ml). Cells were then harvested and lysed for western immunoblotting or co-immunoprecipitation assays.

Luciferase Assay for AT1R Promoter Activity

Human embryonic kidney 293A cells were maintained as previously described.\textsuperscript{5} 3×10^4 cells were seeded into each well of a 24-well plate and grown for 24 h prior to transfection with reporter plasmids and the control pTK-RL plasmid. Cells were transfected using
Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase assays were performed after 24 h using a dual luciferase assay kit (Promega). Specific promoter activity was expressed as the relative ratio of firefly luciferase activity to Renilla luciferase activity. All promoter constructs were evaluated in a minimum of three separate wells per experiment.

**Chromatin Immunoprecipitation (ChIP) Assay and Sequential ChIP**

The chromatin immunoprecipitation (ChIP) assay was carried out as previously described. In brief, VSMCs were treated with 1% formaldehyde for 15 min to cross-link proteins with DNA. The cross-linked chromatin was then prepared and sonicated to an average size of 400-600 bp. The DNA fragments were immunoprecipitated overnight with the anti-KLF4, anti-Sp1 or anti-PPAR-γ antibodies. Following cross-linking reversal, the genomic region of the AT1R flanking the potential KLF4-binding sites TCE3 and TCE4 was amplified by PCR with the following primer pairs: 5’- TTGGAGTCAGTTCATGTGGCC-3’ (forward) and 5’-GGCTCGGCTTTTGACTCCTC-3’ (reverse). Sequential two-step ChIP assays were performed as previously described. In brief, chromatin fragments were immunoprecipitated by overnight incubation with antibodies for KLF4, Sp1, PPAR-γ, or control IgG at 4°C. After several washes, the precipitates were incubated with 50 μl of buffer containing 0.5% SDS and 0.1 M NaHCO₃ for 10 min at 65°C. The supernatant was collected following spinning; diluted with 1 mM EDTA, 150 mM NaCl, 50 mM HEPES, pH 7.5, 0.1% SDS, 1% Triton X-100, and 0.1% sodium deoxycholate; and incubated overnight with antibodies for KLF4, Sp1, PPAR-γ, or control IgG. After washing, the protein-DNA
complexes were eluted from the beads, and treated with proteinase K overnight. DNA was purified using a minicolumn, and the KLF4-binding sites TCE3 and TCE4 of the AT1R promoter were amplified by PCR using the primer described above. Each experiment was replicated three times at least.

DNA Affinity Precipitation Assay

The oligonucleotides containing biotin on the 5'-end of the each strand were used. The sequences of these oligonucleotides were as follows: TCE3, biotin-5'-AGTTCCCTAAATCACCCCTTAAAAGTTTTC-3' (wild type, forward) and biotin-5'-GAAAAACTTTTAAGGGTAGTTAGGAACCT-3' (wild type, reverse), which corresponds to base pairs –362 to –334 of the AT1R promoter region; TCE3 mutant, biotin-5'-AGTTCCCTAAATCCCTTAAAAGTTTTC-3' (forward) and biotin-5'-GAAAAACTTTTAAGGGAGTTAGGAACCT-3' (reverse); TCE4, biotin-5'-GAAGGCAGACACTGGTGACTGGCAGCAG-3' (wild type, forward) and biotin-5'-CCTGCTGCCCAGTCTCCAGTCTGGCTTGCTTC-3' (wild type, reverse), which corresponds to base pairs –184 to –156 of the AT1R promoter region; TCE4 mutant, biotin-5'-GAAGGCAGACACTGGGAGGACTGGCAGCAG-3' (forward) and biotin-5'-CCTGCTGCCCAGTCTCCAGTCTGGCTGGCCTTC-3' (reverse). Each pair of oligonucleotides was annealed following standard protocols. VSMCs treated with or without TGF-β1 (2 ng/ml) for 40 min were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40) containing protease inhibitors. Whole-cell extracts (200 μg) were precleared with ImmunoPure streptavidin-agarose beads (20 μl/sample,
Promega) for 1 h at 4°C. Following centrifugation for 2 min at 12,000 rpm, the supernatant was incubated with 100 pmol of biotinylated double-stranded oligonucleotides and 10 μg of poly (dI-dC)-poly (dI-dC) overnight at 4°C while gently being rocked. Thirty μl of streptavidin-agarose beads was added, followed by an additional 1 h of incubation at 4°C. The protein-DNA-streptavidin-agarose complex was washed four times with lysis buffer, separated on a 10% SDS-PAGE, and subjected to Western immunoblotting with various antibodies.

Cell Proliferation Assay

VSMC proliferation assays were performed with the BrdU Cell Proliferation Assay kit (Millipore) according to the manufacturer’s recommendations. VSMCs were labeled for 6 h with BrdU prior to the termination of TGF-β1 incubation. OD readings were performed at 450 nm. All groups were evaluated in a minimum of three separate wells per experiment.

Statistical Analysis

Data are presented as bar graphs (means ± SEM) of at least three independent experiments. Statistical analyses were performed using Student’s t test. The results were considered statistically significant at p < 0.05.
Supplemental Legends

Supplemental Figure Ⅰ
Rat PASMCs were incubated in serum-free medium for 24 h, followed by treatment with TGF-β1 for the indicated times and doses. A and B, Western blot analysis for AT1R and KLF4 expression. C and D, Relative expression of AT1R or KLF4 mRNA was examined by qRT-PCR and presented after normalizing to GAPDH (means ± SEM; n=3).

Supplemental Figure Ⅱ
HASMCs were incubated in serum-free medium for 24 h, followed by treatment with TGF-β1 for the indicated times and doses. A and B, Western blot analysis for AT1R and KLF4 expression. C and D, Relative expression of AT1R or KLF4 mRNA was examined by qRT-PCR and presented after normalizing to GAPDH (means ± SEM; n=3).

Supplemental Figure Ⅲ
A, VSMCs were pretreated with the PKC-δ inhibitor Rottlerin (5 μmol/l) for 2 h, followed by a 40-min incubation with or without TGF-β1 (2 ng/ml). Cell lysates were used for the detection of the phosphorylated KLF4 in anti-phosphothreonine immunoprecipitates and total levels of KLF4 and AT1R. Left panel: Blots from a representative experiment. Right panel: Quantitation of the phosphorylated KLF4 normalized to total KLF4 (white bars, means ± SEM; n=3) and AT1R normalized to β-actin (gray bars, means ± SEM; n=3). B, VSMCs were transfected with siPKC-δ or siControl for 24 h. Cells were then treated with or without
TGF-β1 (2 ng/ml) for 40 min, after which the phosphorylated (at Thr residue) KLF4 and total forms of KLF4 and AT1R were determined. Left panel: Blots from a representative experiment. Right panel: Quantitation of the phosphorylated KLF4 normalized to total KLF4 (white bars, means ± SEM; n=3) and AT1R normalized to β-actin (gray bars, means ± SEM; n=3). *p < 0.05 vs. untreated group; #p < 0.05 vs. group treated with TGF-β1 alone.

**Supplemental Figure IV**

VSMCs were transfected for 48 h with the indicated vectors and then stimulated with TGF-β1 (2 ng/ml) for 40 min. Top panel: Phosphorylated KLF4 (at Thr residue) was quantitated by densitometry and values were normalized to total KLF4. Middle and bottom panels: Quantitation of KLF4 and Sp1 co-immunoprecipitation. Data are means ± SEM, n=3. *p < 0.05 vs. group treated with vehicle; #p < 0.05 vs. group transfected with KLF4 Wt before TGF-β1 treatment.

**Supplemental Figure V**

A, VSMCs were pretreated with the TβRI inhibitor SB431542 (10 μmol/l) for 2 h, followed by a 40-min incubation with TGF-β1 (2 ng/ml). Cell lysates were used for the detection of the phosphorylated KLF4 in anti-phosphoserine immunoprecipitates and total levels of KLF4 and AT1R. Left panel: Blots from a representative experiment. Right panel: Quantitation of the phosphorylated KLF4 normalized to total KLF4 (white bars, means ± SEM; n=3) and AT1R normalized to β-actin (gray bars, means ± SEM; n=3). *p < 0.05 vs. untreated group; #p < 0.05 vs. group treated with TGF-β1 alone. B, VSMCs were transfected with
siSmad2/siSmad3 or siControl for 24 h. Cells were then treated with or without TGF-β1 (2 ng/ml) for 40 min, after which the phosphorylated KLF4 (at Ser residue) and total forms of KLF4 and AT1R were determined. Left panel: Blots from a representative experiment. Right panel: Quantitation of the phosphorylated KLF4 normalized to total KLF4 (white bars, means ± SEM; n=3) and AT1R normalized to β-actin (gray bars, means ± SEM; n=3). *p < 0.05 vs. untreated group; # p < 0.05 vs. group treated with TGF-β1 alone.
Supplemental References


6. Irie N, Sakai N, Ueyama T, Kajimoto T, Shirai Y, Saito N. Subtype- and species-specific knockdown of PKC using short interfering RNA. *Biochem Biophys Res*
Supplemental Figures

Supplemental Figure  I

rat PASMCs

A  TGF-β1 (2 ng/ml)  0  3  6  12  24 (h)
    AT1R  KLF4  β-actin

B  TGF-β1 (24 h)  0  0.5  1  2  4 (ng/ml)
    AT1R  KLF4  β-actin

C  Relative mRNA level (normalized to GAPDH)

D  Relative mRNA level (normalized to GAPDH)

TGF-β1 Treatment (h)  TGF-β1 Treatment (ng/ml)
HASMCs

A) TGF-β1 (2 ng/ml)
   0 6 12 24 (h)
   AT1R
   KLF4
   β-actin

B) TGF-β1 (24 h)
   0 0.5 1 2 4 (ng/ml)
   AT1R
   KLF4
   β-actin

C) Relative mRNA level (normalized to GAPDH)
   "KLF4" [line with square]
   "AT1R" [line with triangle]
   TGF-β1 Treatment (h)

D) Relative mRNA level (normalized to GAPDH)
   "KLF4" [line with square]
   "AT1R" [line with triangle]
   TGF-β1 Treatment (ng/ml)
Supplemental Figure Ⅲ

A

TGF-β1
IP: p-Thr
WB
WB
WB

DMSO Rottlerin

KLF4
KLF4
AT1R
β-actin

B

TGF-β1
IP: p-Thr
WB
WB
WB

siControl siPKC-δ

KLF4
KLF4
AT1R
β-actin

DMSO
Rottlerin
siControl
siPKC-δ

p-KLF4/KLF4
AT1R/β-actin

Relative levels

- - + +

- - + +

- - + +

- - + +
Supplemental Figure IV

**KLF4**

**Relative phosphorylation**

- **Vehicle**
- **TGF-β1 (2ng/ml)**

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**KLF4/Sp1 co-IP (IP: Sp1)**

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**KLF4/Sp1 co-IP (IP: KLF4)**

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**Supplemental Figure V**

**A**

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**Bar Graphs**

- **Graph A**: Relative levels of p-KLF4/KLF4 and AT1R/β-actin with TGF-β1 treated with DMSO or SB431542.
- **Graph B**: Relative levels of p-KLF4/KLF4 and AT1R/β-actin with TGF-β1 treated with siControl or siSmad2/siSmad3.