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Angiotensin II Stimulates KLF5 Phosphorylation and its Interaction with c-Jun Leading to Suppression of p21 Expression in Vascular Smooth Muscle Cells

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Krüppel-like factor 5 (KLF5) and c-Jun are involved in angiotensin II (Ang II)-induced cell proliferation and play an important role in p21 expression. But the direct and functional implications of KLF5 and c-Jun in regulating p21 expression in vascular smooth muscle cells (VSMCs) are unclear. Here, we show that Ang II upregulated KLF5 and c-Jun expression and inhibited p21 expression in VSMCs, and silencing of KLF5 expression by KLF5-specific small interfering RNA (siRNA) neutralized the inhibitory effects of Ang II on p21 expression. Exposure of VSMCs to Ang II rapidly and strongly stimulated KLF5 phosphorylation, which results in an increase of the interaction of KLF5 with c-Jun. Treating VSMCs with PD98059, the ERK inhibitor, inhibited ERK activation and KLF5 phosphorylation as well as the interaction between KLF5 and c-Jun. Reporter analysis showed that both KLF5 and c-Jun cooperatively repressed the promoter of p21. Furthermore, KLF5 bound to its *cis*-elements in the p21 promoter, and meanwhile interacted with c-Jun in Ang II-induced VSMCs. These results suggest that Ang II induces KLF5 phosphorylation mediated by the ERK signalling in VSMCs, which in turn stimulates the interaction of KLF5 with c-Jun, subsequently leads to the suppression of p21 expression.

Key words: angiotensin II, c-Jun, KLF5, p21 expession, vascular smooth muscle cells.

Abbreviations: Ang II, angiotensin II; VSMC, vascular smooth muscle cell; KLF, Krüppel-like factor; KLF5, Krüppel-like factor 5; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; SMemb, smooth muscle embryonic type myosin heavy chain-B; HDAC1, histone deacetylase 1.

Abnormal proliferation of vascular smooth muscle cells (VSMCs) is a key feature of vascular diseases such as atherosclerosis, restenosis and hypertension (*1*). The proliferation of VSMCs requires the coordinate regulation of many genes, including cell cycle control genes (*2*, *3*), which means that VSMC proliferation is very likely governed by the activity of a transcription factor network.

The Krüppel-like factors (KLFs) are DNA-binding transcriptional regulators with a positive effect on cell cycle progression and proliferation in a number of tissue and cell types (*4*). Krüppel-like factor 5 (KLF5, BTEB2, IKLF) promotes cell proliferation by activating the transcription of several cell cycle promoting gene products, including cyclin D1, cyclin B1 and Cdk1/Cdc2 (*5*–*7*). KLF5 expression is abundant in fetal but not adult VSMCs. However, its expression can be reinduced in adult VSMCs after vascular injury or with angiotensin II (Ang II) treatment (*4*, *8*). Importantly, KLF5 can activate expression of many factors that are well-known to influence the vascular injury response such as PDGF-A/B, Egr-1, PAI-1 and the VEGF receptors (*4*, *9*).

c-Jun is a component of the AP-1 transcription factor complex composed of members of the fos and jun families, and AP-1 binds to a specific target DNA site (5′-TGAG/CTCA-3′) to regulate the expression of potentially hundreds of genes, including cell cycle regulators such as cyclin D1, p53, p21, p16 and p19 (*10*–*12*). p21 is a member of the Cip/Kip family of CKIs, and can block G1/S cell cycle progression by inhibiting the activity of cyclin/cdk complexes (*11*).

Previous studies have shown that KLF5 and c-Jun play a role in Ang II-induced suppression of p21 expression in VSMCs (*2*, *9*, *13*). To learn more about the direct and functional implications of KLF5 and c-Jun in regulating p21 expression, we examined the effect of cooperative interaction of KLF5 with c-Jun on p21 expression in Ang II-induced VSMCs. Here, we reported that Ang II rapidly stimulates KLF5 phosphorylation in VSMCs. Moreover, ERK1/2, which is activated after Ang II stimulation, mediates KLF5 phosphorylation that subsequently leads to increased interaction of KLF5 with c-Jun and to the p21 transcriptional repression.

MATERIALS AND METHODS

Cell Culture and Treatment—VSMCs were isolated from the thoracic aorta of 90–110 g male Sprague-Dawley rats as described previously (*13*). Cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml

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penicillin and 100 μg/ml streptomycin in a humidified 95% air and 5% CO_2 atmosphere. When cell growth reached 70–80% confluence, cells were incubated in serum-free DMEM for 24 h. When used, the cells were stimulated with Ang II (100 nM, Sigma, St Louis, MO, USA) for different times. For the inhibitor studies, cells were pretreated with inhibitor PD98059 (Promega, Madison, WI, USA) in DMSO for 2 h before the application of Ang II.

Western Blot Analysis—Protein extracts were prepared in extraction buffer [1% NP-40, 1% SDS, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and then isolated as previously described (*14*). The protein content was determined with the Bio-Rad Bradford protein assay. Equal amounts of proteins (60–100 μg) were separated by 8% SDS–PAGE, and electro-transferred to a PVDF membrane (Millipore Corp., Bedford, MA, USA). Membranes were blocked with 5% non-fat dry milk in TTBS (500 mM NaCl, 0.05% Tween 20, 50 mM Tris–HCl, pH 7.5) for 2 h at room temperature, and incubated overnight at 4°C with primary antibodies: 1 : 500 rabbit anti-KLF5 (BTEB2) (Santa Cruz, CA, USA), 1 : 500 rabbit anti-ERK1/2 (Santa Cruz), 1 : 500 mouse anti-phospho-ERK1/2 (Santa Cruz), 1 : 1000 rabbit anti-MEK1/2 (Cell Signaling, Danvers, MA, USA), 1 : 1000 rabbit anti-phospho-MEK1/2 (Cell Signaling), 1 : 1000 rabbit anti-p38 (Cell Signaling), 1 : 1000 rabbit anti-phospho-p38 (Cell Signaling), 1 : 500 rabbit anti-c-Jun (Santa Cruz), 1 : 500 mouse anti-p21 (Santa Cruz) and 1 : 1000 mouse anti-β-Actin (Santa Cruz). The membranes were then incubated for 2 h at room temperature with a 1 : 5000 dilution of anti-rabbit/horseradish peroxidase or anti-mouse/horseradish peroxidase (Santa Cruz) and developed with the Chemiluminescence Plus western blot analysis kit (Santa Cruz).

Co-immunoprecipitation Assay—VSMCs were stimulated with Ang II (100 nM) for 0.5, 1 or 3 h. Co-immunoprecipitations were performed as described previously (*15*). Briefly, cell extracts were first pre-cleared with 20 μl of protein A-agarose (50% v/v). The supernatants were immunoprecipitated with 1 μg of anti-KLF5, anti-c-Jun or anti-phosphoserine 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 by centrifugation at 12 000 r.p.m. for 2 min at 4°C. The pellets were washed four times with 500 μl IPH-washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 and 0.1 mM PMSF), for 20 min each time at 4°C. Bound proteins were resolved by SDS–PAGE, followed by western blotting with anti-c-Jun or anti-KLF5 antibodies. The experiments were replicated three times at least.

Cell Transfection—Constitutively active ERK kinase MEK1 plasmid (pCMV-MEK1) was kindly provided by Kun-Liang Guan (Moore's Cancer Center, CA, USA). PMT-KLF5 plasmid was kindly provided by Dr Mandayam N (Emory University, Georgia). Mutation of KLF5 S406 to A (KLF5 S406A) was carried out using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Each mutation was verified by DNA sequence analysis. VSMCs were transfected with these plasmids and KLF5-specific siRNA (5′-AACC CGGAUCUGGAGAAGCGATT-3′) or non-specific siRNA (NS-siRNA) (Takara Bio, Ohtsu, Japan) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and then incubated in DMEM containing 10% FBS for 24 h followed by the application of Ang II.

Reporter Gene Assay—Human embryonic kidney 293A cells (ATCC, CRL-1573) were cultured in DMEM containing 10% FBS. The cells were prepared and transfected by Lipofectamine 2000 (Invitrogen). Briefly, 293A cells in 60-mm dish plates were transfected with 1–2 μg of p21 reporter gene (gifted by Dr Bert Vogelstein, Johns Hopkins Medical Institutions) and/or expression plasmids for c-Jun, KLF5 and MEK1. Twenty-four hours after transfection, the cells were harvested and firefly luciferase activities as well as Renilla luciferase activities were measured with the Dual-Luciferase Reporter System (Promega) according to the manufacturer's protocol. The activity of firefly luciferase was normalized to that of *Renilla* luciferase. The relative luciferase activities compared with the luciferase activities of pRL-TK were presented as mean ± SD.

Oligonucleotide Pull-down Assay with Cell Lysates—Oligonucleotide pull-down assay was performed as described previously (*16*). Briefly, oligonucleotides containing potential KLF5 binding sites in the rat p21 promoter (–275 to –313), with biotin added to their 5′-end were as follows: biotin-5′-GCTCTGCTGGTGGGCTGCGT GACAAGCAGGTGTGGGGGA-3′ (wildtype) and biotin-5′-GCTCTGCTGATGAACTGCGTGACAAGCAGGTATGA AGGA-3′(mutant). Each pair of oligos was annealed following standard protocols. VSMCs treated with 100 nM Ang II or control solution for 1 h under serum-free condition were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) containing protease and phosphatase inhibitors. After cell debris was removed by centrifugation, cell extracts were pre-cleared with ImmunoPure streptavidin–agarose beads (20 μl/sample, Pierce, Rockford, IL, USA) for 1 h at 4°C. After centrifugation for 1 min at 5000 *g*, the supernatant was incubated with 100 pmol of biotinylated double-strand oligonucleotides and 10 μg of poly(dI–dC) for 16 h at 4°C. DNA-bound proteins were collected with 30 μl immobilized streptavidin–agarose beads for 1 h at 4°C, washed with lysis buffer for four times, separated on a 10% SDS–polyacrylamide gel, and subjected to western blotting with different antibodies.

Oligonucleotide Pull-down Assay with Purified GST-KLF5 and GST-c-Jun—Glutathione *S*-transferase (GST)-KLF5 and GST-c-Jun fusion proteins were expressed in *Escherchia coli* by inducing with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 25°C overnight and purified by affinity chromatography on glutathione–agarose beads. Ten microliters of the fusion proteins were used in each oligonucleotide pull-down assay as described above.

In vitro ERK Kinase Activity Assay—ERK1/2 activity was determined using a non-radioactive ERK1/2

activity assay kit (Cell Signaling) according to the manufacturer's instructions. Briefly, the active phosphorylated ERK1/2 was incubated with a GST-KLF5 fusion protein in the presence of ATP. Phosphorylation of KLF5 was measured by western blotting using immunoprecipitation assay.

Statistical Analysis—Results are expressed as means ± SD, and an analysis of variance with Bonferroni's test was used for the statistical analysis of multiple comparisons of data. *P*-values <0.05 were considered statistically significant.

RESULTS

Ang II Induces KLF5 and c-Jun Expression and Inhibits p21 Expression in VSMCs—KLF5 is known to play a role in Ang II-induced VSMC proliferation by activating the transcription of several cell cycle promoting gene products, such as cyclin D1, cyclin B1 and Cdk1 (*5–7*). However, it is not fully understood whether p21, a cell cycle inhibitor, is involved in Ang II-induced VSMC proliferation. To determine whether KLF5 is responsible for the p21 expression in VSMCs treated by Ang II, we first examined the effect of Ang II on the expression of KLF5, c-Jun and p21 by western blot analysis. The results showed that the expression of KLF5 and c-Jun was induced by Ang II in dose- and time-dependent manners (Fig. 1A). On the contrary, p21 expression was reduced by Ang II, especially at 12 h after Ang II stimulation. To further determine whether KLF5 directly inhibits p21 transcriptional activity, we performed a reporter gene assay. 293A cells were transfected with the KLF5 expression plasmids along with p21 promoter/enhancer (–1500 bp) reporter. As shown in Fig. 1B, treating cells with Ang II slightly inhibited p21 promoter activity, whereas KLF5 overexpression resulted in a 42% decrease in p21 promoter activity, indicating that KLF5 inhibited directly p21 gene expression. To further determine whether Ang II-inhibited p21 gene expression was due to the increased expression of KLF5, we transfected VSMCs with rat KLF5-specific small interfering RNA (siRNA) or NS-siRNA to block the endogenous KLF5 expression induced by Ang II. In response to Ang II, the level of KLF5 protein was significantly attenuated in VSMCs transfected with KLF5-specific siRNA as compared with cells transfected with NS-siRNA. Importantly, Ang II-elicited suppression of p21 expression was abrogated by transfection with KLF5-specific siRNA (Fig. 1C). These results suggested that Ang II inhibits p21 expression by inducing KLF5 expression.

KLF5 and c-Jun Cooperatively Inhibit p21 Promoter Activation—The above findings suggest that KLF5 is a transcription inhibitor of the p21 gene. Previous studies have demonstrated that the p21 promoter-enhancer is a transcriptional target for c-Jun (*17*). Then, do these two transcription factors have synergistic effect on p21 expression induced by Ang II? To determine this, we first detected the interaction between KLF5 and c-Jun. The cell lysates were immunoprecipitated with anti-KLF5 antibody, and then the precipitate was detected by western blotting with anti-c-Jun antibody. As shown in Fig. 2A, treatment of VSMCs with Ang II resulted in a significant increase in the interaction of KLF5 with c-Jun, which reached a maximum at 1 h after Ang II stimulation, and then began to reduce at 3 h. Crossing Co-IP identified the same result (Fig. 2B). To determine the functional significance of the association of KLF5 with c-Jun, reporter gene assay was done. 293A cells were cotransfected with the p21 promoter reporter, along with expression vectors for KLF5 or/and c-Jun. KLF5 overexpression resulted in a 42% decrease in p21 transcriptional activity, while KLF5 and c-Jun overexpression resulted in a 61% decrease in activity, suggesting that KLF5 and c-Jun synergistically repress the p21 promoter activation (Fig. 2C). We also tested whether KLF5 binds to the p21 promoter. In the rat p21 promoter region, there are two sequences of GTGGG-like motifs that belong to the consensus KLF5 binding element TCE, but no AP-1 binding sites. We performed oligo pull-down experiments for TCE elements using cell lysates from VSMCs treated with Ang II. Biotin-labelled oligonucleotides for wildtype and mutant TCE sequence were incubated with lysates of VSMCs treated with and without Ang II, followed by pull-down with streptavidin–agarose beads and western blot analysis. As shown in Fig. 2D, an increased binding of KLF5 to the TCE oligo was observed in VSMCs treated with Ang II, and also the increased interaction of c-Jun with KLF5 could be detected. Mutation in the TCE element abolished or dramatically decreased its binding to KLF5, with or without Ang II treatment (Fig. 2D). Furthermore, we performed an oligo pull-down assay using GST-KLF5 or/and GST-c-Jun fusion protein. GST-KLF5 bound to TCE oligo, but GST-c-Jun did not bind to it and no binding was detected when mutant TCE oligo was used. However, c-Jun could directly interact with KLF5 (Fig. 2D and E). These results suggested that KLF5 binds to its *cis*-elements in the p21 promoter, and meanwhile interacts with c-Jun in Ang II-induced VSMCs.

Ang II Increases the Interaction Between KLF5 and c-Jun by Inducing KLF5 Phosphorylation—To understand whether the change in KLF5 phosphorylation is responsible for the increased interaction between KLF5 and c-Jun, we detected the levels of phospho-KLF5 in VSMCs treated with Ang II. As shown in Fig. 3A, Ang II stimulation rapidly induced phosphorylation of KLF5 within 0.5 h, and KLF5 phosphorylation reached the maximum at 1 h and persisted for up to 3 h. Further, it is known that activation of MAP kinases by Ang II has been implicated in the expression of KLF5 (*9*). To determine whether activation of MEK and ERK mediates KLF5 phosphorylation induced by Ang II, we examined the levels of phospho-MEK and phospho-ERK by western blotting using phospho-specific antibodies. As shown in Fig. 3C, Ang II markedly induced phosphorylation of ERK1/2 and MEK within 0.5 h, which falls slightly at 1 h and returns to basal level at 3 h, without affecting the levels of ERK1/2 and MEK expression. But, Ang II did not affect the p38 phosphorylation. These results clearly demonstrate that the ERK1/2 signalling plays a key role in Ang II-induced phosphorylation of KLF5 in VSMCs.

ERK1/2 Inhibitor PD98059 Inhibits Ang II-Induced Phosphorylation of KLF5—Since Ang II treatment leads

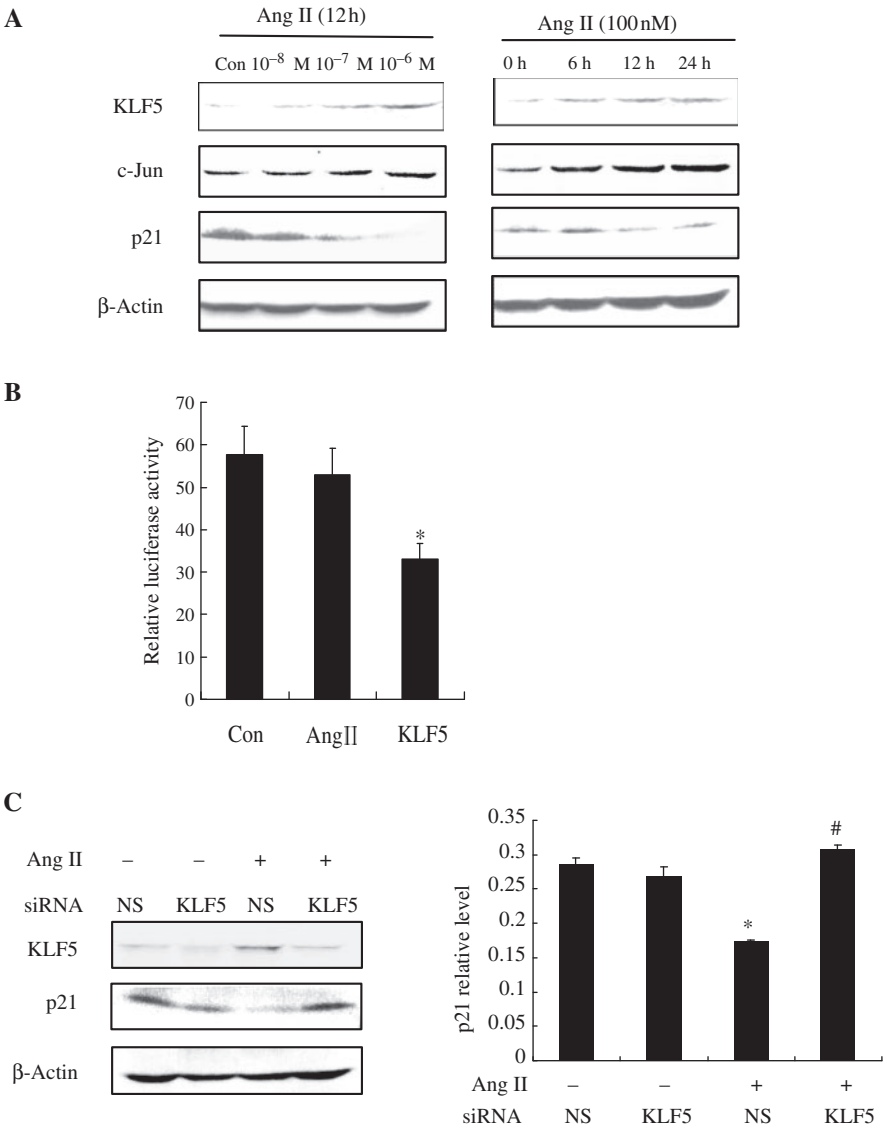

Fig. 1. **Effect of Ang II on expression of KLF5, c-Jun and p21.** (A) VSMCs were stimulated with Ang II (100 nM) for different times (0, 6, 12 and 24 h) or treated with 10^{-6}, 10^{-7} and 10^{-8} M of Ang II for 12 h. The cell lysates were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-KLF5, anti-c-Jun and anti-p21 antibodies. β-Actin was used as loading control. (B) Effect of KLF5 on p21 transcriptional activity. 293A cells were transfected with an equal amount of p21 promoter–reporter plasmids and pRL-TK, pGL3-Basic, pMT-KLF5 for 24 h. Luciferase activity was determined using the dual-luciferase reporter assay system and normalized to pRL-TK plasmids. The data are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, compared with control. (C) VSMCs were transfected with KLF5-specific siRNA or NS-siRNA, and after 20 h of transfection, cells were treated with 100 nM of Ang II for 12 h. The cell lysates were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-KLF5 and anti-p21 antibodies. β-Actin was used as loading control. Left, blots from a representative experiment are shown. Right, densitometry analysis was carried out and normalized to β-Actin. $^{*}P < 0.05$, compared with control. $^{\#}P < 0.05$, compared with non-specific siRNA (NS-siRNA).

to ERK1/2 phosphorylation, we presumed that activated ERK1/2 may be involved in Ang II-induced KLF5 phosphorylation that subsequently leads to the increase of the interaction between KLF5 and c-Jun in VSMCs. The effects of ERK inhibitor PD98059 on Ang II-induced KLF5 phosphorylation and the interaction of KLF5 with c-Jun were detected. As shown in Fig. 4A, KLF5 phosphorylation induced by Ang II was significantly inhibited by PD98059 pretreatment. We also tested the effect of constitutively active ERK kinase MEK1 on KLF5 phosphorylation. As shown in Fig. 4B, transfection of VSMCs with MEK1 expression plasmid substantially increased KLF5 phosphorylation, strongly suggesting that the activation of ERK1/2 pathway induces phosphorylation of KLF5 in VSMCs. As expected, in vitro ERK kinase assay using KLF5 as a substrate of ERK1/2 showed that ERK1/2 directly phosphorylated KLF5 (Fig. 4C). It has been reported that KLF5 DNA binding domain (DBD) was sufficient for its interaction with other transcriptional factors and that phosphorylation site P8 (S406) is important for the interaction between two proteins (*18*, *19*). To demonstrate that phosphorylation of

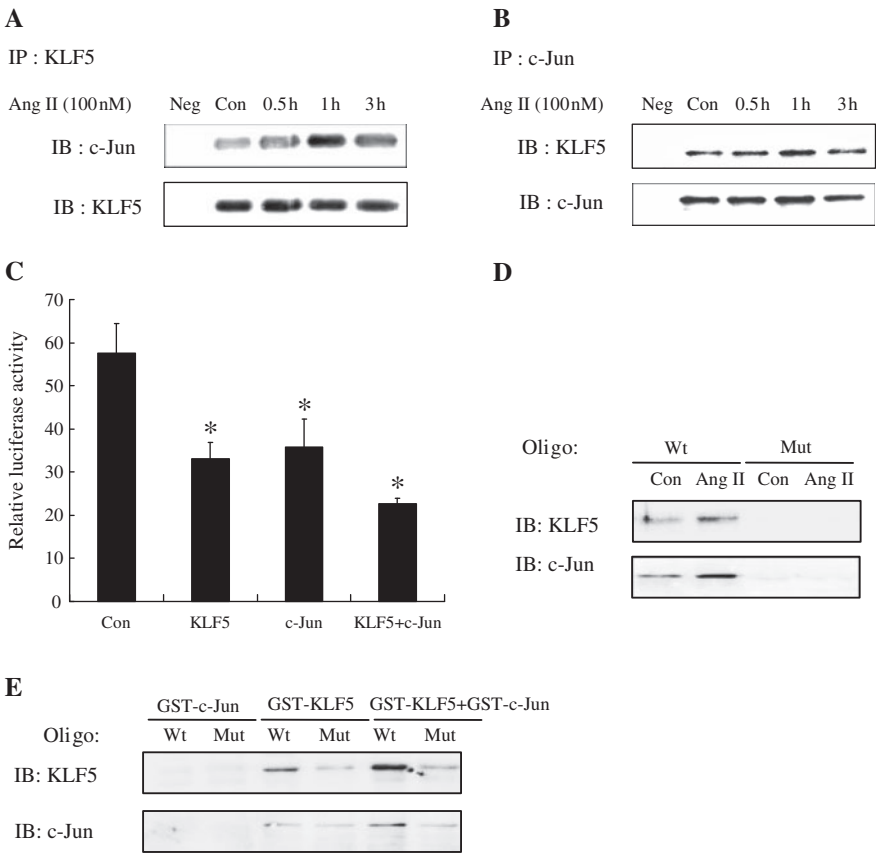

Fig. 2. **Ang II increases the association of KLF5 with c-Jun.** (A) VSMCs were stimulated with Ang II (100 nM) for different times (0, 0.5, 1 and 3 h) and then lysed by lysis buffer. The lysates were immunoprecipitated with anti-KLF5 antibody. Pellets were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-c-Jun and anti-KLF5 antibodies. The supernatants were used as negative control. (B) The lysates of Ang II-stimulated (0, 0.5, 1 and 3 h) VSMCs were immunoprecipitated with anti-c-Jun antibody. The pellets were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-KLF5 or anti-c-Jun antibodies. The supernatants were used as negative control. (C) Effects of KLF5 and c-Jun on p21 transcriptional activity. 293A cells were transfected with an equal amount of p21 promoter-reporter plasmids and pRL-TK, pGL3-Basic, pMT-KLF5 or/and c-Jun expression plasmids for 24 h. Luciferase activity was determined using the dual-luciferase reporter assay system and normalized to pRL-TK plasmids. The data are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, compared with control. (D) Binding of KLF5 to wildtype TCE (Wt) but not mutant TCE (Mut) element in the p21 promoter, as detected by oligo pull-down assay combined with western blot analysis. Mutant TCE oligos (Mut) was used as controls for wildtype oligos (Wt). Ang II treatment was at 100 nM for 1 h. (E) Binding of GST-KLF5 but not GST-c-Jun fusion protein to the TCE site, as detected by oligo pull-down assay.

the DBD of KLF5 is important for its interaction with c-Jun, we mutated phosphorylation site (P8, S406A) within the DBD of KLF5 and examined the significance of this site for KLF5 interaction with c-Jun. We found that the interaction between KLF5 and c-Jun was reduced significantly when the serine in the potential phosphorylation site P8 was mutated to alanine (S406A) (Fig. 4D). To further determine the potential role of ERK1/2 signalling in regulating the interaction between KLF5 and c-Jun induced by Ang II, VSMCs were pretreated for 2 h with PD98059, and then stimulated with Ang II. Figure 4E shows that treatment of VSMCs with the ERK1/2 inhibitor PD98059 markedly attenuated the interaction between KLF5 and c-Jun induced by Ang II, suggesting that activation of ERK pathway is involved in Ang II-induced KLF5 phosphorylation and the interaction between KLF5 and c-Jun in VSMCs.

Activation of the ERK Pathway by Ang II Inhibits p21 Expression—293A cells were cotransfected with the p21 promoter-reporter plasmids, along with various combinations of expression vectors for KLF5, c-Jun or MEK1. As expected, the strongest suppression was observed when all three expression vectors for KLF5, c-Jun and MEK1 were cotransfected. The p21 promoter activity returned to baseline after PD98059 treatment (Fig. 5A and B), further suggesting that Ang II stimulates functional interaction between KLF5 and c-Jun through ERK pathway, subsequently leading to synergistic suppression of the p21 transcriptional activity.

DISCUSSION

In the present study, we have shown that Ang II induced KLF5 phosphorylation via ERK pathway in VSMCs, which results in an increase of the interaction of KLF5 with c-Jun. In addition, we found that KLF5 and c-Jun cooperatively inhibit p21 expression in Ang II-stimulated VSMCs. These results may represent

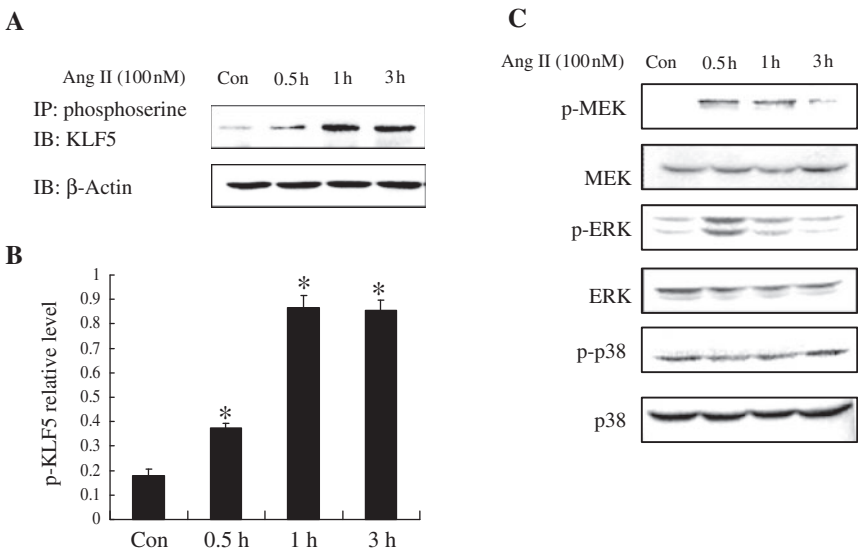

Fig. 3. **Ang II induces KLF5 phosphorylation by activating ERK1/2 signalling pathway.** (A) Cell extracts were immunoprecipitated by anti-phosphoserine antibody and immunoblotted with anti-KLF5 antibody. β-Actin was detected by immunoblotting with anti-β-Actin antibody. (B) Densitometric scanning. Data are mean ± SD from three independent experiments. $^{*}P < 0.05$, compared with control (0 h). (C) VSMCs were stimulated with Ang II (100 nM) for 0, 0.5, 1 and 3 h and then lysed by lysis buffer. The whole cell lysates were analyzed by western blotting with antibodies against phospho-MEK, MEK, phospho-ERK1/2, ERK1/2, phospho-p38 and p38.

an important mechanism for Ang II effects on VSMC proliferation.

KLF5 is a transcription factor that promotes proliferation of multiple cell types, including the intestinal epithelium (*6*) and SMCs (*4*). Recent studies have shown that regulation of KLF5 activity can occur on multiple levels, including interaction with cofactors and modification (*e.g.* phosphorylation and acetylation), which results in additional regulation affecting the specificity of actions of KLF5. For example, a physical association between KLF5 and PIAS1 was documented by both yeast two-hydrid and co-immunoprecipitation. PIAS1 binds to both the amino- and carboxyl-terminal regions of KLF5 and significantly increases KLF5-regulated cyclin D1 and Cdc2 promoter activities, thus influences the ability of KLF5 to regulate cell cycle-related genes (*20*). Nagai and his colleagues found that the oncogenic regulator SET inhibits KLF5-mediated transactivation of the KLF5-responsive smooth muscle embryonic type myosin heavy chain-B (SMemb/NMHC-B) gene promoter and PDGF-A promoter via interacting with DBD of KLF5. On the other hand, the coactivator/acetylase p300 interacts with acetylated KLF5 DBD, and activates its transcription (*18*). Consequently, a novel finding was reported that deacetylase (histone deacetylase 1, HDAC1) binds to the first zinc finger of KLF5, which is the same region where p300 interacts with KLF5, and negatively regulates the transcriptional activity of KLF5 via inhibiting interaction of p300 with KLF5 (*21*). Although several lines of evidence suggest that Ang II affects the expression of KLF5 and c-Jun (*9*, *13*), it remains unclear whether there is a functional interaction of KLF5 with c-Jun in regulating cell cycle-related genes.

In our study, we identified that Ang II rapidly upregulated the expression of transcription factors KLF5 and c-Jun, and exposure of VSMCs to Ang II rapidly and strongly stimulated KLF5 phosphorylation and the interaction of KLF5 with c-Jun in a time-dependent manner. Furthermore, Ang II-induced KLF5 phosphorylation is mediated through ERK signal pathway, and the active ERK1/2 directly phosphorylate KLF5. We demonstrated that phosphorylation site P8 (S406) is important for the interaction between KLF5 and c-Jun, which is consistent with recent reports (*18*, *19*). Treating VSMCs with PD98059, the ERK inhibitor, inhibited ERK activation and KLF5 phosphorylation as well as the interaction between KLF5 and c-Jun, subsequently leading to the repression of p21 expression.

Because there are the several Sp1-binding sites in the human p21 promoter region and c-Jun can regulate p21 expression through Sp1 sites (*17*), we investigated whether there is any cross-talk between KLF5 and c-Jun in repression of p21 expression by Ang II. Reporter analysis showed that both KLF5 and c-Jun synergistically repressed the promoter of p21. We speculate that KLF5 binds to its *cis*-elements in the p21 promoter, and meanwhile interacts with c-Jun and synergistically represses the p21gene expression in Ang II-induced VSMCs. We also found that silencing of KLF5 expression by KLF5-specific siRNA neutralized the inhibitory effects of Ang II on p21 expression. This interaction of KLF5 with c-Jun in binding complexes on DNA elements may be important for maximal genes repression. p21 has growth-permissive effects in VSMCs at low levels by promoting cdk–cyclin complex formation, but growth-inhibitory effects at higher levels (*2*, *22*, *23*). Thus, the inhibition of p21 by Ang II is involved in Ang II-induced

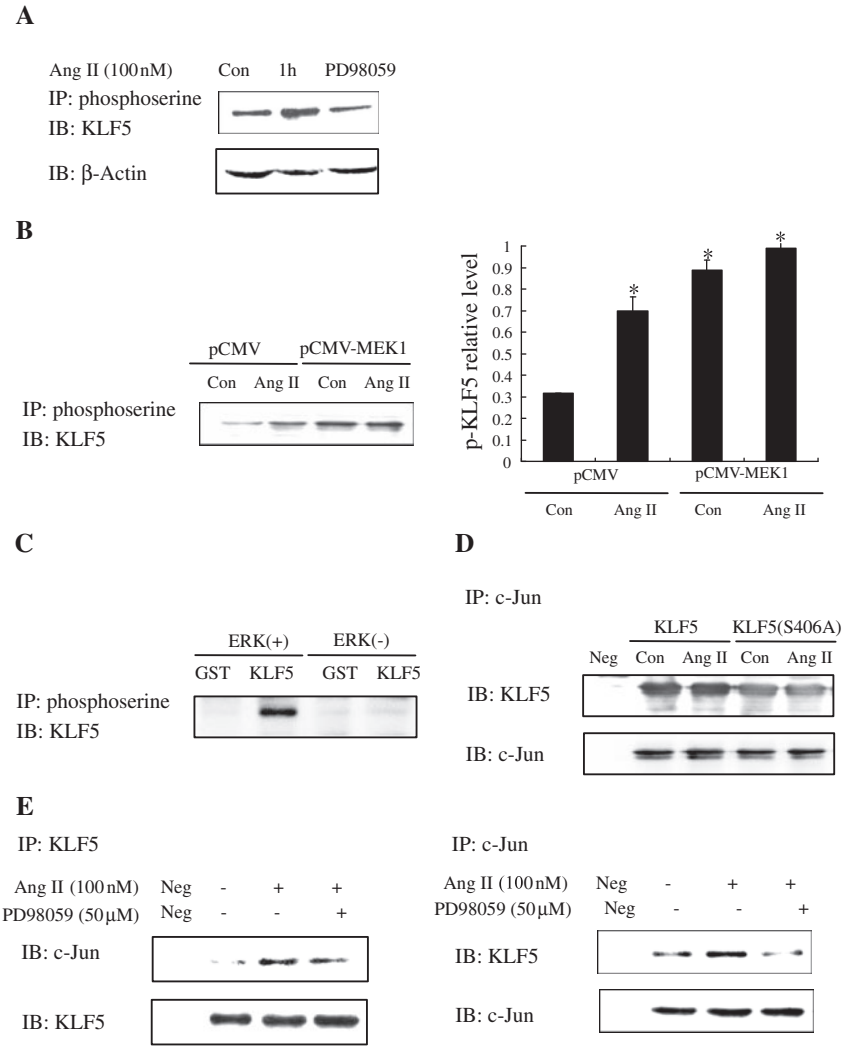

Fig. 4. **Effects of the ERK inhibitor PD98059 on Ang II-induced KLF5 phosphorylation and interaction of KLF5 with c-Jun.** (A) Growth-arrested VSMCs were pre-treated without or with PD98059 (50 μM) for 2 h before stimulation with Ang II (100 nM) for 1 h, cell extracts were immunoprecipitated with anti-phosphoserine antibody, and immunoblotted with anti-KLF5 antibody. β-Actin was used as loading control. (B) Cultured VSMCs were transfected with pCMV-MEK1 or pCMV for 24 h, and then stimulated with Ang II (100 nM) for 1 h. Cells were harvested, and immunoprecipitation was performed to detect the phospho-KLF5. The empty vector (pCMV) was used as a control. Left, blots from a representative experiment are shown. Right, densitometry analysis was carried out. $^{*}P < 0.05$, compared with control. (C) The GST-KLF5 fusion protein as a substrate was incubated with the active ERK for 15 min at 37°C, then the mixture were immunoprecipitated by anti-phosphoserine antibody and immunoblotted with anti-KLF5 antibody. (D) PMT-KLF5 or PMT-KLF5 S406A (P8 mutant) were transfected into VSMCs. Interaction between KLF5 or KLF5 S406A and c-Jun was examined by co-immunoprecipitation and western blot analysis with the indicated antibodies. (E) Interaction between KLF5 and c-Jun was examined by co-immunoprecipitation and western blot analysis with the indicated antibodies.

VSMC proliferation. Yang and colleagues found that KLF4 and KLF5 can upregulate the p21 expression following UV irradiation in TE2 cells (*24*). Several reports indicate that AP-1 not only mediates the response to environmental stimuli but also exerts a specific role during the cell cycle (*10–12*). c-Jun has been reported to directly regulate the p21 promoter, both positively and negatively, via an Sp1 site (*11*, *17*, *25*). Presumably because of time and space constraints, functional interaction of protein–protein may be different depending on cell types. In addition to the p21 promoter, this process may also be important for activation of other cell cycle genes, including cyclin D1, which also contain AP-1--binding sites and GC rich elements in their respective promoters.

In conclusion, we have identified that Ang II induced KLF5 phosphorylation mediated by the ERK signalling in VSMCs, which in turn stimulates the interaction of KLF5 with c-Jun, subsequently leads to the suppression of p21 expression. Further experiments will determine how MEK/ERK signalling regulates the interaction of KLF5 with c-Jun as well as whether altered phosphorylation of KLF5 affects the DNA binding activity of KLF5.

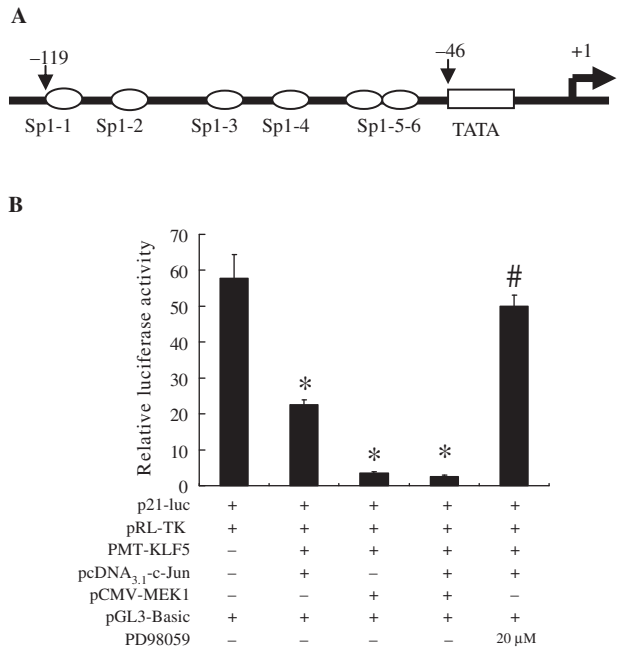

Fig. 5. **Activation of ERK1/2 pathway inhibits p21 promoter activity.** (A) Schematic representation of the Sp1-binding sites in the p21 promoter region. (B) 293A cells were transfected with the p21 promoter–reporter plasmids, pRL-TK, pGL3-Basic and expression plasmids for KLF5, c-Jun, or MEK1 for 6 h, and then stimulated with Ang II (100 nM) for 24 h in the presence or absence of PD98059 (20 μM). Luciferase activity was determined using the dual-luciferase reporter assay system and normalized to pRL-TK plasmids. The data are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, compared with control. $^{\#}P < 0.05$, compared with 293A cells transfected by PMT-KLF5 and pcDNA$_{3.1}$–c-Jun.

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CONFLICT OF INTEREST

None declared.

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