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Krüppel-like Factor 4 is Required for the Expression of Vascular Smooth Muscle Cell Differentiation Marker Genes Induced by All-Trans Retinoic Acid

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Krüppel-like factor 4 (KLF4) is involved in phenotypic modulation of vascular smooth muscle cells (VSMCs). All-trans retinoic acid (ATRA) inhibits VSMC proliferation and induces VSMC differentiation. However, the role of KLF4 in ATRA-elicited VSMC phenotypic modulation remains unclear. Here, we show that treatment of VSMCs with ATRA resulted in significant inhibition of proliferation and migration of VSMCs, as well as up-regulation of KLF4 and the VSMC differentiation marker genes SM22α and SM α-actin (α-SMA). At the same time, the KLF4 target gene p53 was up-regulated, while the VSMC dedifferentiation marker gene nonmuscle myosin heavy chain-B (SMemb) was down-regulated. We also show that overexpression of KLF4 in VSMCs increased the expression of p53, SM22α and α-SMA, but decreased the expression of SMemb and VSMC proliferation and migration. Silencing of KLF4 expression by KLF4-specific small interfering RNA (siRNA) abrogated the inducing effects of ATRA on p53, SM22α and α-SMA expression and neutralized the inhibitory effects of ATRA on SMemb expression and VSMC proliferation and migration. Thus, our data suggest that KLF4 is required for the expression of VSMC differentiation marker genes induced by ATRA and that this transcription factor is one of the key mediators of retinoid actions in VSMCs.

Key words: all-trans retinoic acid, expression, KLF4, marker gene, VSMC.

Abbreviations: ATRA, all-trans retinoic acid; α-SMA, SM α-actin; DMEM, Dulbecco's modified Eagles medium; DMSO, dimethyl sulfoxide; KLF4, Krüppel-like factor 4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl; SMemb, smooth muscle embryonic type myosin heavy chain-B; siRNA, small interfering RNA; VSMCs, vascular smooth muscle cells.

Vascular smooth muscle cells (VSMCs) retain remarkable plasticity and can undergo reversible changes in phenotype in response to changes in local environmental cues (*1–6*). In normal mature blood vessels, VSMCs are mainly differentiated cells which express smooth muscle (SM)-specific contractile proteins α-SM actin (α-SMA) and SM22α (*2*). In response to pathogenic vascular injuries, such as atherosclerosis and restenosis after angioplasty, VSMCs can down-regulate SM contractile proteins and revert to dedifferentiated phenotype. In the dedifferentiated VSMCs, the expression of embryonic type MHC (SMemb/NMHC) gene (dedifferentiated marker gene) is up-regulated (*3*). Indeed, phenotypic switching involves the changes in the expression of many genes; however, the exact mechanisms by which the phenotypic modulation of VSMCs is regulated have not been fully understood (*4*). Therefore, exploring the molecular mechanisms of regulation of VSMC phenotypic switching is helpful for therapy of these remodelling cardiovascular diseases.

All-trans retinoic acid (ATRA), a derivative of vitamin A, is known to regulate growth and induce differentiation of normal and malignant cells (*7*, *8*). Many retinoids have been pursued from therapeutic standpoints with the aim of chemotherapy of cancer, leukemia and prevention of atherosclerosis (*9*). Recent studies have shown that retinoids can also inhibit proliferation of human coronary smooth muscle cells (*10*) and in-stent neointima formation in rabbit stent-placement models (*11*). Thus, ATRA may also serve as a potential anti-proliferative and a differentiation-promoting agent in VSMCs.

Krüppel-like factors (KLFs) are a subclass of the zinc finger family of transcription factors. Many studies have revealed that KLFs play important roles in cell growth, proliferation, differentiation and embryogenesis as well as carcinogenesis (*12*). Previous studies show that both KLF4 and KLF5 are notable factors that have been implicated in developmental as well as pathological vascular processes. KLF4 is highly expressed in terminally differentiated, post-mitotic epithelial cells of the intestinal tract (*13–15*). In serum-deprived quiescent NIH3T3 cells, the level of KLF4 mRNA is high, while in exponential proliferating cells, it is nearly undetectable. When serum-deprived cells are stimulated into proliferation by the addition of fresh serum, the level of KLF4 mRNA decreases (*13*). KLF4 is up-regulated by platelet-derived growth factor (PDGF)-BB in cultured VSMCs and in response to vascular injury *in vivo* (*16*). A recent study showed that KLF4 can activate the transcription of

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p53 and inhibit VSMC proliferation (*17*). Therefore, KLF4 may be a key effector of ATRA-induced phenotypic switching of VSMCs.

ATRA can inhibit VSMC proliferation and induce VSMC differentiation, while KLF4 is involved in VSMC phenotypic switching. However, the role of KLF4 in ATRA-elicited VSMC phenotypic modulation remains unclear. Here, we report that ATRA inhibited VSMC proliferation and migration by up-regulating expression of KLF4 and VSMC differentiation marker genes SM22α and α-SMA. Our data suggest that KLF4 is required for the expression of VSMC differentiation marker genes SM22α and α-SMA induced by ATRA.

MATERIALS AND METHODS

Construction of recombinant plasmids and adenovirus vectors—The mouse SM22α promoter region from –527 to +174 was subcloned into the pGL3-Basic vector to generate the reporter construct pGL3-SM22α-luc. Full-length cDNA of mouse KLF4 was subcloned into a mammalian expression vector, pEGFP-N1, to generate pEGFP-KLF4. The full-length cDNA of mouse KLF4 was also subcloned into pAd/CMV/V5-DEST Gateway Vector (Invitrogen, Carlsbad, CA) to make the KLF4 adenovirus Ad-KLF4, according to the manufacturer's protocol. All of these clones were verified by sequencing (data not shown).

Cell culture and stimulation by ATRA—VSMCs were isolated from the thoracic aorta of 90–110 g male Sprague–Dawley rats as described previously (*18*). VSMCs were grown in low glucose Dulbecco's-modified Eagle's medium (DMEM) (Invitrogen), and the 293A cell line was maintained in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.075% sodium bicarbonate and 200 μg/ml L-glutamine. All the cells were maintained in 5% CO_2 humidified atmosphere at 37°C.

Vascular smooth muscle cells were grown to 70% confluence before serum deprivation for 16 h, then treated with 5, 10 or 20 μM of ATRA or ethanol as a control for the stated periods in DMEM containing 5% FBS.

Transfection and infection—The cultured VSMCs were grown to 60% confluence, and then transfected with rat KLF4-specific siRNA (5′-CACCUUGCCUUACACAU GA-3′) or non-specific NS-siRNA (sc-37007, Santa Cruz) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Twenty hours after transfection, the VSMCs were treated with ATRA or vehicle for 48 h. Alternatively, VSMCs were infected with KLF4 adenovirus (Ad-KLF4) or control virus (Ad) for 48 h. Then cells were collected for assays, as described.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described previously (*19*). In brief, VSMCs (2×10^7 cells) were treated with 1% formaldehyde for 15 min at room temperature to cross-link associated protein to DNA. Then the crosslinking was terminated by the addition of glycine to a final concentration of 0.125 M. Cells were lysed with 300 μl of radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate and protease inhibitors). Cell lysates were then sonicated to yield chromatin fragments of ~600 bp, as assessed by agarose gel electrophoresis. After centrifugation at 13,000 rpm for 10 min, the supernatants were precleared for 15 min at 4° by incubation with 30 μl of protein A-Sepharose beads (Sigma) and sheared salmon sperm DNA. After centrifugation for 5 min at 13,000 rpm, the supernatants were divided into three equal shares: one was used to extract DNA as input, the other were subjected to immunoprecipitation by rocking overnight at 4°C with or without 2 μg KLF4 antibody (sc-20691, Santa Cruz). Immune complexes were then precipitated with protein A-Sepharose beads and sheared salmon sperm DNA. The beads were collected by centrifugation and washed twice sequentially with radioimmune precipitation assay wash buffer I (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), wash buffer II (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), wash buffer III (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA), and wash buffer IV (10 mM Tris-HCl, pH 8.1, and 1 mM EDTA). The immunoprecipitates were eluted by adding 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) to the washed beads followed by incubation at 65° overnight. After centrifugation for 10 min at 13,000 rpm, DNA was extracted from the supernatants by phenol–chloroform extraction and ethanol precipitation. PCR was performed with the following primers: SM22α, 5′-CAAGGAAGGTTTTCGTGGTC-3′ and 5′-AAG GCTTGGTCGTTTGTGG-3′ (−307∼+65); α-SMA, 5′-ATCC CCATAAGCAGCTGAAC-3′ and 5′-CTTACCCTGATGGC GACTG-3′ (−229∼+52).

Luciferase assays—293A cells were grown to 70% confluence, and then transfected in triplicate with pGL3-SM22α-luc, pEGFP-KLF4 or pGL3-Basic, along with pRL-TK. After 48 h of transfection, the luciferase activity was measured with the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) according to the manufacturer's manual. The relative luciferase activities compared with the luciferase activities of pRL-TK were presented as mean ± S.E.

Western blotting—Lysates from VSMCs were prepared with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40). Equal amounts of protein (60 ∼ 100 μg) were separated by 12% SDS-PAGE, and electro-transferred to a PVDF membrane. Membranes were blocked with 5% BSA for 2 h at room temperature, and incubated with the primary antibodies, KLF4 (1 : 400 dilution, sc-20691, Santa Cruz), SM22α (1 : 300 dilution, sc-50446, Santa Cruz), α-SMA (1 : 500 dilution, sc-32251, Santa Cruz), SMemb (1 : 300 dilution, sc-33729, Santa Cruz), p53 (1 : 300 dilution, sc-126, Santa Cruz) or β-actin (1 : 1000 dilution, sc-47778, Santa Cruz) overnight, respectively, and then with the respective secondary antibody for 2 h. Proteins were detected with the enhanced chemiluminescence (ECL) detection system. The experiments were replicated three times.

RNA preparation and quantitative real-time RT-PCR—Total RNA from cultured VSMCs was isolated using Trizol reagent (Invitrogen) according to

the manufacturer's protocol. RNA (2 μg) was reverse-transcribed using PrimeScript™ RT reagent Kit (DRR037S, TaKaRa Bio, Japan), and the single stranded cDNA was amplified by quantitative real-time RT-PCR using SYBR® Premix Ex Taq™ Kit (DRR041S, TaKaRa Bio, Japan) on an ABI PRISM 7000 Sequence Detection PCR System (Applied Biosystems, U.S.A.). The following primers were used: KLF4, 5′-GGCTGATGGGCAAG TTTGTG-3′ (sense) and 5′-CAAGTGTGGGTGGCTGTT CT-3′ (antisense); SM22α, 5′-ATCCAAGCCAGTGAAG GTGC-3′ (sense) and 5′-CCTCTGTTGCTGCCCATTTG-3′ (antisense); α-SMA, 5′-CGATAGAACACGGCATCATCA-3′ (sense) and 5′-TTCGTAGCTCTTCTCCAGGGA-3′ (antisense); p53 5′-GTCATCTTCCGTCCCTTCTC-3′ (sense) and 5′-ACAAACACGAACCTCAAAGC-3′ (antisense); SMemb, 5′-CGCTTTGGCAAGTTTATCCG-3′ (sense) and 5′-CAGCACTGAAGACACGACTT-3′ (antisense); β-actin, 5′-CAGGGTGTGATGGTGGG-3′ (sense) and 5′-GGAAG AGGATGCGGCAG-3′ (antisense). The mRNA expression of the target gene was normalized to β-actin.

Cell migration assay—VSMCs from different treatment groups were subjected to wound healing assays and transmembrane migration assays as described previously (*20*). The VSMC migration activity was expressed as the number of cells migrating into the wound and the lower surface of membrane.

MTT assay—VSMCs cultured in 96-well plates were treated as indicated in the figures. MTT (20 μl, 5 mg/ml) was added to each well for 4 h at 37°C. The supernatant was removed and cells were washed once with PBS. DMSO (200 μl) was added to each well, including control wells, the plate was swirled gently for 10 min, and then the absorbance was measured at 570 nm by microplate reader. The experiment was repeated three times.

Data statistics—Data are presented as means ± S.E. Statistical analyses were carried out with the SPSS 11.5 statistical software package (Chicago, IL). Our primary statistical test was the one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

ATRA inhibits proliferation and migration of VSMCs—To elucidate the mechanism whereby ATRA induces VSMC differentiation and inhibits VSMC proliferation and migration, we first determined the effects of ATRA on VSMC proliferation and migration by MTT and cell migration assays. As shown in Fig. 1A, treatment of

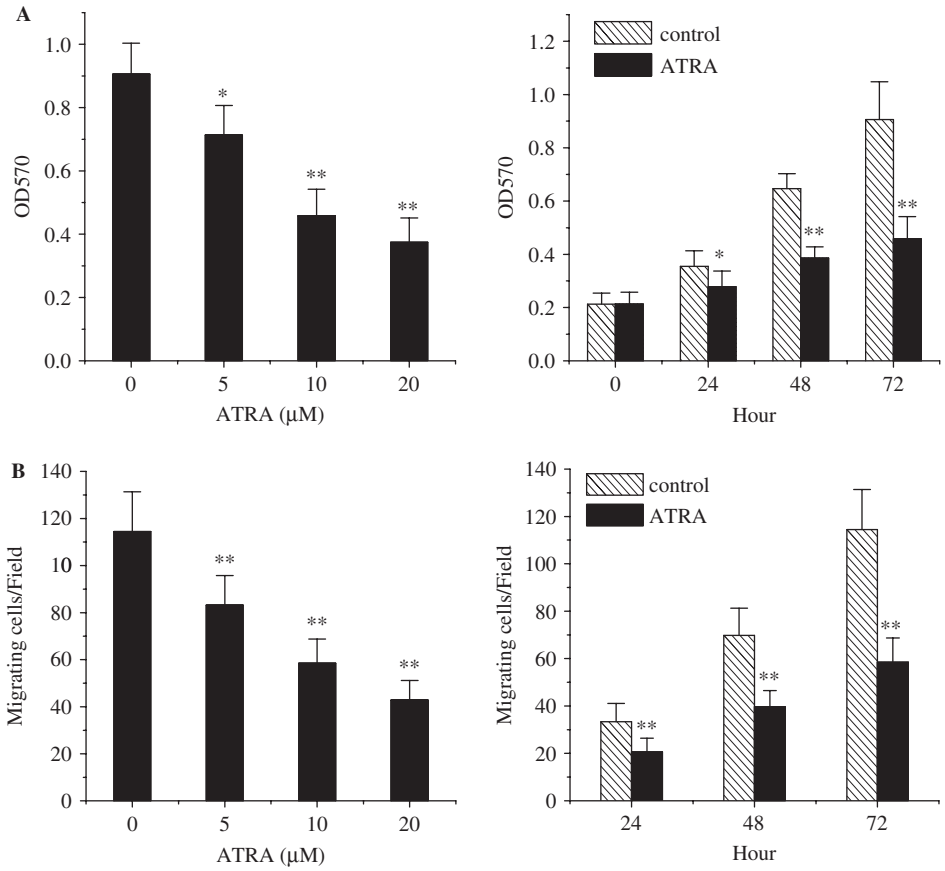

Fig. 1. **The effects of ATRA on VSMC proliferation and migration.** (A) VSMCs were seeded in 96-well plates and grown to 70% confluence, followed by serum deprivation for 16 h, and then treated with 5, 10 or 20 μM of ATRA or ethanol control for 72 h in DMEM containing 5% FBS (left); or 10 μM of ATRA for 24, 48 and 72 h. Thereafter, MTT assays were performed as described in the Materials and Methods, respectively. $^{*}P < 0.05$ or $^{**}P < 0.01$, compared with ethanol control. (B) Confluent VSMCs grown on coverslips in DMEM containing 5% FBS were scraped and treated with ATRA or ethanol control as indicated in the figures. Cells migrating into the wound were counted at various times. $^{**}P < 0.01$, compared with ethanol control.

VSMCs with ATRA from 5 to 20 μM for 72 h (left) resulted in a significant reduction of cell proliferation in a dose-dependent manner ($P < 0.05$ or $P < 0.01$, $n = 6$). A significant inhibition of proliferation was observed by treating VSMCs with 10 μM of ATRA. Therefore, we used a concentration of 10 μM of ATRA to perform a time-course study (right). VSMCs were treated with 10 μM of ATRA or ethanol as a control for 24, 48 or 72 h, and then the cell proliferation was determined by MTT assay. The results showed that ATRA markedly repressed VSMC proliferation in a time-dependent fashion ($P < 0.05$ or $P < 0.01$, $n = 6$). Figure 1B shows that ATRA was also capable of inhibiting VSMC migration, as measured by a wound healing assay, in a dose-dependent and time-dependent fashion ($P < 0.01$, $n = 6$).

ATRA enhances the expression of KLF4, p53 and differentiation marker genes, and simultaneously suppresses the expression of dedifferentiation marker gene SMemb—To determine the molecular mechanism by which ATRA inhibits VSMC proliferation and migration, we investigated the effects of ATRA on VSMC phenotype by assessing the expression of KLF4, p53, SMemb, SM22α and α-SMA. As shown in Fig. 2A, treatment of VSMCs for 72 h with a range of ATRA concentrations from 0 to 20 μM resulted in increased protein levels of KLF4, p53, SM22α and α-SMA in a dose-dependent manner, and decreased level of dedifferentiation marker SMemb. In addition, when VSMCs were treated with 10 μM of ATRA from 0 to 72 h, the protein levels of KLF4, p53, SM22α and α-SMA sustainedly increased in a time-dependent manner, at the same time, the level of SMemb was decreased. As expected, these changes were also reflected in their mRNA level as measured by quantitative real-time PCR (Fig. 2B and C). These results suggest that ATRA inhibits VSMC proliferation by inducing VSMC differentiation via increasing the expression of differentiation marker genes and decreasing the expression of dedifferentiation marker gene, and that KLF4 and p53 are involved in differentiation processes in VSMCs.

Overexpression of KLF4 up-regulates the expression of p53 and differentiation markers—The expression of SM22α and α-SMA, VSMC differentiation marker genes, was induced by ATRA in VSMCs. KLF4, known as a transcription factor that controls the expression of VSMC marker genes, was also up-regulated by ATRA. However, the role of KLF4 in the expression of VSMC differentiation marker genes induced by ATRA is still unknown. In order to reveal the effect of KLF4 on VSMC differentiation marker genes, we constructed an adenovirus expression system which was used to overexpress the KLF4 gene in VSMCs. As shown in Fig. 3A, dramatically increased expression of KLF4 was observed in VSMCs infected with Ad-KLF4. Furthermore, we demonstrated that overexpression of KLF4 in VSMCs significantly increased mRNA and protein expression of SM22α, α-SMA and p53, and significantly decreased SMemb expression, as shown by real-time RT-PCR and Western blotting, respectively (Fig. 3A and B).

KLF4 is known to bind a CACCC-like motif present in both the rat and mouse α-SMA and SM22α promoter regions, where the first 445 base pairs have been shown

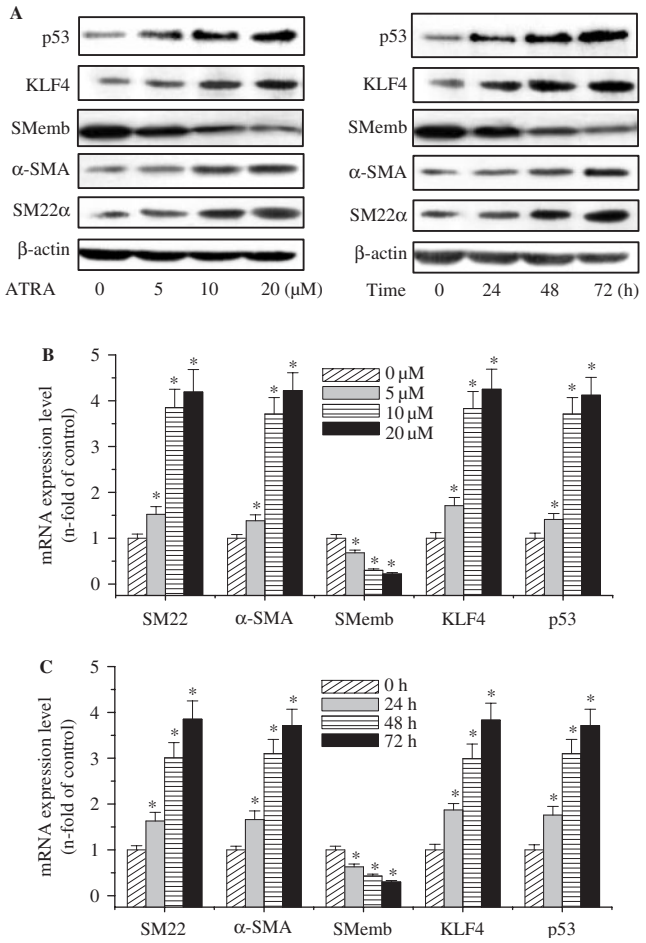

Fig. 2. **The effects of ATRA on the expression of KLF4, p53, SM22α, α-SMA and SMemb in cultured VSMCs.** (A) Western blot analysis shows expression of KLF4, p53, SM22α, α-SMA and SMemb. VSMCs were treated for 72 h with 0 to 20 μM of ATRA (left), or 10 μM of ATRA for 24, 48 or 72 h (right). A 100 μg of protein was loaded per lane and examined by Western blot using antibodies against KLF4, p53, SM22α, α-SMA, SMemb or β-actin (as a loading control). (B and C) Real-time RT-PCR analysis shows relative mRNA levels of KLF4, p53, SM22α, α-SMA and SMemb. Treatments of VSMCs with ATRA were the same as above. Total RNA was isolated from VSMCs and subjected to real-time RT-PCR analysis by using specific primers of KLF4, p53, SM22α α-SMA and SMemb. β-actin was used as an internal control. The relative mRNA levels of each gene were expressed as fold increase over control. $^{*}P < 0.01$, compared with ethanol control or 0 h control.

to be sufficient to drive the expression of a linked reporter gene in all three muscle lineages (*21–23*). The promoter region of α-SMA and SM22α containing a CACCC-like motif and its reverse orientation sequence (*i.e.* GTGGG) previously shown to bind KLF4 (*24*) is illustrated in Fig. 3C. To investigate whether KLF4 binds both the α-SMA and SM22α promoters within intact chromatin, ChIP assays were performed. Chromatin DNA was immunoprecipitated with anti-KLF4 antibody, and recovered DNA was analysed by PCR using primers specific for the α-SMA and SM22α promoters containing CACCC-like motifs. As shown in Fig. 3D, DNA fragments containing CACCC-like sequence could

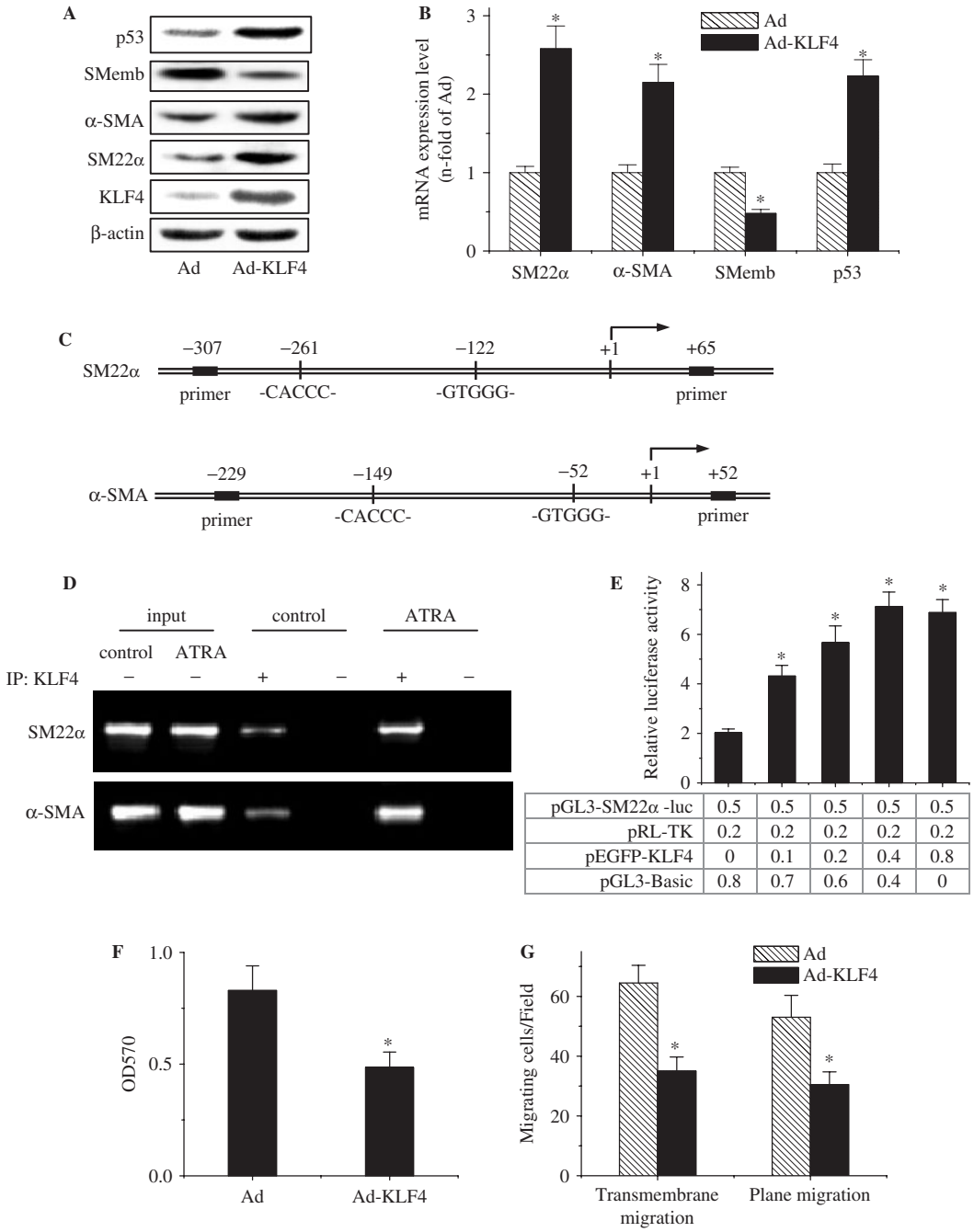

Fig. 3. **The effects of KLF4 overexpression on VSMC marker gene expression and proliferation and migration.** (A) 60 μg of protein from VSMCs infected with Ad-KLF4 or control Ad was subjected to Western blotting using antibodies against KLF4, p53, SM22α, α-SMA, SMemb or β-actin (as a loading control). (B) Real-time RT-PCR analysis shows relative mRNA levels of SM22α, α-SMA, SMemb and p53. VSMCs infected with Ad-KLF4 or control Ad were subjected to real-time RT-PCR analysis. β-actin was used as an internal control. The relative mRNA levels of each gene were expressed as fold increase over Ad control. $^{*}P < 0.01$, compared with Ad control. (C) Schematic representation of the KLF4-binding sites in SM22α and α-SMA promoter regions. The PCR primers used in ChIP assays were also schematically represented. (D) ChIP assays. VSMCs were treated with 10 μM ATRA or ethanol control for 48 h. ChIP assays were performed as described in Materials and Methods. The total input DNA was the supernatant from the no-antibody control. (E) Luciferase assays. 293A cells were transfected in triplicate with increasing amounts of pEGFP-KLF4 and the indicated amounts of pRL-TK, pGL3-SM22α-luc and pGL3-Basic. Cell lysates were subjected to luciferase activity assays, and the luciferase activity was normalized to pRL-TK activity. Values are the means ± S.E. $^{*}P < 0.01$, compared with control (0 μg of pEGFP-KLF4). (F) The effect of KLF4 overexpression on VSMC proliferation. VSMCs were transfected with Ad-KLF4 or control Ad for 48 h. MTT assay was performed as described in Materials and Methods. $^{*}P < 0.01$, compared with the cells transfected with Ad control. (G) The effect of KLF4 overexpression on VSMC migration. VSMCs transfected with Ad-KLF4 or control Ad for 48 h were subjected to wound healing assays and transmembrane migration assays. The cells migrating into the wound and the lower surface of membrane were counted. $^{*}P < 0.01$, compared with the cells transfected with Ad control.

be detected in the immunoprecipitates pulled by anti-KLF4 antibody. An increased binding of KLF4 to the α-SMA and SM22α promoters was observed in VSMCs treated by ATRA. As KLF4 binds the SM22α and α-SMA promoters, we then performed a luciferase reporter gene assay to assess whether KLF4 can stimulate the activity of the SM22α promoter. For this experiment, we co-transfected 293A cells with pEGFP-KLF4 and pGL3-SM22α-luc containing the SM22α promoter and a luciferase reporter gene, along with pRL-TK as an internal control. Figure 3E shows that KLF4 significantly increased the SM22α promoter activity in a dose-dependent fashion ($P < 0.01$, $n = 6$).

We further assessed the effects of overexpression of KLF4 on VSMC proliferation and migration by MTT and VSMC migration assays. As shown in Fig. 3F and G, the overexpression of KLF4 in VSMCs significantly decreased VSMC proliferation and migration by at least 40%, as compared with the cells infected with Ad control ($P < 0.01$, $n = 6$). These results suggest that KLF4 is a crucial mediator of ATRA-elicited VSMC differentiation marker gene expression and that proliferation and migration activity in the differentiated VSMCs is lower than the dedifferentiated cells.

Down-regulation of KLF4 mediated by siRNA abrogates the effects of ATRA on the expression of differentiation and dedifferentiation markers—The above findings showed that ATRA inhibits VSMC proliferation and migration by inducing the expression of VSMC differentiation marker genes. Interestingly, the overexpression of KLF4 in VSMCs also produced the same effects. To further determine whether ATRA-stimulated differentiation marker gene expression in VSMCs was due to the increased expression of KLF4, we transfected VSMCs with rat KLF4-specific siRNA or non-specific siRNA (NS-siRNA) to block the increase in endogenous KLF4 expression induced by ATRA. Figure 4A shows that in response to ATRA, the level of KLF4 protein was significantly attenuated in VSMCs transfected with KLF4-specific siRNA as compared to cells transfected with NS-siRNA. Importantly, ATRA-elicited increases in protein and mRNA of p53, α-SMA and SM22α were also reduced significantly in the cells transfected with KLF4-specific siRNA. In contrast, SMemb expression was up-regulated in the cells with suppression of KLF4 by siRNA. However, these changes were not observed in the cells treated without ATRA. (Fig. 4B and C). Figure 4D shows that KLF4-specific siRNA neutralized the cytostatic action of ATRA as shown by MTT assay. Fig. 4E shows that silencing of KLF4 expression in VSMCs by KLF4-specific siRNA significantly increased transmembrane and plane migration activities. These results suggest that VSMC phenotype modulation is closely related to KLF4 expression up-regulation.

DISCUSSION

Phenotypic modulation is a prerequisite for proliferation and migration of differentiated VSMCs and play a key role in vascular diseases. These diseases are characterized by changes in the gene expression of VSMC contractile proteins such as SM22α and α-SMA, as well as the pathophysiological proliferation and migration of VSMCs (*1*, *2*, *5*, *6*). Although much progress has been made in this field, the mechanisms controlling VSMC differentiation and phenotypic modulation are still poorly understood.

ATRA modulates growth, differentiation and apoptosis of cells (*7*, *25*). Previous studies have demonstrated that ATRA inhibits significantly cell proliferation, and this inhibitory effect is primarily due to an arrest in the G1 to S transition of the cell cycle (*7*). Recent studies show that synthetic retinoid Am80 suppresses smooth muscle phenotypic modulation and in-stent neointima formation by inhibiting KLF5 (*11*). In the present study, we have shown that KLF4 is also one of the key mediators of retinoid actions in VSMCs and is required for the expression of VSMC differentiation marker genes induced by ATRA.

KLF4 and KLF5 are two closely related members of the Krüppel-like factor family of transcription factors. They bind to a similar DNA sequence that has either a CACCC homology or is rich in GC content (*12*, *26*) and exert important biological effects on cellular proliferation and differentiation *in vivo* and *in vitro*. Despite a close homology and similar developmental and tissue patterns of expression, the two KLFs exert very different, often opposing, effects on regulation of gene transcription and cellular proliferation (*15*). For example, *in vitro*, expression of KLF4 is associated with a process of growth arrest (*13*), while that of KLF5 mainly accompanies cellular proliferation (*27*). In NIH3T3 cells, KLF4 is not expressed in differentiating cells and is preferentially expressed in growth-arrested states, but when cells are induced to enter the cell cycle, a decrease in expression levels are seen (*13*). In addition, KLF5 is involved in phenotypic modulation induced by ATRA in VSMC (*11*, *28*).

In our study, treatment of VSMCs with ATRA resulted in an phenotypic modulation from dedifferentiation to differentiation state, judging from up-regulation of differentiation marker genes SM22α and α-SMA, and down-regulation of dedifferentiation marker gene SMemb, as well as inhibition of cellular proliferation and migration (Figs. 1 and 2). We further demonstrated overexpression of KLF4 in VSMCs also exerted the same effects as ATRA (Fig. 3), while knockdown KLF4 by using siRNA suppressed ATRA-induced up-regulation of VSMC differentiation marker gene expression (Fig. 4). Since KLF4 was highly expressed in ATRA-treated VSMCs and KLF4 gene silencing abrogated the induction of ATRA on VSMC marker gene expression, we speculate that ATRA-induced up-regulation of VSMC differentiation marker gene expression is at least in part through up-regulation of KLF4.

So how does KLF4 regulate VSMC marker gene expression? One possibility is that KLF4 might positively regulate SM22α and α-SMA on one hand and negatively regulate SMemb on the other, which in turn controls the phenotype and differentiation state of VSMCs. It is also likely that KLF4 controls other unidentified genes, which may cumulatively affect VSMC phenotype.

Because p53 is essential for proliferation processes, the effect of ATRA and KLF4 on p53 gene expression was also investigated. The results showed that p53 mRNA

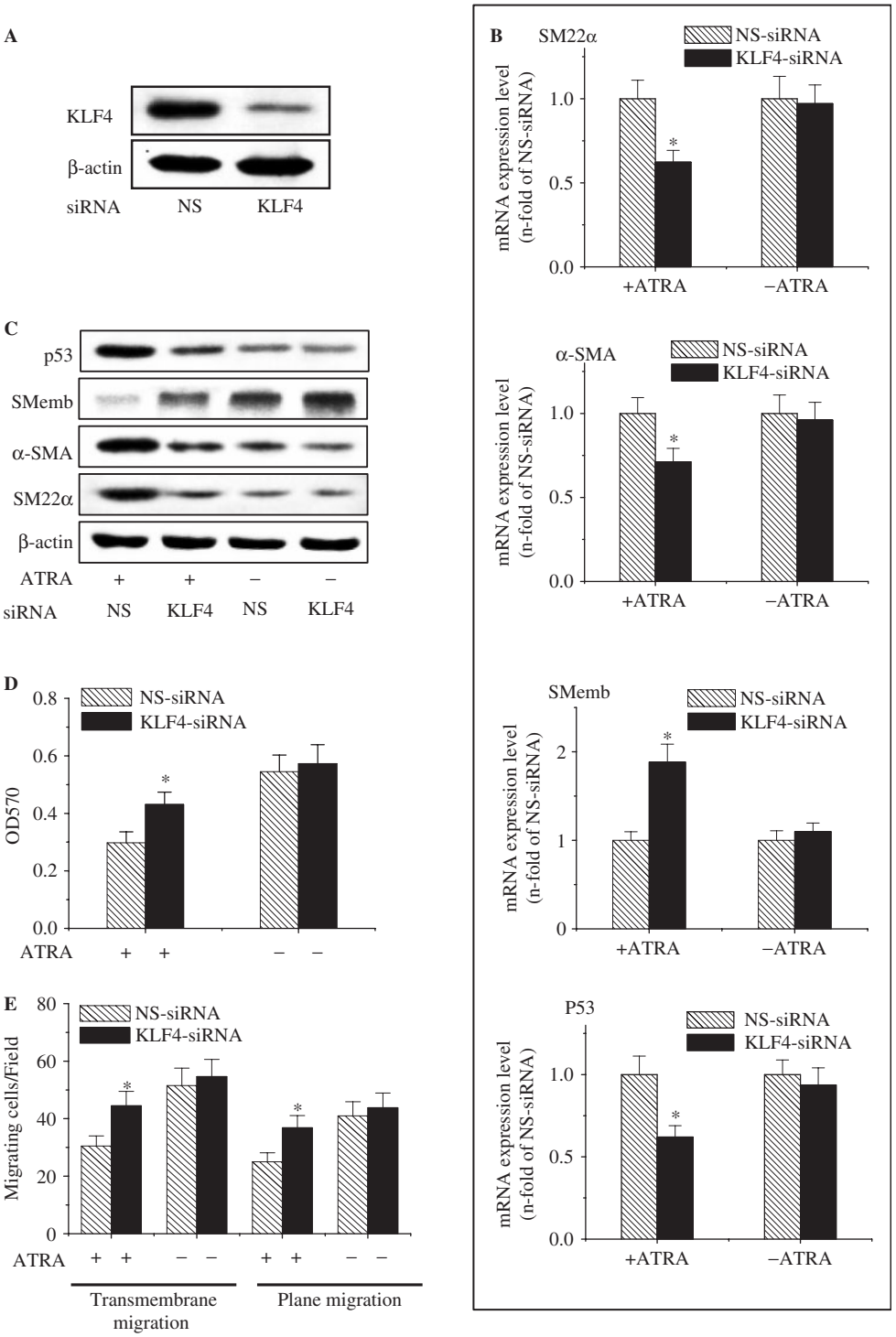

Fig. 4. **The effects of knockdown of KLF4 expression by siRNA on VSMC marker gene expression and proliferation and migration.** VSMCs were transfected with KLF4-specific siRNA or NS-siRNA, and after 20 h of transfection, cells were treated with 10 μM of ATRA or ethanol control for 48 h. (A) The cells were harvested and subjected to Western blot assay to determine the level of KLF4. (B) Real-time RT-PCR analysis shows relative mRNA levels of SM22α, α-SMA, SMemb and p53. β-actin was used as an internal control. The relative mRNA levels of each gene were expressed as fold increase over NS-siRNA control. $^{*}P < 0.01$, compared with NS-siRNA control. (C) The cells were harvested and lysed by lysis buffer, and cell lysates were subjected to Western blotting using antibodies against p53, SM22α, α-SMA, SMemb or β-actin (as a loading control). (D) The effect of KLF4 gene silencing by siRNA on VSMC proliferation. VSMCs were transfected with KLF4-specific siRNA or Ns-siRNA for 20 h, and then treated with 10 μM ATRA or ethanol control. MTT assay was performed as described in Materials and Methods. $^{*}P < 0.01$, compared with the cells transfected with NS-siRNA. (E) The effect of KLF4 gene silencing VSMC migration. VSMCs were treated as above. Cells migrating into the wound and the lower side of membrane were counted. $^{*}P < 0.01$, compared with the cells transfected with NS-siRNA.

and protein levels were increased by KLF4 overexpression and ATRA induction and decreased by KLF4 gene silencing. In VSMCs, p53 is a downstream target gene of KLF4, and studies have shown that KLF4 overexpression led to an enhanced p53 promoter activity and increased expression of the p53 target gene $P21^{WAF1/Cip1}$ (*17*). Therefore, p53 may be an essential mediator of KLF4-induced differentiation and KLF4-inhibited proliferation processes, indicating that KLF4 induces inhibition of proliferation of VSMCs which is mechanistically linked to a KLF4-induced enhancement of the expression of p53. Previous studies indicated that p53 is able to suppress the expression of matrix metalloproteinase-9 (MMP-9), collagenase-1 (MMP-1) and collagenase-3 (MMP-13) in some cell lines (*29*, *30*). In addition, since the promoter regions of MMP-9 and MMP-2 include some putative KLF4-binding sites, these genes might also be target genes of KLF4. This may be one of the reasons why ATRA treatment and KLF4 overexpression inhibits VSMC migration.

In conclusion, our study has characterized the differentiation-promoting effects of ATRA on cultured VSMCs *in vitro*, and we are the first to prove that KLF4 is required for VSMC differentiation induced by ATRA.

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