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All-trans retinoic acid increases KLF4 acetylation by inducing HDAC2 phosphorylation and its dissociation from KLF4 in vascular smooth muscle cells

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ABSTRACT

The zinc finger transcription factor Krüppel-like factor 4 (KLF4) has been implicated in vascular smooth muscle cell differentiation induced by all-trans retinoic acid (ATRA). However, the molecular mechanism whereby ATRA regulates KLF4 activity is still poorly understood. Here, we show that ATRA-induced histone deacetylase 2 (HDAC2) phosphorylation at Ser424 in VSMCs and inhibited the interaction of HDAC2 with KLF4. Inhibiting JNK by JNK inhibitor SP600125 or knockdown of JNK by JNK siRNA abrogated ATRA-induced HDAC2 phosphorylation and reversed ATRA-induced suppression of the interaction of HDAC2 with KLF4. We further demonstrated that HDAC2 directly deacetylated KLF4, and that KLF4 acetylation and binding activity of KLF4 to the SM22α promoter were significantly increased in ATRA-treated VSMCs. Collectively, our results indicate that ATRA induces HDAC2 phosphorylation mediated by JNK signaling, and thus causes HDAC2 dissociation from KLF4, subsequently leading to the increase in KLF4 acetylation.

Introduction

The Krüppel-like factors (KLFs) are DNA-binding transcriptional regulators that regulate a diverse array of cellular processes, including development, differentiation, proliferation and apoptosis [1,2]. KLF4 (GKLF/EZF), a member of this family, might function as an anti-proliferative factor or a prodifferentiation factor depending on the interaction partner and the cellular context in vascular smooth muscle cells (VSMCs). We and the others have previously shown that all-trans retinoic acid (ATRA) can induce KLF4 expression and inhibit proliferation and migration of VSMCs as well as suppress neointima formation after balloon injury [3]. Given the importance of KLF4 in the regulation of cellular functions, control of its activity has become the subject of considerable investigation. Like other zinc finger-containing proteins, the KLF4 activity may be regulated not only by de novo synthesis but also by posttranslational modification and protein–protein interactions. Recent studies suggest that KLF4 can be acetylated by the acetylase p300/CBP, and acetylation of KLF4 increases its transactivation [4,5]. Histone deacetylases (HDACs) play an important role in the regulation of transcription via triggering deacetylation of histones and some transcription factors in gastrointestinal tumors and cell lines [6]. It is not fully understood whether HDACs affect KLF4 activity and expression of differentiation marker genes in VSMCs. However, recent evidence shows that HDAC1 interacts with KLF5 and suppresses KLF5-dependent promoter activation [7]. HDAC2 is one of the most thoroughly studied HDACs, and can undergo different posttranslational modifications such as phosphorylation [8–10], acetylation [11], ubiquitination [12] and sumoylation [13]. However, little is known of the role of HDAC2 in the regulation of KLF4 activity in ATRA-induced VSMC differentiation.

In the present studies, we showed that ATRA increases KLF4 acetylation and its DNA-binding activity via inducing HDAC2 phosphorylation and dissociation from KLF4. We reported for the first time that JNK signaling mediates ATRA-induced HDAC2 phosphorylation.

Materials and methods

Cell culture and treatment. VSMCs were isolated from the thoracic aorta of 90–100 g male Sprague–Dawley rats as described previously [14]. VSMCs were maintained and passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin. In all experiments, only cell passages 3–5 were used. All experiments were initiated when the cells reached 70% confluence, except if stated otherwise. Cultured VSMCs were growth-arrested by incubation in serum-free DMEM for 24 h and then treated by 10 μM ATRA (Sigma) as indicated. For the inhibitor studies, cells were pretreated with 20 μM PD98059, 20 μM LY294002 (Promega) or 20 μM SP600125 (Promega) in DMSO for 2 h before the application of 10 μM ATRA.

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GST pull-down assays. Glutathione *S*-transferase (GST)–KLF4 fusion proteins were expressed in *Escherichia coli* and purified with GST beads (glutathione–agarose from Sigma) [15]. The cell lysates were incubated with equal amounts of GST beads containing GST–KLF4 or GST proteins for 2 h at 4 °C in the following buffer: 20 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 mM dithiothreitol, 0.4% Nonidet P-40, 0.5 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Beads were washed three times with the above-described buffer and then once with the same buffer containing 150 mM NaCl but lacking bovine serum albumin. After washing, bound proteins were resolved by SDS–PAGE and detected by Western blotting.

Western blot analysis. The cells were lysed with 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholic acid, and Complete Protease Inhibitor Cocktail Tablets (Roche). The protein content was determined with the Bio-Rad Bradford protein assay. Total protein (100 μg) from each sample was separated by 8% SDS–PAGE and electrotransferred onto a PVDF membrane (Millipore Corp.). The membranes were blocked with 5% milk in TTBS for 2 h at room temperature, and incubated overnight at 4 °C using the following primary antibodies: 1:500 rabbit anti-KLF4 antibody (Santa Cruz Biotechnology), 1:800 rabbit anti-HDAC2 antibody (Santa Cruz Biotechnology), 1:200 rabbit anti-phospho-Akt1/2/3 antibody (Santa Cruz Biotechnology), 1:500 mouse anti-Akt antibody (Santa Cruz Biotechnology), 1:800 rabbit anti-phospho-JNK antibody (Cell Signaling), 1:500 rabbit anti-JNK antibody (Cell Signaling), 1:500 mouse anti-phospho-ERK1/2 antibody (Santa Cruz Biotechnology), 1:500 rabbit anti-ERK1/2 antibody (Santa Cruz Biotechnology), 1:500 mouse anti-acetylated lysine antibody (Santa Cruz Biotechnology), 1:800 mouse anti-GFP antibody (Santa Cruz Biotechnology) and 1:1000 mouse anti-β-actin antibody (Santa Cruz Biotechnology). The membranes were then incubated for 1 h at room temperature with a 1:5000 dilution of anti-rabbit/horseradish peroxidase or anti-mouse/horseradish peroxidase (Santa Cruz Biotechnology) and developed with the Chemiluminescence Plus Western blot analysis kit (Santa Cruz Biotechnology) according to the manufacturer's instructions.

Co-immunoprecipitation assays. Co-immunoprecipitation was performed as described previously [16]. Briefly, cell extracts were first precleared with 20 μl of protein A-agarose (50% v/v). The supernatants were immunoprecipitated with mouse anti-phosphoserine antibody (Santa Cruz Biotechnology), anti-KLF4 antibody or anti-HDAC2 antibody 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*g* for 20 s at 4 °C. The pellets were washed four times with 500 μl IPH buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM PMSF) for 20 min each time at 4 °C. Bound proteins were resolved by SDS–PAGE, followed by Western blotting with the anti-acetylated lysine antibody, anti-HDAC2 antibody or anti-KLF4 antibody. The experiments were replicated three times at least.

Knockdown of JNK and HDAC2. siRNAs specific for rat JNK, rat HDAC2 and non-specific siRNA (NS siRNA) were designed and synthesized by Sigma. VSMCs were transfected with JNK siRNA (50 nM), HDAC2 siRNA (50 nM) or NS siRNA (50 nM) as control. Transfections were done using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions. Twenty-hours after transfections, the VSMCs were treated with or without 10 μM ATRA for 1 h. Then cells were harvested and lysed for Western blotting or co-immunoprecipitation assays.

Plasmids and transfection. Human HDAC2 plasmid was kindly gifted by Dr. Kim (Sungkyunkwan University School of Medicine). pEGFP-N1-HDAC2 plasmids that express GFP-tagged HDAC2 were constructed by standard recombinant DNA methods. Mutation of HDAC2 S424 to A ($HDAC2^{S424A}$) was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. All constructs were verified by DNA sequence analysis. Transfections were done, and cells were harvested and lysed as described above.

Chromatin immunoprecipitation (ChIP) assays. Chromatin immunoprecipitation (ChIP) assays were performed as described previously [17]. In brief, the crosslinking of associated protein to DNA in VSMCs was performed by treating cells with 1% formaldehyde for 10 min at room temperature. VSMCs 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). The lysates were then sonicated to yield chromatin fragments of ~600 bp. After centrifugation at 13,000*g* for 10 min, the supernatants were precleared for 15 min at 4 °C by incubation with protein A-Sepharose beads and sheared salmon sperm DNA. After centrifugation for 5 min at 13,000*g*, 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 anti-KLF4 antibody. Immune complexes were then precipitated with protein A-Sepharose beads and sheared salmon sperm DNA. The beads were collected by centrifugation and washed as described previously [3]. The immunoprecipitated chromatins were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). DNA was purified by phenol–chloroform extraction and ethanol precipitation. PCR was performed with primers spanned −307 to +65 of the SM22α promoter. The primer sequence was 5′-CAAGGAAGGTTTTCGTGGTC-3′ and 5′-AAGGCTTGGTCGTTTGTGG-3′.

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 less than 0.05 were considered statistically significant.

Results

ATRA induces HDAC2 phosphorylation via JNK-dependent signal pathway in VSMCs

To determine whether HDAC2 plays a role in ATRA signaling and function, we first studied the phosphorylation of HDAC2 in VSMCs in response to ATRA stimulation. The lysates of VSMCs treated with ATRA for 15, 30 and 60 min were immunoprecipitated with anti-phosphoserine antibody, and then phospho-HDAC2 was detected by Western blotting with anti-HDAC2 antibody. As shown in Fig. 1A, ATRA treatment time-dependently increased the levels of phospho-HDAC2 within 60 min in VSMCs. During the course of ATRA treatment, there was no significant change of HDAC2 expression. To determine which signal pathway mediates HDAC2 phosphorylation in VSMCs treated with ATRA, we examined the phosphorylation of ERK1/2, JNK and Akt by Western blotting with phosphospecific antibodies. Fig. 1B shows that ATRA markedly increased levels of phospho-JNK at 15 min after ATRA treatment, but did not affect levels of total JNK. In contrast, ATRA did not affect the phosphorylation of ERK and Akt. To further identify whether JNK activation mediates HDAC2 phosphorylation induced by ATRA, VSMCs were pretreated for 2 h with 20 μM PD98059 (a specific inhibitor of ERK), 20 μM LY294002 (a specific inhibitor of Akt) or 20 μM SP600125 (a specific inhibitor of JNK), and then treated with 10 μM ATRA for 1 h. As shown in Fig. 1C, inhibition of JNK by SP600125 blocked the ATRA-induced phosphorylation of HDAC2, whereas PD98059 and LY294002 had no significant effect on HDAC2 phosphorylation induced by ATRA. To further examine the role of JNK signaling in HDAC2 phosphorylation, endogenous JNK was knocked down from VSMCs using JNK siRNA. Western blot analysis indicated that both endogenous JNK and phospho-JNK

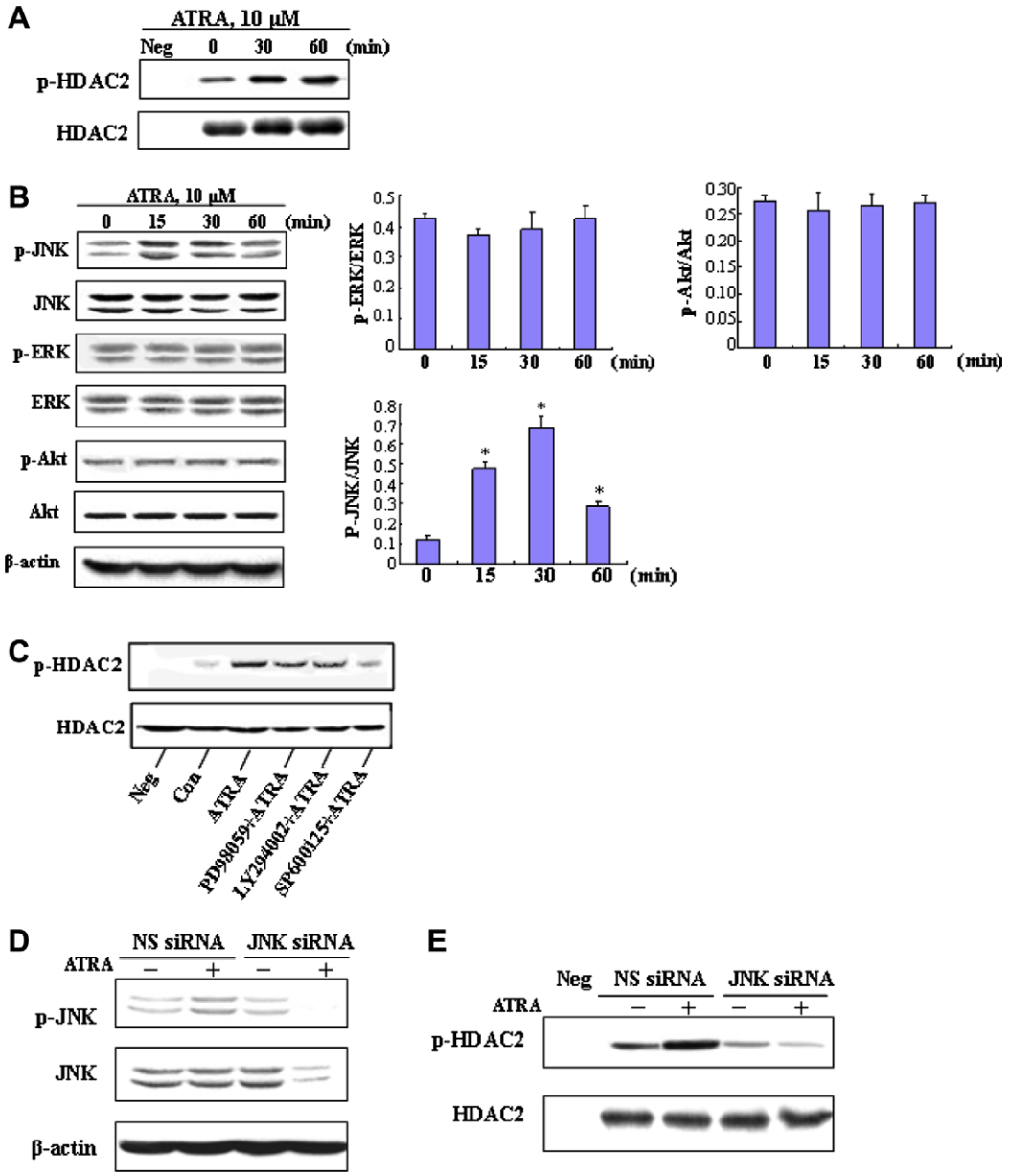

Fig. 1. ATRA induces HDAC2 phosphorylation through JNK activation in VSMCs. (A) ATRA-induced the phosphorylation HDAC2 in VSMCs, as determined by immunoprecipitation using anti-phosphoserine antibody. Blots for total HDAC2 are also shown. (B) Phospho-JNK, phospho-ERK, phospho-Akt were analyzed by Western blotting with their respective antibodies. Total protein of JNK, ERK and Akt were also shown. (C) Phospho-HDAC2 was detected in ATRA-induced VSMCs pretreated with PD98059, LY294002 or SP600125, respectively. (D) JNK protein was depleted in VSMCs transfected with JNK siRNA. (E) Phospho-HDAC2 was detected in ATRA-induced VSMCs transfected with JNK siRNA. Bar graphs represent the density ratio of phospho-bands to total protein bands ($n = 3$), $^{*}P < 0.05$ compared with the control.

(p-JNK) were decreased to a hardly detectable level after cells were transfected with JNK siRNA and treated by ATRA (Fig. 1D). Simultaneously, phospho-HDAC2 (p-HDAC2) was significantly reduced after transfection with JNK siRNA and ATRA treatment (Fig. 1E). Together, these results suggest that JNK-, but not ERK- and Akt-dependent signal pathway, is involved in the ATRA-induced HDAC2 phosphorylation in VSMCs.

ATRA inhibits the interaction between KLF4 and HDAC2

Because HDAC2 is known to interact with KLF4 [18], we wanted to determine if HDAC2 phosphorylation affects its interaction with KLF4. To do this, we performed a co-immunoprecipitation (Co-IP) assay by using crossing Co-IP. As shown in Fig. 2A, treating cells with ATRA for 30 and 60 min significantly reduced HDAC2 levels in the precipitates pulled down with anti-KLF4 antibody. Likewise, ATRA also reduced the levels of co-immunoprecipitated KLF4 pulled down with anti-HDAC2 antibody (Fig. 2B), suggesting that ATRA treatment inhibits the interaction of KLF4 with HDAC2. GST pull-down assays further identified that the interaction between KLF4 and HDAC2 was significantly inhibited in VSMCs treated with ATRA in a time-dependent manner (Fig. 2C). To further understand whether the change in HDAC2 phosphorylation is responsible for the decreased interaction between KLF4 and HDAC2, the effects of JNK inhibitor SP600125 on ATRA-induced suppression of the interaction between KLF4 and HDAC2 were detected. As shown in Fig. 2D, inhibiting JNK by SP600125 substantially reversed ATRA-induced suppression of the interaction between KLF4 and HDAC2, again suggesting that activation of JNK signaling is involved in ATRA-induced HDAC2 phosphorylation that subsequently leads to

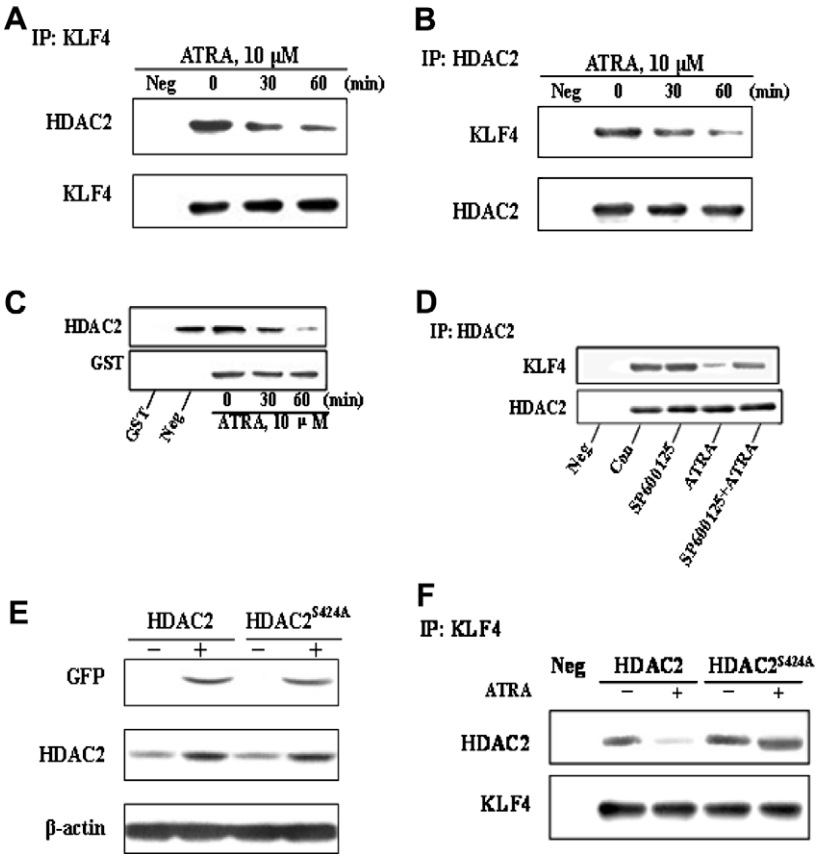

Fig. 2. ATRA inhibits the interaction between KLF4 and HDAC2. (A,B) The interaction between KLF4 and HDAC2 was reduced in VSMCs treated with ATRA for 0, 30 and 60 min respectively, as determined by co-immunoprecipitation assays using anti-KLF4 or anti-HDAC2 antibodies. (C) GST pull-down assays were performed to detect the ATRA effect on HDAC2 interaction with GST–KLF4 in vitro. (D) The interaction between KLF4 and HDAC2 was detected by co-immunoprecipitation assays in VSMCs treated with SP600125. (E) The expression of GFP and total HDAC2 protein in VSMCs transfected with pEGFP-N1-HDAC2 or pEGFP-N1-HDAC2^{S424A} plasmids were analyzed by Western blotting. (F) Phospho-HDAC2 in ATRA-induced VSMCs transfected previously with pEGFP-N1-HDAC2 or pEGFP-N1-HDAC2^{S424A} plasmids were examined by co-immunoprecipitation assays as described above.

the dissociation of KLF4 from HDAC2 in VSMCs. To determine whether KLF4 only binds to dephosphorylated HDAC2, we mutated the phosphorylation site (S424A) of HDAC2 and examined the significance of this site for KLF4 interaction with HDAC2. Western blot analysis showed that both GFP-HDAC2 and HDAC2^{S424A} were expressed in VSMCs after transfection with pEGFP-N1-HDAC2 or pEGFP-N1-HDAC2^{S424A} plasmids (Fig. 2E). The interaction of HDAC2 ^{S424A} with KLF4 was significantly increased after ATRA stimulation compared with wild HDAC2 (Fig. 2F), suggesting that HDAC2 phosphorylation induced by ATRA stimulates its dissociation from KLF4 and only dephosphorylated HDAC2 can bind to KLF4.

ATRA prevents the deacetylation of KLF4 by inhibiting the interaction between KLF4 and HDAC2

Because ATRA treatment inhibited the interaction between KLF4 and HDAC2, we decided to test whether the dissociation of KLF4 from HDAC2 affected KLF4 acetylation and KLF4-DNA-binding activity. As shown in Fig. 3A, the acetylation level of KLF4 time-dependently increased within 60 min after ATRA treatment, with a ~2-fold increase at 30 min and an ~18-fold increase at 60 min after ATRA treatment, and total KLF4 had no change during the course of ATRA stimulation in VSMCs. In addition, treating cells with SP600125 abrogated the ATRA-suppressed deacetylation of KLF4 through blocking JNK signaling (Fig. 3B), further suggesting that ATRA prevents KLF4 deacetylation by inducing HDAC2 phosphorylation mediated by JNK signaling as well as by leading to the dissociation of KLF4 from HDAC2. To examine whether HDAC2 directly deacetylate KLF4, endogenous HDAC2 was knocked down with HDAC2 siRNA (Fig. 3C). We found that the acetylation level of KLF4 significantly increased after endogenous HDAC2 was depleted (Fig. 3D), strongly suggesting that HDAC2 directly deacetylates KLF4.

Our previous study showed that ATRA induces the expression of VSMC differentiation marker genes, such as SM22α and SM α-actin by up-regulating KLF4 expression [5]. To determine whether the increase in KLF4 acetylation induced by ATRA is associated with a concomitant increase in binding of KLF4 to the SM22α promoter, ChIP assays were performed. As shown in Fig. 3E, DNA fragments containing the KLF4-responsive element within the SM22α promoter could be hardly detected in the immunoprecipitates pulled by anti-KLF4 antibody. An increased binding of KLF4 to the SM22α promoter was observed in VSMCs treated by ATRA. This suggests that ATRA-induced upregulation of SM22α gene expression is at least in part responsible for the increase in KLF4 acetylation induced by ATRA.

Discussion

Previous report revealed that ATRA can activate multi-signaling pathways including cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) in the human acute promyelocytic leukemia (APL) cell during ATRA-induced differentiation [19–22]. These results suggest that signal-

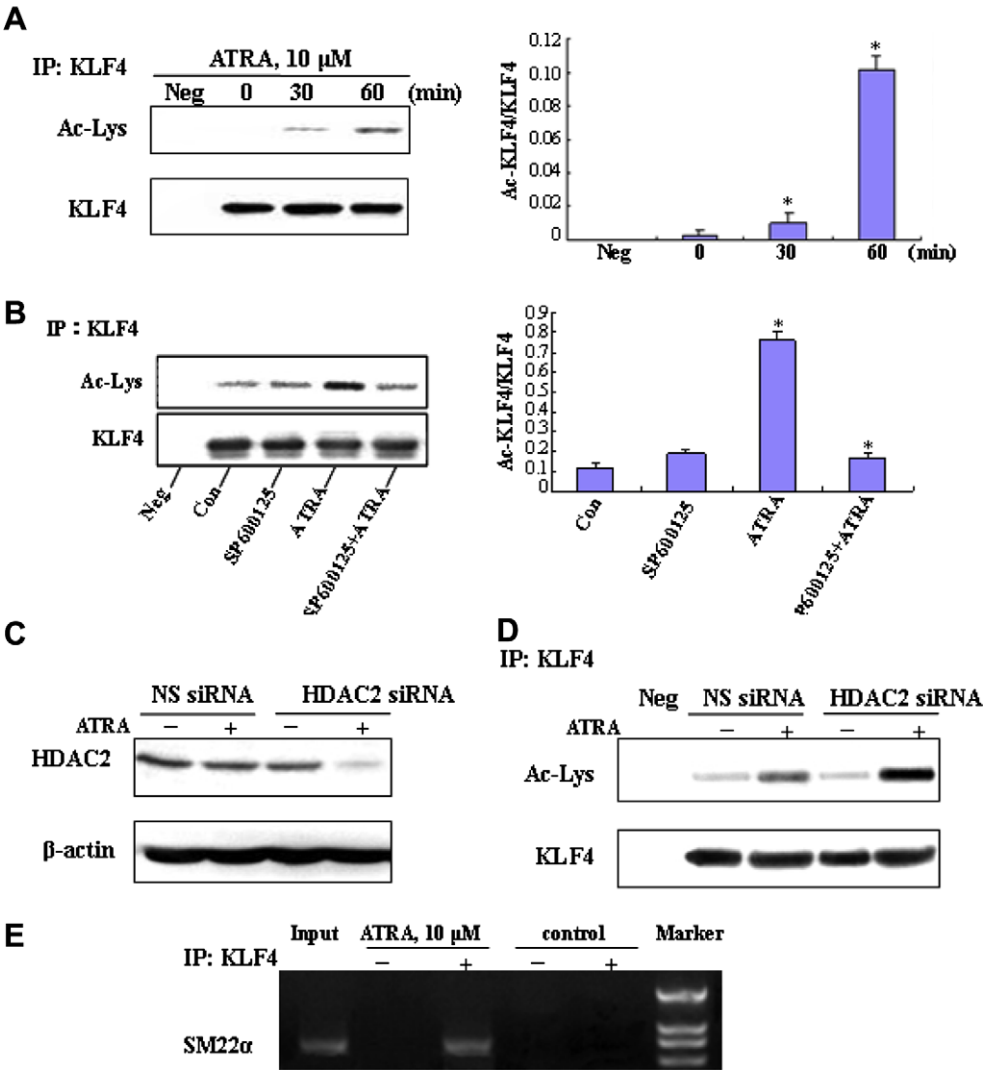

Fig. 3. ATRA prevents the deacetylation of KLF4 by inhibiting the interaction between KLF4 and HDAC2. (A) ATRA-induced KLF4 acetylation in VSMCs, as determined by immunoprecipitation using anti-acetylated lysine antibody. Blots for total KLF4 are also shown. (B) KLF4 acetylation in VSMCs pretreated with SP600125 was analyzed by immunoprecipitation assays. (C) The expression of HDAC2 was examined in VSMCs transfected with HDAC2 siRNA or NS siRNA and then stimulated with or without ATRA. (D) KLF4 acetylation was detected in VSMCs which were treated with or without ATRA after cells were transfected with HDAC2 siRNA or NS siRNA. (E) VSMCs were treated with ATRA or ethanol control. ChIP assays were performed and the SM22α promoter region was amplified by PCR. Bar graphs represent the ratio of the acetylated KLF4 to KLF4 ($n = 3$), $^{*}P < 0.05$ compared with the control.

ing pathways activated by ATRA in the different cell types might be different.

In present study, we showed for the first time that JNK-dependent signal pathway mediates ATRA-induced HDAC2 phosphorylation in VSMCs and suggested that JNK and HDAC2 are implicated in the regulation of VSMC differentiation marker gene expression. It is a novel finding that ATRA induces HDAC2 phosphorylation, which in turn leads to the dissociation of HDAC2 from KLF4 complexes, and prevents KLF4 deacetylation by HDAC2.

Four classes of HDACs have been identified in mammalian cells [23]. HDAC2 belongs to class I and is a core component of multiprotein corepressor complexes like Sin3 and NuRD, in which their activities are modulated via interactions with other proteins while being recruited by transcription factors to specific promoters [24]. Our results showed that only dephosphorylated HDAC2 could bind to KLF4, and phosphorylated HDAC2 dissociated from the KLF4 complexes in ATRA-induced VSMCs. On the other hand, KLFs have in common three contiguous C_2H_2-type zinc fingers at the carboxyl terminus which contain the DNA-binding domain (DBD). The DBD of KLF5 is known to interact with HDAC1 and p300. A recent study revealed that as the opposing cofactor to p300, HDAC1 interacts directly with KLF5 and represses KLF5-dependent promoter activation through competitive binding to the DBD with p300 [7]. Our data show that ATRA promotes the dissociation of KLF4 from HDAC2 by inducing HDAC2 phosphorylation at Ser424 in VSMCs, and thus prevents KLF4 deacetylation by HDAC2. We think that treating VSMCs with ATRA leads to the increase in KLF4 acetylation possibly because HDAC2 dissociates from KLF4 or because binding of p300 to KLF4 increases. It is supposed that p300, as a competitor, may compete with HDAC2 for binding to the first zinc finger of KLF4. Then, the binding activity of acetylated KLF4 to SM22α promoter is increased, and the acetylated KLF4 plays an important role in VSMC differentiation marker gene expression. A recent report revealed that KLF4 recruits HDAC2 to VSMC-specific gene promoter, and the later deacetylates histone H4 and reduces SRF/myocardin binding to CArG elements in VSMCs induced by PDGF-BB [25]. Interestingly, our results confirmed that ATRA stimulates the dissociation of HDAC2 from KLF4 through inducing HDAC2

phosphorylation, subsequently leading to the increase in KLF4 acetylation, and that the binding activity of acetylated KLF4 to SM22α promoter is increased.

In conclusion, we have demonstrated that ATRA promotes the dissociation of HDAC2 from KLF4 by inducing HDAC2 phosphorylation mediated by JNK signaling, leading to the increase in KLF4 acetylation. Further studies are needed to understand a potential role of JNK-HDAC2 pathway in the regulation of the interaction of p300 with KLF4.

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