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Retinoic Acid Receptor α Mediates All-*trans*-retinoic Acid-induced *Klf4* Gene Expression by Regulating *Klf4* Promoter Activity in Vascular Smooth Muscle Cells*[S]

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Background: KLF4 is essential for VSMC differentiation induced by ATRA.
Results: RARα interacts with KLF4-Sp1-YB1 bound to the *Klf4* promoter and transactivates *Klf4* in VSMCs in a RARE-independent manner.
Conclusion: RARα functions as a co-activator of KLF4-Sp1-YB1 complex.
Significance: These results described a novel mechanism of regulation of *Klf4* by ATRA and RARα.

The transcription factor Krüppel-like factor 4 (KLF4) plays a critical role in vascular smooth muscle cell (VSMC) differentiation induced by all-*trans*-retinoic acid (ATRA). Although it has been demonstrated that ATRA stimulation augments both KLF4 protein and mRNA levels in VSMCs, the molecular mechanisms by which ATRA regulates *Klf4* transcription are unknown. In this study, we examined the roles of ATRA-selective nuclear retinoic acid receptors (RARs) in the transcriptional regulation of *Klf4*. The introduction of small interfering RNA and an RAR antagonist demonstrated that RARα, but not RARβ or RARγ, mediated ATRA-induced *Klf4* expression. A luciferase assay for the *Klf4* promoter showed that three GC boxes in the proximal *Klf4* promoter were indispensible for ATRA-induced *Klf4* transcription and that RARα enhanced *Klf4* promoter activity in a GC box-dependent manner. Furthermore, chromatin immunoprecipitation and oligonucleotide pulldown assays demonstrated that the transcription factors KLF4, Sp1, and YB1 directly bound to the GC boxes of the proximal *Klf4* promoter. Upon RARα agonist stimulation, RARα was recruited to the *Klf4* promoter through its interaction with KLF4, Sp1, and YB1 to form a transcriptional activation complex on the three GC boxes of the *Klf4* promoter. These results suggest that RARα serves as an essential co-activator for ATRA signaling and that the recruitment of RARα to the KLF4-Sp1-YB1 complex, which leads to *Klf4* expression in VSMCs, is independent of a retinoic acid response element.

Krüppel-like factor 4 (KLF4),[2] a zinc finger-containing transcription factor, is expressed in a variety of tissues. KLF4 both activates and represses the transcription of different genes depending on the cellular context and thereby regulates numerous biological processes including proliferation, differentiation, development, inflammation, and apoptosis (1, 2). In addition, KLF4 plays an important role in reprogramming differentiated somatic cells into induced pluripotent stem cells in both mouse and human (1). The pleiotropic properties of KLF4 and its roles in human cancer and cardiovascular diseases are thus eliciting significant attention.

Previous studies have shown that the function of KLF4 may be regulated not only at the transcription level but also by post-translational modifications, such as phosphorylation, acetylation, sumoylation, and ubiquitylation (3). Furthermore, accumulating evidence suggests that KLF4 can be induced by a variety of stimuli, including serum starvation, oxidative stress, platelet-derived growth factor-BB, butyrate, cyclosporine, selenium, TGF-β, interferon-γ, cAMP, and all-*trans*-retinoic acid (ATRA) (4–14). We have found in our laboratory that in addition to induction of KLF4 expression, ATRA promotes KLF4 acetylation and phosphorylation, which subsequently transactivates SM22α and SM α-actin and promotes the differentiation process in vascular smooth muscle cells (VSMCs) (15, 16). Other studies also indicated that transcription factor stimulating protein-1 (Sp1) participates in the transcription regulation of *Klf4* in VSMCs (17). Moreover, Yang and co-workers (18) showed that KLF4 is subjected to autoregulation by its own gene product.

ATRA, a metabolite of dietary vitamin A (retinol), is a major bioactive retinoid in the body (19). ATRA directly transactivates downstream target genes by binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which belong to the nuclear receptor superfamily and which promote VSMC differentiation (20–22). RARs bind to retinoic acid response elements (RAREs) and, when bound by ligands, recruit a protein complex to activate transcription (23); in the

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[2] The abbreviations used are: KLF, Krüppel-like factor; ATRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; si-NS, nonspecific siRNA; Sp1, transcription factor stimulating protein-1; VSMC, vascular smooth muscle cell; YB1, Y box-binding protein 1.

absence of ligands, RARs associate with a co-repressor complex that silences transcription (24). With other transcription factors, including Sp1/Sp3 and STAT5 (signal transducer and activator of transcription 5), RARs cooperatively transactivate the target genes (25–27). RARs are now considered to be an attractive research target for treatment of VSMC proliferation disease (28, 29). Clinical applications of ATRA have successfully been applied in human diseases such as leukemia, cancer, restenosis, and plaque formation (29, 30). Despite these advances, the mechanisms by which ATRA functions to induce *Klf4* transcription are still largely unknown.

In this study, we aimed to elucidate the molecular mechanisms of ATRA signaling in the transactivation of *Klf4* expression in VSMCs. We show that RARα, but not RARβ or RARγ, mediated ATRA-induced *Klf4* expression in VSMCs. RARα was recruited to the *Klf4* promoter via its interaction with KLF4, Sp1, and Y box-binding protein 1 (YB1), which are associated with GC boxes at the site, to cooperatively activate *Klf4* transcription. ATRA promoted the interaction of RARα with KLF4, Sp1, and YB1. Accordingly, we reveal a novel mechanism by which ATRA-activated RARα, as a co-activator, promoted *Klf4* transactivation in a RARE-independent manner in VSMCs.

EXPERIMENTAL PROCEDURES

Cells, Cell Culture, and Treatment—VSMCs were extracted from the thoracic aorta of male Sprague-Dawley rats (90–100 g) as described previously (31). The cells were maintained in DMEM supplemented with 10% FBS (HyClone, Logan, UT) in a humidified atmosphere with 5% CO_2 at 37 °C; cells used in this study were passaged for three to six generations. Prior to ATRA stimulation, VSMCs were maintained in serum-free DMEM for 24 h. They were then cultured in DMEM containing 5% FBS and 10 μM ATRA (Sigma-Aldrich) for the indicated times. To introduce inhibitors, thet cells were pretreated with the indicated inhibitors at a final concentration of 20 μM for 2 h before the addition of 10 μM of ATRA. The A293 cells and CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in high glucose DMEM supplemented with 10% FBS.

Adenovirus Expression Vector and Plasmid Construction—pEGFP-KLF4 and pCMV-RARα have been described previously (32). The RARα cDNA was amplified and subcloned into the pEGFP (Clontech), pCMV-FLAG (Sigma-Aldrich), and pGEX (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) vectors. For the adenovirus expression vector, the RARα cDNA was cloned into the pAD/CMV/V5-DEST vector (Invitrogen) to create the RARα adenovirus pAd-RARα. The resulting constructs were packaged in A293 cells by transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Culture supernatants from A293 cells were used to infect VSMCs. The cells were passaged after 24 h and selected with 300 μg/ml G418 for 14 days. The expression plasmid for Sp1 (pPac-Sp1) was a generous gift from Dr. Tijan (University of California, Berkeley, CA). The full-length Sp1 cDNA was subcloned into the pEGFP and pGEX vectors. The pGEX-YB1 plasmid was kindly provided by Dr. Kiyoshi Higashi (Sumitomo Chemical, Konohana-ku, Osaka, Japan), and the YB1 cDNA was subcloned into the pEGFP vector. Full-length cDNA of mouse MEF2C was subcloned into the pGEX vector to generate pGEX-MEF2C. For the promoter assay, the luciferase reporter plasmid constructs bearing the mouse *Klf4* promoter region was kindly provided by Dr. Walden Ai (University of South Carolina, Columbia, SC). Truncation promoter constructs were similarly generated using different 5′-primers.

Site-directed Mutagenesis—Site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Agilent Technologies-Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers used to generate mutation in the putative GC box 1 (GC1) were 5′-GGGGGCTGCGGGAAGGAAAGGAGAAGAAAGGCAGG-3′ (sense) and 5′-CCTGCCTTTCTTCTCCTTTCCTTCCCGCAGCCCCC-3′ (antisense). Primers for mutation in the GC box 2 (GC2) were 5′-AGAAAGGCAGGGGTTTTGGCCTGGCGGCGG-3′ (sense) and 5′-CCGCCGCCAGGCCAAAACCCCTGCCTTTCT-3′ (antisense). The primers for mutation in the GC box 3 (GC3) were 5′-GCCACAGGGAGGAGGAAAGGAGCAAGCGAGCGAG-3′ (sense) and 5′-CTCGCTCGCTTGCTCCTTTCCTCCTCCCTGTGGC-3′ (antisense). All of the constructs were verified by sequencing.

siRNA Transfection—Small interfering RNA (siRNA) targeting *Klf4* were synthesized by Sigma-Aldrich, as previously described (7). The siRNAs against the rat sequences *RARα*, *RARβ*, and *RARγ* were designed and synthesized by Sigma-Aldrich. The siRNA sequences against *RARα* (si-*RARα*) were, 5′-CUCAGAACAACGUGUCUCU-3′ and 5′-AGAGACACGUUGUUCUGAG-3′. The siRNA sequences against *RARβ* (si-*RARβ*) were 5′-GCCAUUUCAUGCCACCAGA-3′ and 5′-UCUGGUGGCAUGAAAUGGC-3′. The siRNA sequences against *RARγ* (si-*RARγ*) were 5′-GGUCUAUAAGCCGUGCUUU-3′ and 5′-AAAGCACGGCUUAUAGACC-3′. Nonspecific siRNA (si-NS) and the siRNAs specific for rat *Sp1* and *YB1* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection was performed using Lipofectamine reagent (Invitrogen) following the manufacturer's instructions. Twenty-four hours after transfection, VSMCs were treated with 10 μM of ATRA for indicated times. The cells were then harvested and lysed for Western blot.

RNA Preparation and Quantitative RT-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative PCR of *Klf4* was performed using Platinum SYBR Green quantitative PCR SuperMix UDG Kit (Invitrogen). As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene primers were used for RNA template normalization. All of the PCRs were performed in triplicate. The relative expression level was calculated using the following equation: relative gene expression $= 2^{-(\Delta Ct\ \mathrm{Sample} - \Delta Ct\ \mathrm{Control})}$. The following primers were used: *Klf4*, 5′-CGGGAAGGGAGAAGACACTGC-3′ (sense) and 5′-GCTAGCTGGGGAAGACGAGGA-3′ (antisense); and *GAPDH*, 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TTCACCACCCTGTTGCTGTA-3′ (antisense).

Western Blotting—Crude proteins were extracted from VSMCs as described previously (33), resolved by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were blocked with

5% milk in Tris-buffered saline with Tween 20 for 2 h at 37 °C and then incubated overnight at 4 °C with the following primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit anti-KLF4, rabbit anti-RARα, rabbit anti-RARβ, rabbit anti-RARγ, rabbit anti-SP1, rabbit anti-GST, mouse anti-GFP, or mouse anti-β-actin. Additional primary antibodies were: rabbit anti-YB1 (Abcam, Cambridge, MA), rabbit anti-MEF2C (Proteintech, Chicago), or rabbit anti-FLAG (Sigma-Aldrich). After incubation with the appropriate secondary antibody, the immunoreactive signal of antibody antigens were visualized using the Chemiluminescence Plus Western blot analysis kit (Santa Cruz).

Luciferase Assay—CHO-K1 cells, maintained in high glucose DMEM supplemented with 10% FBS, were seeded in each well (3×10^4 cells/well) of a 24-well plate and grown for 24 h prior to transfection with reporter plasmids or the control reporter plasmid pRL-TK. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after treatment with ATRA, the cells were lysed, and luciferase assays were performed using a dual luciferase assay kit (Promega, Madison, WI). Specific promoter activity was expressed as the relative activity ratio of firefly luciferase to *Renilla* luciferase. All of the promoter constructs were evaluated in ≥3 separate wells/experiment.

Co-immunoprecipitation Assay—Co-immunoprecipitation was performed as described previously (15, 34). Briefly, cell lysates were first precleared with 25 μl of protein A-agarose (50% v/v, Santa Cruz). The supernatants were immunoprecipitated with 2 μg of anti-RARα 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 pelleted by centrifugation at 12,000 × *g* 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 each time at 4 °C. Bound proteins were resolved by SDS-PAGE, followed by Western blot with antibodies against KLF4, Sp1, YB1, and RARα.

GST Pulldown Assay—GST, GST-KLF4, GST-Sp1, GST-YB1, and GST-MEF2C fusion proteins were produced by BL21 *Escherichia coli* under induction by isopropylthio-β-galactoside at 30 °C. Proteins were purified by affinity absorption using glutathione-Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden). The recombinant GST, GST-KLF4, GST-Sp1, GST-YB1, or GST-MEF2C proteins on the glutathione beads were incubated with total cell lysates of VSMCs at 4 °C overnight followed by extensive washing. Proteins on the beads were resolved by SDS-PAGE and probed by Western blot with anti-KLF4, anti-Sp1, anti-YB1, and anti-RARα antibodies.

Chromatin Immunoprecipitation Assay—The ChIP assay was carried out as described previously (15). Briefly, VSMCs were treated with 1% formaldehyde for 10 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-RARα, anti-KLF4, anti-Sp1, or anti-YB1 antibodies. After reversal of cross-linking, the genomic region of *Klf4* flanking the GC boxes (the region between −179 to +20) was amplified by PCR with the following primers (positive primers): 5′-CCACGTGCGCCGAGTTTGTTT-3′ (sense) and 5′-GCTCTTTCGGCCGGGGAACTG-3′ (antisense). A negative control region upstream of the *Klf4* promoter (the region between −1063 to −821) was amplified with the following primers (negative primers): 5′-AACTGGAGAGTGCGAGTGCGT-3′ (sense) and 5′-GGACGGGTAAGAATCTCAGAAGC-3′ (antisense).

Oligonucleotide Pulldown Assay—The oligonucleotide pulldown assay was carried out as described previously (12). Oligonucleotides containing the wild-type or mutant GC1/2 or GC3 box sequence in the rat *Klf4* promoter with biotin added to their 5′-end were as follows: GC1/2 (−128 to −84), biotin-5′-CTGCGGGAAGGCGGGGAGAAGAAAGGCAGGGGGCGGGGCCTGGCG-3′ (wild type) and biotin-5′-CTGCGGGAAGTTTTGGAGAAGAAAGGCAGGGGTTTTGGCCTGGCG-3′ (mutant); and GC3 (−70 to −34), biotin-5′-CGCCACAGGGAGGAGGCGGGGAGCGAACGAGCGAGAA-3′ (wild type) and biotin-5′-CGCCACAGGGAGGAGGAAAGGAGCGAACGAGCGAGAA-3′ (mutant). Each pair of oligonucleotides was annealed following standard protocols.

VSMCs treated with or without 10 μM of ATRA for 1 h 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. VSMCs lysates or purified recombinant proteins (GST, GST-KLF4, GST-Sp1, GST-YB1, and GST-RARα) were precleared with ImmunoPure streptavidin-agarose beads (20 μl/sample; Pierce) for 1 h at 4 °C. After centrifugation at 12,000 × *g* for 60 s at 4 °C, the supernatant was incubated with 100 pmol of biotinylated double-strand oligonucleotides and 10 μg of poly(deoxyinosinic-deoxycytidylic) for 16 h at 4 °C. DNA-bound proteins were enriched with 30 μl of immobilized streptavidin-agarose beads for 1 h at 4 °C. After washing with lysis buffer four times, bound proteins were separated by SDS-PAGE and subjected to Western blot.

Isolation and Identification of Factors Mediating ATRA-induced Klf4 Expression—Oligonucleotides containing the *Klf4* proximal promoter region −179 to +20 sequence was amplified by PCR with 5′-biotinylated primers: biotin-5′-CCACGTGCGCGGAGTTTGTTT-3′ (sense) and biotin-5′-TCTCTTGGCCGGGGAACTG-3′ (antisense). The biotinylated oligonucleotide (−179/+20) was used as a probe. VSMCs treated with or without 10 μM ATRA for 1 h were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40) containing protease inhibitors. The whole cell lysates were precleared and incubated with 200 pmol of biotinylated double-stranded oligonucleotide containing the *Klf4*-proximal promoter region (−179/+20). DNA-bound proteins were enriched with 50 μl of immobilized streptavidin-agarose beads, and the protein components were eluted with Laemmli loading buffer. After separation by SDS-PAGE following staining with Coomassie Brilliant Blue, target bands were excised and subjected to in-gel trypsin digestion. Peptides were analyzed by high capacity ion trap mass spectrometry. Spectra were formatted and searched against mammalian sequences in the Swiss Protein Database by using the Mascot search engine (Matrix Science, Boston, MA).

Statistical Analyses—The data presented as bar graphs are the means ± S.E. of at least three independent experiments. Statistical analyses were performed using Student's *t* test. The results were considered statistically significant at $p < 0.05$.

RESULTS

RARα-mediated ATRA-induced Klf4 Expression—Previous studies showed that in VSMCs ATRA induces KLF4 expression and its post-translational modifications, including phosphorylation and acetylation (15, 16, 35). However, the underlying mechanism by which ATRA regulates *Klf4* transcription in VSMCs remains largely unknown. ATRA functions by binding to RARs and RXRs, which act as transcription factors upon ligand binding (20). There are three types of RARs: RARα, RARβ, and RARγ, each of which is encoded by their respective genes.

To explore the actual relationship between KLF4 and RARs in ATRA-stimulated VSMCs, we first examined the expression of KLF4 and RARs in response to ATRA signaling. ATRA increased KLF4 and RARα expressions in a concentration- (supplemental Fig. S1) and time-dependent manner (Fig. 1*A*). As shown in Fig. 1*A*, 6 h after 10 μM of ATRA treatment, KLF4 and RARα levels were elevated, and this increase was maintained until 24 h after treatment. However, unlike RARα, the expressions of RARβ and RARγ were little changed by the ATRA treatment.

To further define which RAR isoform mediated the induction of KLF4 by ATRA, we knocked down endogenous RARs by transfecting VSMCs with siRNAs against *RARα* (si-*RARα*), *RARβ* (si-*RARβ*), *RARγ* (si-*RARγ*), or a control (si-NS), respectively. Transfection of si-*RARα*, si-*RARβ*, and si-*RARγ* downregulated endogenous RARα, RARβ, and RARγ level, respectively. Although ATRA was able to induce both KLF4 and RARα in the cells with RARβ and RARγ knockdown, the induction of KLF4 by ATRA was significantly reduced in mRNA and protein levels in the cells with RARα knockdown (Fig. 1, *B* and *C*). Furthermore, when VSMCs were treated with a RARα antagonist (Ro 41-5253) prior to the addition of ATRA, the blockade of RARα signaling partially reduced the response of KLF4 to ATRA (Fig. 1*D*).

To validate the roles of RARα in ATRA-induced KLF4 expression, RARα overexpression was introduced by infection of the adenovirus vector pAd-GFP-RARα. RARα overexpression further increased the induction of KLF4 by ATRA (Fig. 1*E*). To examine whether the RARα-mediated KLF4 induction was dependent on its elevated promoter activity, CHO-K1 cells were transiently co-transfected with a *Klf4* promoter-reporter construct and RARα, RARβ, or RARγ expression plasmid (GFP-RARα, GFP-RARβ, or GFP-RARγ). The luciferase activity assay showed that RARα overexpression significantly increased the activation of the *Klf4* promoter under ATRA treatment (Fig. 1*F*). The inductive effect of ATRA was partially compromised by the presence of increasing amounts of RARα antagonist Ro 41-5253, whereas pretreated with RARβ antagonist LE135 or RARγ antagonist MM11253 did not affect ATRA-induced *Klf4* transcription (Fig. 1*G*). Collectively, these results suggest that RARα, but not RARβ or RARγ, is essential for induction of *Klf4* transcription by ATRA.

RARα-mediated ATRA-induced Klf4 Expression via Three Proximal GC Boxes in Klf4 Promoter—To identify the RARα-responsive elements in the *Klf4* promoter region, we constructed progressive 5′-deletion constructs of the *Klf4* promoter fused to the luciferase reporter gene. Using transfection of these constructs, we examined the ability of ATRA to activate the expression of each, in CHO-K1 cells. The results showed that the *Klf4* promoter-proximal region −179 to +20 exhibited the greatest increase in luciferase activity, compared with control, in response to ATRA treatment (Fig. 2*A*). Sequence analysis revealed that there were three GC boxes in the *Klf4* promoter region within this region (Fig. 2*B*).

To assess whether these GC boxes were required for ATRA-induced *Klf4* expression, we transfected the proximal promoter constructs (−179/+20) encoding various GC box mutations with ATRA stimulation. We observed that the mutation of a single GC box reduced the ATRA-stimulated activation of *Klf4* promoter activity by 64–80%. The simultaneous mutation of two GC boxes decreased ATRA-induced *Klf4* promoter activity by ~85%. Furthermore, the elimination of all three GC boxes completely abrogated the response of the *Klf4* promoter to ATRA (Fig. 2*C*). This implies that the response to ATRA by the three GC boxes in the proximal region of the *Klf4* promoter was synergistic.

To investigate whether RARα directly bound to the three GC boxes in ATRA-stimulated VSMCs, we carried out the ChIP assay. The results showed that ATRA stimulation significantly promoted the binding of RARα to the proximal region of the *Klf4* promoter (Fig. 2*D*); no binding of RARα was detected when distal *Klf4* promoter region was amplified. Consistent with the results of the ChIP assay, the oligonucleotide pulldown assay showed that the binding of RARα to the GC1/2 or GC3 box was increased by ATRA stimulation, whereas mutation in the GC boxes interrupted binding (Fig. 2*E*), indicating that the binding of RARα to the GC boxes is specific.

To further determine whether the binding of RARα to GC boxes affected *Klf4* promoter activity, CHO-K1 cells were co-transfected with GFP-RARα and the *Klf4* promoter-reporter construct (−179/+20 wt) or with GFP-RARα and the *Klf4* promoter-reporter mutated construct (−179/+20 mutant). As shown in Fig. 2*F*, RARα overexpression significantly elevated the activities of the *Klf4*-proximal promoter with three integrative GC boxes in response to ATRA stimulation. However, when all three GC boxes were mutated, the response of the K*lf4* promoter to RARα overexpression and ATRA was almost completely abolished. Taken together, these results indicate that RARα-mediated ATRA-induced *Klf4* expression in a GC box-dependent manner.

Interaction of RARα with Sp1, YB1, or KLF4 Cooperatively Activated Klf4 Promoter Activity—Several recent studies have reported that RARs can exert their effect via RARE-independent regulatory mechanisms by interacting with other transcription factors (26, 36). We reasoned that, as a nuclear receptor mediating the effect of ATRA, RARα could interact with one or more GC box-binding factors to regulate KLF4 expression. Several transcription factors, including Sp1 and KLF4, have been described as binding with the proximal *Klf4* promoter (17, 18).

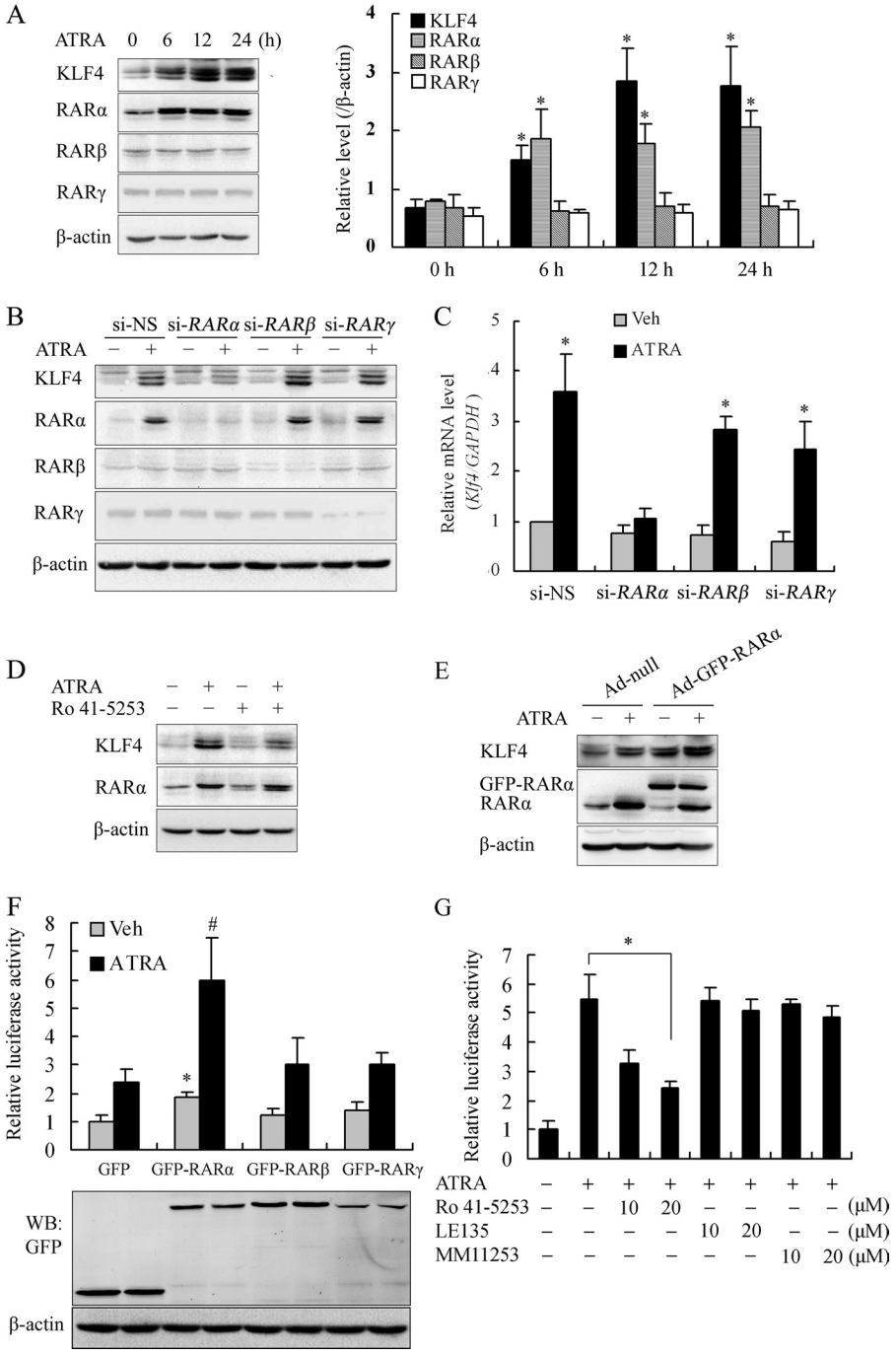

FIGURE 1. **RARα mediated ATRA-induced *Klf4* expression.** *A*, VSMCs were treated with 10 μM of ATRA for 0, 6, 12, or 24 h. Crude proteins were extracted from the treated cells and then subjected to Western blot with antibodies against KLF4, RARα, RARβ, or RARγ. β-Actin was used as a control for equal protein loading. *Left*, blots from a representative experiment. *Right*, densitometry; results were normalized to β-actin. The *bars* represent the means ± S.E. from three independent experiments. *, $p < 0.05$ *versus* ATRA-free group. *B*, VSMCs were transfected with si-*RARα*, si-*RARβ*, si-*RARγ*, or si-NS for 24 h and then treated with 10 μM of ATRA for 24 h. The cell lysates were analyzed by Western blot with antibodies against KLF4, RARα, RARβ, or RARγ. β-Actin was the loading control. *C*, VSMCs were transfected with si-*RARα*, si-*RARβ*, si-*RARγ*, or si-NS for 24 h and then treated with 10 μM of ATRA for 24 h. Total RNA was isolated and subjected to quantitative RT-PCR. The *bars* represent the means ± S.E. from three independent experiments. *, $p < 0.05$ *versus* si-NS-treated and ATRA-free group (*first bar*). *D*, VSMCs were pretreated with 20 μM of Ro 41-5253 for 2 h prior to exposure to ATRA (10 μM) for 24 h. Crude proteins from cell lysates were analyzed by Western blot with antibodies against KLF4 and RARα. β-Actin was the loading control. *E*, VSMCs were infected with Ad-null or Ad-GFP-RARα for 24 h prior to the exposure to ATRA (10 μM) for 24 h. Crude proteins from cell lysates were analyzed by Western blot with antibodies against KLF4 or RARα. β-Actin was the loading control. *F*, CHO-K1 cells were co-transfected with a *Klf4* promoter-reporter construct and RARα, RARβ, or RARγ expression plasmid (GFP-RARα, GFP-RARβ, or GFP-RARγ) for 24 h and then treated with 10 μM of ATRA for 24 h. Cell lysates were subjected to luciferase activity assays using the dual luciferase reporter assay system, and the luciferase activity was normalized to pRL-TK activity. The *bars* represent the means ± S.E. from three independent experiments. * and #, $p < 0.05$ *versus* the respective control group. Expression level of GFP, GFP-RARα, GFP-RARβ, and GFP-RARγ was assessed by Western blot analysis. β-Actin was used as a control for equal protein loading. *G*, CHO-K1 cells were pretreated with Ro 41-5253, LE135, or MM11253 (antagonists of RARα, RARβ, or RARγ, respectively) for 2 h and then stimulated with 10 μM of ATRA for 24 h. Luciferase activity of the *Klf4* promoter-reporter construct was measured as described above. *, $p < 0.05$ *versus* ATRA-treated group (*second bar*).

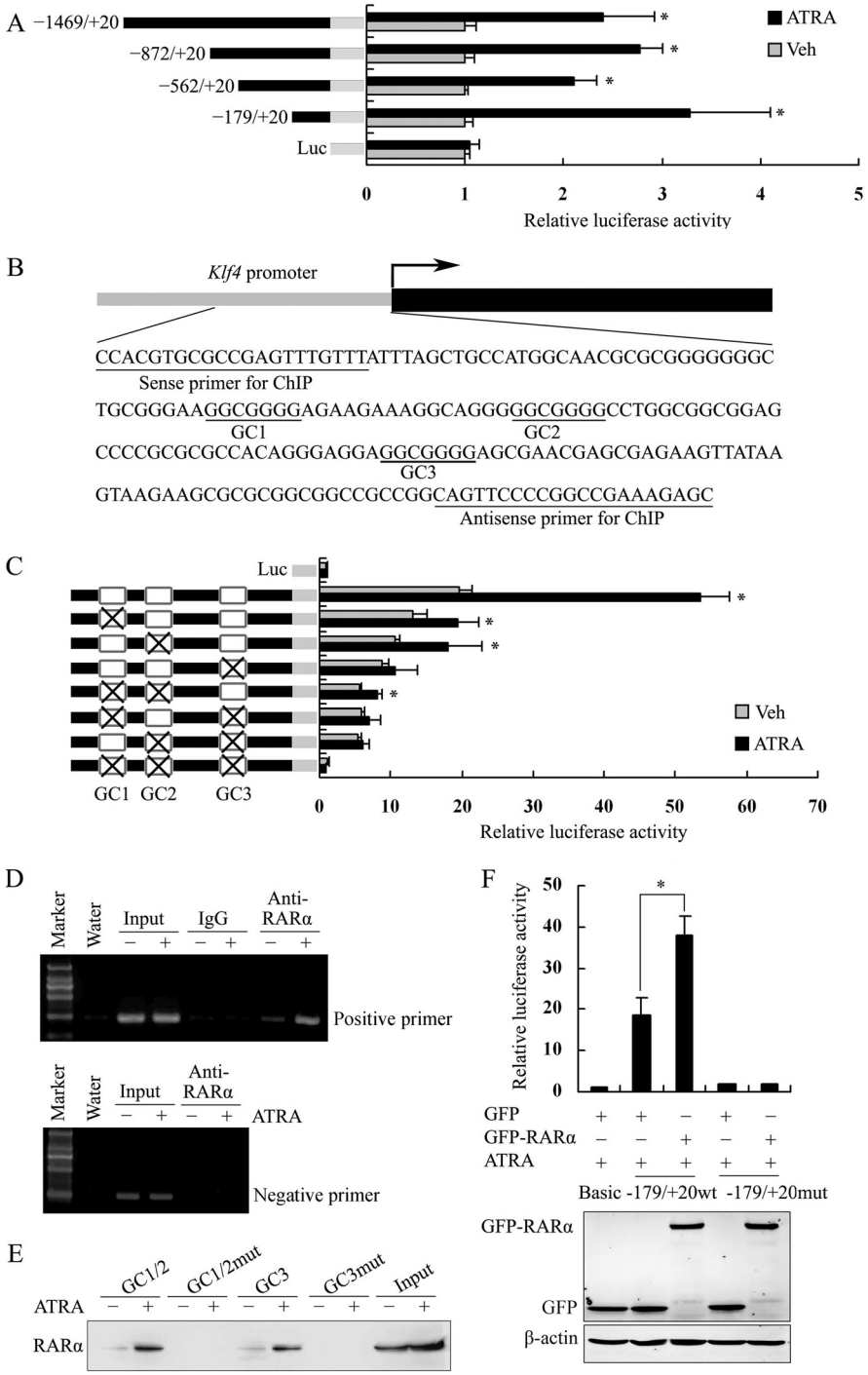

FIGURE 2. **RARα mediated ATRA-induced *Klf4* expression via the proximal three GC boxes in the *Klf4* promoter.** *A*, CHO-K1 cells were transfected with the *Klf4* promoter-reporter plasmid containing various 5′ deletion fragments for 24 h and then treated with 10 μM of ATRA for 24 h. The cell lysates were subjected to luciferase activity assay. The data represent relative *Klf4* promoter activity normalized to pRL-TK activity. The *bars* indicate the means ± S.E. from three independent experiments. *, $p < 0.05$ *versus* the respective control group. *B*, a schematic map of the *Klf4* promoter region −179 to +20 showing position of GC boxes. The *underlined* sequences were primers for the ChIP assay. *C*, CHO-K1 cells were transfected with the *Klf4* proximal promoter-reporter constructs (−179/+20) containing various GC box mutations for 24 h and then treated with 10 μM of ATRA for 24 h. × indicates the mutation of GC boxes. Luciferase activity assay was performed with total cell lysates as described above. *, $p < 0.05$ *versus* the respective control group. *D*, VSMCs were exposed to ATRA (10 μM) for 1 h. ChIP assay was then performed with antibody against RARα. Nonimmune IgG was used as negative control for immunoprecipitation. Immunoprecipitated DNA was amplified by PCR using primers spanning the proximal region of rat *Klf4* promoter containing the three GC boxes (positive primers) or using primers spanning the distal *Klf4* promoter region −1063 to −821 (negative primers). *E*, VSMCs were treated with or without ATRA (10 μM) for 1 h, and the whole cell lysates were subjected to oligonucleotide pulldown assay with biotinylated double-stranded oligonucleotides containing wild-type GC1 and GC2 box sequences (GC1/2), mutated GC1 and GC2 (GC1/2mut), wild-type GC3 (GC3), or mutated GC3 (GC3mut) as probes. DNA-bound proteins were collected with streptavidin-agarose beads and analyzed by Western blot with anti-RARα antibody. *F*, CHO-K1 cells were co-transfected with a GFP-RARα expression vector and the *Klf4*-proximal promoter-reporter plasmid containing the three wild-type or mutated GC boxes for 24 h. The cells were then treated with vehicle or ATRA (10 μM) for 24 h. The cell lysates were prepared for luciferase assay as described above. *, $p < 0.05$ *versus* GFP-RARα plasmid-untransfected group. Expression level of GFP and GFP-RARα was assessed by Western blot analysis. β-Actin was used as a control for equal protein loading. *Veh*, vehicle; *WB*, Western blot.

To find new factors mediating ATRA-stimulated KLF4 expression, we affinity-purified DNA-binding transcription factors from VSMC extracts using as a probe a biotinylated double-stranded DNA fragment harboring nucleotides −179 to +20 of the *Klf4* promoter. Fig. 3*A* shows that one protein of 36 kDa was pulled down by the *Klf4* (−179 to +20) promoter fragment after ATRA stimulation. High capacity ion trap mass spectrometry analysis with a database search and further confirmation of the amino acid sequence by post-source decay peptide sequencing showed that this protein was YB1 (supplemental Fig. S2).

A co-immunoprecipitation assay showed that ATRA stimulation significantly increased the interaction of RARα with KLF4, Sp1, and YB1 in VSMCs *in vivo* (Fig. 3*B*). To reveal whether RARα directly interacted with YB1, Sp1, and KLF4 *in vitro*, GST pulldown with overexpressed GFP-RARα was performed *in vitro*. Apparently, GFP-RARα physically bound to GST-KLF4, GST-Sp1, or GST-YB1 *in vitro* but not GST alone; ATRA treatment increased their binding (Fig. 3*C*). The binding of RARα to MEF2C, a negative control, could not be detected *in vivo* and *in vitro* (Fig. 3, *B* and *C*). In addition, the interactions of RARβ and RARγ with these transcription factors were also examined by GST pulldown assay. As shown in supplemental Fig. S3, the interaction of GFP-RARβ and GFP-RARγ with KLF4, Sp1, and YB1 could hardly be detected.

Furthermore, the oligonucleotide pulldown assay demonstrated that the binding affinity of KLF4, Sp1, and YB1 to GC boxes (GC1/2 or GC3) in the proximal *Klf4* promoter were increased in ATRA-stimulated VSMCs, whereas the mutation in GC boxes abrogated the binding (Fig. 3*D*). The ChIP assay also showed that ATRA promoted the association of KLF4, Sp1, and YB1 with the proximal *Klf4* promoter in VSMCs (Fig. 3*E*).

Finally, to evaluate the additive effects of the interaction of RARα with KLF4, Sp1, or YB1 on *Klf4* promoter activity, CHO-K1 cells were co-transfected with a *Klf4* promoter reporter, along with various combinations of expression plasmids for KLF4, Sp1, YB1, and RARα and then treated with or without 10 μM of ATRA and followed by a luciferase assay. As shown in Fig. 3*F*, transient expression of KLF4, Sp1, or YB1 alone, as well as two or three combinations of these expression plasmids, increased *Klf4* promoter activity to a certain extent. The strongest activation was observed when all four expression plasmids for KLF4, Sp1, YB1, and RARα were co-transfected in the presence of ATRA (Fig. 3*F*). Altogether, these results demonstrated that the interaction of RARα with KLF4, Sp1, or YB1 in the *Klf4* promoter region facilitated promoter activity.

Association of KLF4, Sp1, or YB1 with GC Boxes Facilitated Klf4 Promoter Activities—To examine the physical binding of KLF4-Sp1-YB1 to the GC boxes, we performed an oligonucleotide pulldown assay using GST-KLF4, GST-Sp1, GST-YB1, or GST-RARα with biotinylated double-stranded oligonucleotides containing the wild-type or mutant GC1/2 or GC3 box. As shown in Fig. 4 (*A* and *B*), GST-KLF4 or GST-Sp1 directly bound to the GC1/2 or GC3 boxes, but not their mutants, in a concentration-dependent manner. Importantly, GST-YB1 was also associated with biotinylated GC1/2 or GC3 probes, in that a higher dose of the probe caused a stronger binding of YB1 to the GC box, and the mutation of the GC box sequence interrupted its binding activity (Fig. 4*C*). These results strongly suggested that, *in vitro*, YB1 bound to the GC box directly. However, we did not observe the physical binding of GST-RARα to biotinylated probes containing GC boxes *in vitro* (Fig. 4*D*).

To further investigate the effect of KLF4, Sp1, and YB1 on *Klf4* promoter activity, CHO-K1 cells were co-transfected with a constant amount of pPac-Sp1 plasmid and increasing amounts of pEGFP-KLF4 plasmid, along with the *Klf4* promoter-reporter construct pGL3-*Klf4*-luc. As shown in Fig. 4*E*, the stimulatory effect of Sp1 on the *Klf4* promoter gradually increased with increasing amounts of pEGFP-KLF4. Likewise, when CHO-K1 cells were co-transfected with a constant amount of pEGFP-KLF4 and increasing amounts of pPac-Sp1, Sp1 enhanced the stimulatory effect of KLF4 on the *Klf4* promoter in a concentration-dependent manner (Fig. 4*F*). These results suggested that both KLF4 and Sp1 bound to the *Klf4* promoter to co-operatively activate its transcription. In addition, as shown in supplemental Fig. S4, both Sp1 and YB1, as well as both KLF4 and YB1, also cooperated with each other to activate the *Klf4* promoter.

Next, we knocked down endogenous *RARα* by transfecting VSMCs with si-*RARα* (Fig. 4*G*). The ChIP assay showed that ATRA promoted the binding of KLF4, Sp1, and YB1 to the GC box region of the *Klf4* promoter in si-NS-treated VSMCs; down-regulation of endogenous RARα by si-RARα reduced ATRA-induced recruitments of these factors to the *Klf4* promoter (Fig. 4*H*). The results suggest that the presence of RARα or ATRA stimulation facilitated the association of KLF4, Sp1, or YB1 with the GC box region of the *Klf4* promoter.

ATRA Promoted Binding of RARα to Klf4 Promoter in KLF4-Sp1-YB1-dependent Manner—To reveal the roles of ATRA in the interactions between RARα and KLF4, Sp1, or YB1, we next performed a GST pulldown assay *in vitro*. We observed that although KLF4, Sp1, and YB1 formed a complex without ATRA treatment, the addition of ATRA stimulation promoted the binding of RARα to the KLF4-Sp1-YB1 complex. (Fig. 5, *A*–*C*). Furthermore, to analyze the interactions between KLF4, Sp1, and YB1, GST pulldown assays with overexpressed GFP-KLF4, GFP-Sp1, or GFP-YB1 were performed *in vitro*. Apparently, GFP-KLF4, GFP-Sp1, and GFP-YB1 physically bound to any of the other factors (Fig. 5*D*). Simultaneously, we also analyzed the effect of ATRA on some other target genes of KLF4, Sp1, or YB1. As shown in supplemental Fig. S5, the protein level of SM22α, p53, and p21, the target genes of KLF4, YB1, and Sp1, significantly increased in VSMCs after ATRA stimulation for 24 h.

To further examine whether RARα recruitment to the proximal *Klf4* promoter depended on the binding of the KLF4-Sp1-YB1 complex to GC boxes on the *Klf4* promoter, CHO-K1 cells were co-transfected with expression constructs for KLF4, Sp1, YB1, or RARα, and then oligonucleotide pulldown assays were performed using GC1/2 or GC3 box sequence as probes. The results showed that RARα bound to GC1/2 or GC3 box at the

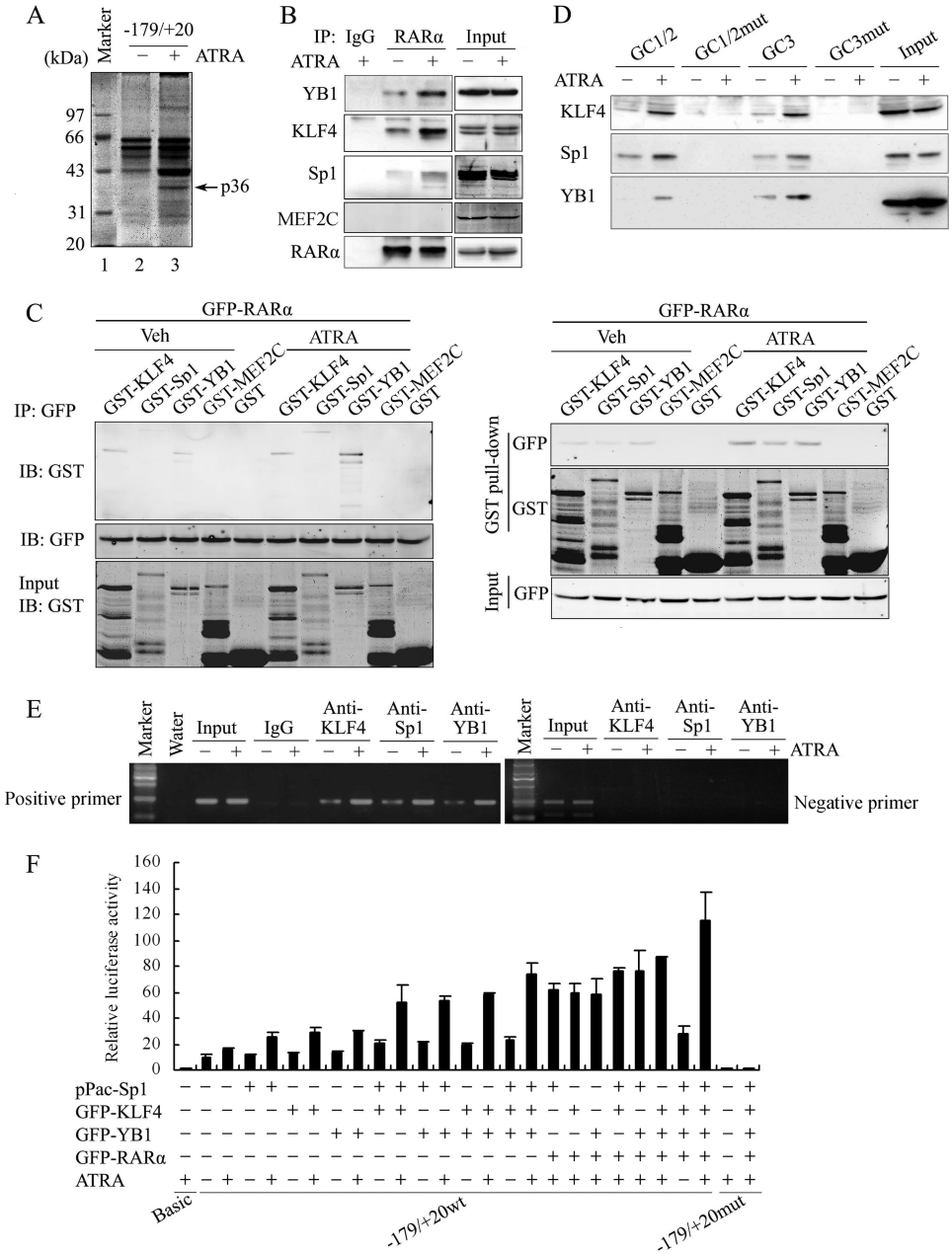

FIGURE 3. **Interaction of RARα with Sp1, YB1, or KLF4 cooperatively activated *Klf4* promoter activity.** *A*, YB1 associated with the *Klf4*-proximal promoter region in response to ATRA stimulation. VSMCs were treated with ATRA (10 μM) for 1 h. Oligonucleotide pulldown was carried out with biotinylated double-stranded oligonucleotide containing *Klf4* promoter region −179 to +20 as a probe. DNA-bound proteins were collected with streptavidin-agarose beads and analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. *p36*, 36-kDa band. *B*, VSMCs were treated with 10 μM of ATRA for 1 h. The cell lysates were immunoprecipitated with anti-RARα antibody and analyzed by Western blotting using anti-YB1, anti-KLF4, anti-Sp1, anti-MEF2C, or anti-RARα antibodies, respectively. Nonimmune IgG was used as negative control. *C*, GST, GST-KLF4, GST-Sp1, GST-YB1, and GST-MEF2C fusion proteins were purified from isopropylthio-β-galactoside-induced BL21 *E. coli* using glutathione-Sepharose 4B beads. CHO-K1 cells were transfected to express GFP-RARα protein for 24 h and then treated with 10 μM of ATRA for 1 h. *Left*, CHO-K1 cell lysates were incubated with the recombinant GST, GST-KLF4, GST-Sp1, GST-YB1, and GST-MEF2C proteins at 4 °C for 2 h, followed by immunoprecipitation with anti-GFP antibody. Proteins on the beads were eluted and detected by Western blot with anti-GST antibody. *Right*, the whole cell lysates of CHO-K1 cells overexpressing exogenous GFP-RARα were also used to perform GST pulldown assay. The recombinant GST, GST-KLF4, GST-Sp1, GST-YB1, and GST-MEF2C proteins on the glutathione beads were incubated with the cell lysates, respectively, overnight at 4 °C, followed by extensive washing. Proteins on the beads were eluted and detected by Western blot with anti-GFP antibodies. *D*, VSMCs were treated with 10 μM of ATRA for 1 h. Oligonucleotide pulldown assay was performed with biotinylated double-stranded oligonucleotides containing wild-type GC1 and GC2 box sequences (GC1/2), mutated GC1 and GC2 (GC1/2mut), wild-type GC3 (GC3), and mutated GC3 (GC3mut) as probes. DNA-bound proteins were collected with streptavidin-agarose beads and analyzed by Western blot with anti-KLF4, anti-Sp1, or anti-YB1 antibodies. *E*, VSMCs were treated with 10 μM of ATRA for 1 h, and ChIP assays were performed using antibodies against KLF4, Sp1, or YB1. Primers spanning the proximal region of the *Klf4* promoter, which contains the three GC boxes (positive primers), or the distal region of the *Klf4* promoter −1063 to −821 (negative primers) were used to amplify the precipitated DNA by PCR. *F*, CHO-K1 cells were co-transfected with the *Klf4* proximal promoter-reporter construct containing the three wild-type or mutated GC boxes, along with various combinations of expression plasmids for KLF4, Sp1, YB1, and RARα as indicated. Luciferase activity assay was performed on total cell lysates as described above. *IB*, immunoblot; *IP*, immunoprecipitation; *Veh*, vehicle.

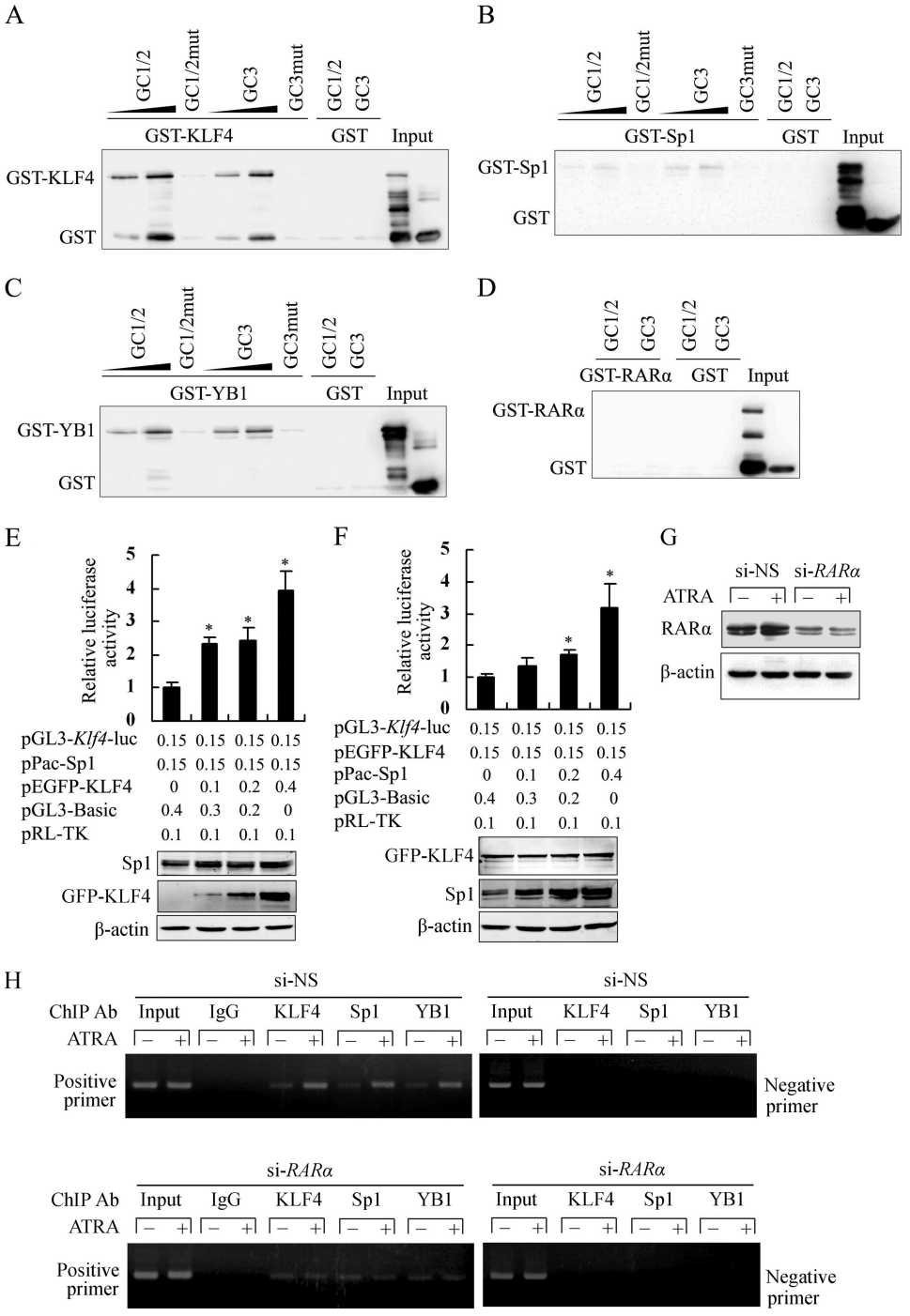

FIGURE 4. **Association of KLF4, Sp1, or YB1 with GC boxes facilitated *Klf4* promoter activities.** *A–D*, oligonucleotide pulldown assay was performed to analyze the binding of recombinant KLF4 (*A*), Sp1 (*B*), YB1 (*C*), or RARα (*D*) to the *Klf4* promoter *in vitro*. DNA-bound proteins were collected with streptavidin-agarose beads and analyzed by Western blot with anti-GST antibody. *E*, CHO-K1 cells were co-transfected with a constant amount of pPac-Sp1 and increasing amounts of pEGFP-KLF4, along with *Klf4* promoter-reporter construct pGL3-*Klf4*-luc. Luciferase activity was measured on total cell lysates as described above. *, $p < 0.05$ *versus* pEGFP-KLF4-untransfected group (*first bar*). Expression level of Sp1 and GFP-KLF4 was assessed by Western blot analysis. β-Actin was used as a control for equal protein loading. *F*, CHO-K1 cells were co-transfected with the indicated constructs, and then luciferase activity of *Klf4* promoter-reporter construct was analyzed as described above. *, $p < 0.05$ *versus* the pPac-Sp1-untransfected-group (*first bar*). The expression level of GFP-KLF4 and Sp1 was assessed by Western blot analysis. β-Actin was used as a control for equal protein loading. *G*, VSMCs were transfected with si-*RARα* or si-NS for 24 h and then treated with 10 μM of ATRA for 1 h. Expression of RARα was assessed by Western blot analysis. β-Actin was used as a control for equal protein loading. *H*, VSMCs were transfected with si-RARα or si-NS for 24 h and then treated with 10 μM of ATRA for 1 h. ChIP assays were performed using antibodies against KLF4, Sp1, or YB1. Primers spanning the proximal region of the *Klf4* promoter containing the three GC boxes (positive primers) or the distal region of the *Klf4* promoter −1063 to −821 (negative primers) were used to amplify the precipitated DNA by PCR.

lower level, but the co-expression of KLF4, Sp1, or YB1 significantly increased the binding of RARα to GC boxes when ATRA was present (Fig. 5, *E–G*). These results suggested that the binding of RARα to the *Klf4* promoter was KLF4-Sp1-YB1 complex-dependent and that ATRA stimulation promoted the interaction of RARα with KLF4, Sp1, and YB1.

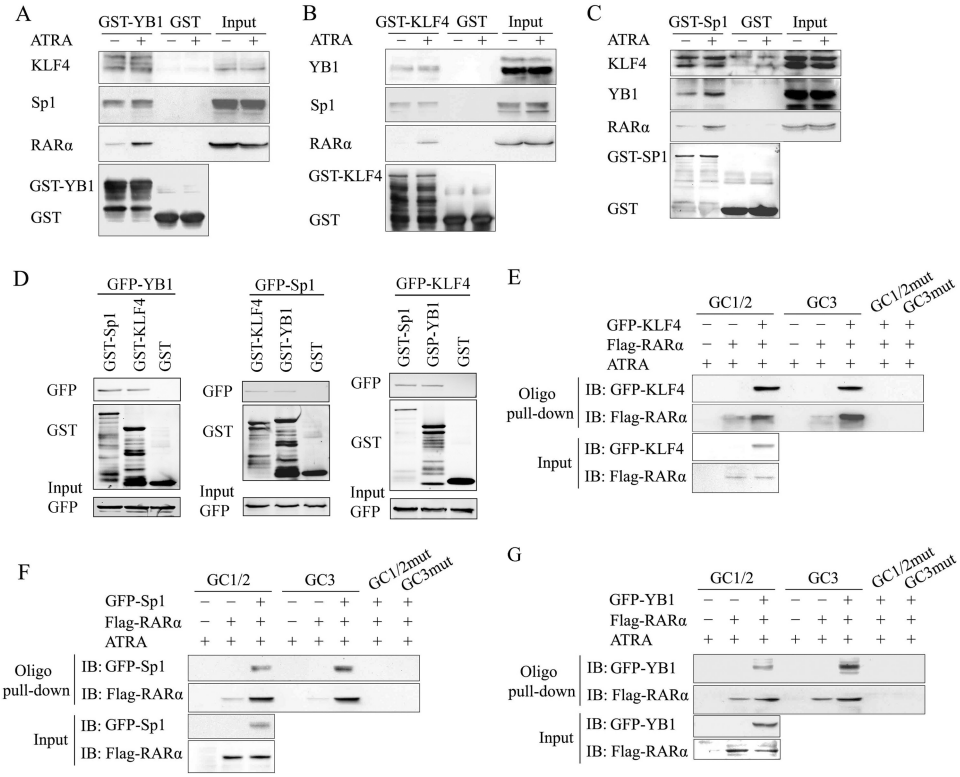

FIGURE 5. **ATRA promoted the binding of RARα to the *Klf4* promoter in a KLF4-Sp1-YB1-dependent manner.** *A*, the purified recombinant GST and GST-YB1 proteins on the glutathione beads were incubated with total cell lysates of VSMCs treated with 10 μM ATRA for 1 h, followed by extensive washing. Proteins on the beads were subjected to Western blot with anti-KLF4, anti-Sp1, or anti-RARα antibodies. *B*, GST pulldown assay of recombinant GST-KLF4 or GST with lysates from VSMCs treated with ATRA for 1 h. Proteins on the beads were subjected to Western blot with anti-YB1, anti-Sp1, and anti-RARα antibodies. *C*, GST pulldown assay of recombinant GST-Sp1 or GST with lysates from VSMCs treated with ATRA for 1 h. Proteins on the beads were subjected to Western blot with anti-KLF4, anti-YB1, or anti-RARα antibodies. *D*, CHO-K1 cells were transfected for 24 h to express GFP-YB1 (*left*), GFP-Sp1 (*middle*), or GFP-KLF4 (*right*) protein and then treated with 10 μM of ATRA for 1 h. The whole cell lysates of CHO-K1 cells were used to perform GST pulldown assay. The recombinant GST, GST-KLF4, GST-Sp1, and GST-YB1 proteins on the glutathione beads were incubated with the cell lysates, respectively, overnight at 4 °C, followed by extensive washing. Proteins on the beads were eluted and detected by Western blot with anti-GFP antibody. *E–G*, CHO-K1 cells were co-transfected to express indicated factors, with a FLAG tag attached to RARα and with a GFP tag attached to KLF4 (*E*), Sp1 (*F*), or YB1 (*G*). The cell lysates were subjected to an oligonucleotide pulldown assay with biotinylated double-stranded oligonucleotides containing wild-type GC1 and GC2 box sequences (GC1/2), mutated GC1 and GC2 (GC1/2mut), wild-type GC3 (GC3), and mutated GC3 (GC3mut) as probes. The precipitates were analyzed by Western blot with antibodies against the FLAG tag or GFP tag.

Knockdown of Endogenous KLF4, Sp1, and YB1 Disrupted Recruitment of RARα to Klf4 Promoter—To further test whether the binding of RARα to the *Klf4* promoter depended on KLF4, Sp1, and YB1, we performed a ChIP assay in VSMCs where KLF4, Sp1, or YB1 was knocked down by RNA interference (siRNA). As shown in Fig. 6*A*, higher occupancy of RARα on the *Klf4* promoter was observed under ATRA stimulation. Knockdown of endogenous KLF4, Sp1, or YB1 by siRNA significantly attenuated the binding of RARα to the *Klf4* promoter; no binding of RARα was detected when one pair of negative control primers was used (Fig. 6*A*).

To further validate the above results, we next transfected VSMCs with siRNAs targeting *Klf4*, *Sp1*, or *YB1* or with an adenovirus expression vector for RARα, followed by ATRA treatment. Overexpression of RARα significantly enhanced KLF4 expression induced by ATRA. Knockdown of endogenous KLF4, Sp1, or YB1, together with RARα overexpression, abrogated the induction of KLF4 expression by ATRA in VSMCs (Fig. 6, *B* and *C*). Altogether, these results confirmed that KLF4, Sp1, and YB1 were indispensible for the recruitment of RARα to the *Klf4* promoter.

DISCUSSION

KLFs form a large family of transcription factors that share in common a transcriptional activation/repression domain, a nuclear localization signal, and three Krüppel-like zinc fingers (37). To date, at least 20 KLFs have been identified in mammals. Each individually has important biological functions in cell proliferation, apoptosis, development, and oncogenic processes (3, 38). KLF4 has been shown to play a key role in pathological vascular processes and is considered a molecular switch, regulating the function of VSMCs under pathophysiological conditions (3).

Recently, we demonstrated that KLF4 is required for VSMC differentiation induced by ATRA (16). Because the effect of ATRA is mediated by RARs and RXRs, we then speculated that the role of RARs in up-regulating KLF4 expression may be important. In the present study, we characterized the regulation of *Klf4* by ATRA and RARα. We found that ATRA regulated *Klf4* expression through the three GC boxes in the proximal promoter of the gene and that this regulation required interaction between its nuclear receptor RARα and the KLF4-Sp1-YB1 complex. These results therefore provide evidence for

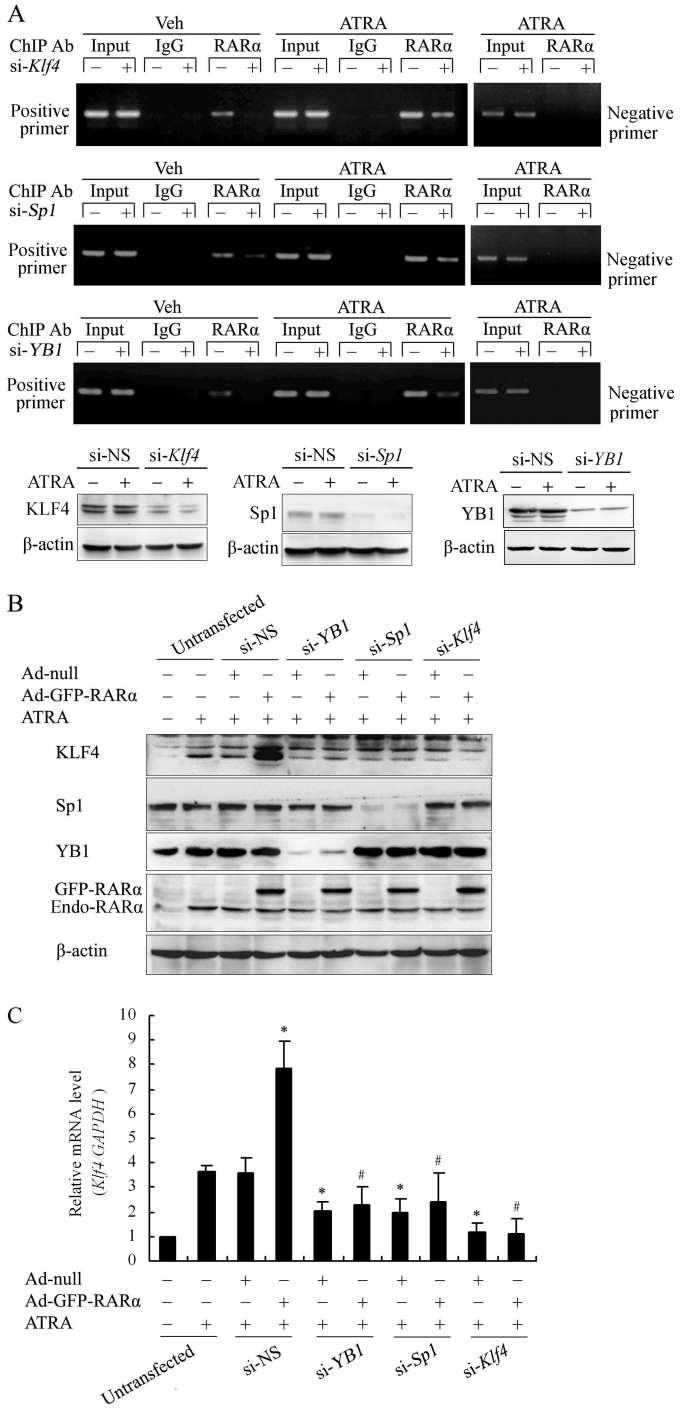

FIGURE 6. **Knockdown of endogenous KLF4, Sp1, and YB1 disrupted the recruitment of RARα to the *Klf4* promoter.** *A*, VSMCs were transfected with siRNA targeting *KLF4* (si-*Klf4*), *Sp1* (si-*Sp1*), or *YB1* (si-*YB1*) for 24 h and then treated with 10 μM of ATRA for 1 h. ChIP assay was performed using anti-RARα antibody. Primers spanning the proximal region of the *Klf4* promoter containing the three GC boxes (positive primers) or the distal *Klf4* promoter region −1063 to −821 (negative primers) were used to amplify the precipitated DNA by PCR. Knockdown of level of KLF4, Sp1, or YB1 in VSMCs was assessed by Western blot analysis. β-Actin was used as a control for equal protein loading. *B*, VSMCs were transfected with siRNA targeting KLF4 (si-*Klf4*), Sp1 (si-*Sp1*), YB1 (si-*YB1*), or NS-siRNA (si-NS) and infected with Ad-null or Ad-GFP-RARα for 24 h. The cells were treated with 10 μM of ATRA for 24 h and then lysed. Western blot was performed with anti-KLF4, anti-Sp1, anti-YB1, or anti-RARα antibodies. β-Actin was the loading control. *Endo-RARα* indicated endogenous RARα in VSMCs. *C*, VSMCs were transfected with si-*Klf4*, si-*Sp1*, si-*YB1*, or si-NS for 24 h and infected with Ad-null or Ad-GFP-RARα for 24 h. The cells were then treated with 10 μM of ATRA for 24 h. Total RNA was isolated and subjected to quantitative RT-PCR. The *bars* represent the means ± S.E. from three independent experiments. *, $p < 0.05$ *versus* si-NS and Ad-null-infected group (*third bar*). #, $p < 0.05$ *versus* si-NS and Ad-GFP-RARα-infected group (*fourth bar*).

the first time that RARα activates the *Klf4* promoter in an RARE-independent manner in VSMCs.

RARα has been shown to distinctly mediate the growth inhibitory effect of retinoids and therefore is a potential target of research for preventive treatment of vascular proliferation disease (24). In VSMCs, we discovered that treatment with ATRA induced the expressions of KLF4 and RARα. Silencing of the *RARα* gene or inhibiting RARα with its antagonist Ro 41-5253 abrogated the ATRA-induced *Klf4* transcription, indicating that RARα plays a critical role in the induction of *Klf4* by ATRA and cannot be substituted by other RARs.

Although retinoid signaling is considered responsible for the direct binding of RARs/RXRs to RAREs in target gene promoters, other evidence has shown that RARs exert their effect by interacting with other general transcription factors, such as Sp1, Ets-1, cAMP response element-binding protein, and KLF5 (24, 26, 36, 39). There are three GC boxes in the proximal region of the *Klf4* promoter, which may play a vital role in *Klf4* transactivation (9, 18). Yang and co-workers (40) reported that KLF4 activates its own gene promoter, whereas KLF5 suppresses the *Klf4* promoter through the same three closely spaced GC boxes within the *Klf4* promoter. Owens and co-workers (17) demonstrated that Sp1 regulates the expression of KLF4 in platelet-derived growth factor-BB-stimulated VSMCs via the GC boxes in the proximal *Klf4* promoter. Their results demonstrate that GC boxes in the *Klf4* promoter execute important roles in *Klf4* transcriptional activities.

Our data showed that ATRA stimulation increased RARα binding to the proximal *Klf4* promoter. Luciferase reporter gene assays revealed that when all three of the GC boxes were mutated, ATRA-dependent *Klf4* promoter transactivation was abrogated, and overexpression of RARα failed to activate *Klf4* expression. These results suggest that the three GC boxes in the proximal *Klf4* promoter are required for RARα-dependent *Klf4* transcription.

Interestingly, using the oligonucleotide pulldown assay, we found that YB1 specifically binds to the *Klf4* promoter region in response to ATRA stimulation. ChIP and oligonucleotide pulldown assays demonstrated that YB1 interacted with DNA in the *Klf4* proximal promoter region with the GC boxes motif (−179 to +20). It is well known that YB1 belongs to the evolutionarily conserved group of CCAAT-binding proteins that control the expression of a large number of gene products. It classically binds to DNA containing a Y box or inverted CCAAT box (CTGATTGGCCAA) and activates genes associated with proliferation and cancer such as cyclin A, cyclin B1, DNA polymerase α, and the multidrug resistance 1 gene (41–44). Importantly, the present data for the first time provide *in vivo* and *in vitro* evidence that YB1 directly binds to the GC box (GGCGGGG) and transactivates the *Klf4* promoter in ATRA-stimulated VSMCs. Therefore, the novel binding motif of YB1 implies an unrevealed signaling pathway involving YB1 in VSMCs in response to ATRA stimulation.

Because RARα has been shown to synergistically interact with other transcription factors, it is conceivable that besides a direct activation, RARα might also cooperate with other transcription factors in the regulation of *Klf4* gene transcription. The present study showed that KLF4, Sp1, and YB1 formed a protein complex without ATRA treatment. When cells were treated with the RAR-selective antagonist, RARα was recruited to the three GC boxes in the *Klf4* promoter by its association with KLF4, Sp1, and YB1. Knockdown of endogenous YB1 led to a significant decrease of RARα occupancy in the *Klf4* promoter in ATRA-stimulated VSMCs. YB1 transactivates CC chemokine ligand 5 gene transcription by binding to the CCAAT box on the chemokine ligand 5 promoter in VSMCs and contributes to neointimal hyperplasia (45). This implicates YB1 as a critical factor in the regulation of the differentiation and proliferation of VSMCs, and our findings further suggest that YB1 may serve as a key mediator of VSMC differentiation. This might be due to selective binding of YB1 to different specific DNA elements in a cell context-dependent manner.

In summary, we showed that ATRA signaling up-regulated *Klf4* gene transcription through functional interaction of RARα with KLF4, Sp1, and YB1 bound to the GC boxes of the *Klf4* promoter. The present results thus describe a novel mechanism of regulation of *Klf4* by ATRA and RARα, which is critical toward the understanding of the biological functions of retinoids during VSMC phenotypic modulation.

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