Biochemical and Biophysical Research Communications 437 (2013) 625–631

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Key role of microRNA-15a in the KLF4 suppressions of proliferation and angiogenesis in endothelial and vascular smooth muscle cells

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ARTICLE INFO

Article history:
Received 4 July 2013
Available online 15 July 2013

Keywords:
Krüppel-like factor 4
miR-15a
Proliferation
Cell cycle
Cyclin D1

ABSTRACT

While recent insights indicate that the transcription factor Krüppel-like factor 4 (KLF4) is indispensable for vascular homeostasis, its exact role in proliferation and angiogenesis and how it functions remain unresolved. Thus, the aim of the present study was to evaluate the role of KLF4 in the proliferations of endothelial and vascular smooth muscle cells, as well as the angiogenesis. The overexpression of KLF4 in endothelial cells significantly impaired tube formation. KLF4 inhibited the formation of a vascular network in implanted Matrigel plugs in nude mice. Importantly, we found that KLF4 significantly upregulated the miR-15a expression in endothelial cells and vascular smooth muscle cells, and conversely, KLF4 depletion reduced the amount of miR-15a. Furthermore, KLF4 blocked cell cycle progression and decreased cyclin D1 expression in endothelial cells and vascular smooth muscle cells through the induction of miR-15a. Intriguingly, the delivery of a miR-15a antagomir to nude mice resulted in marked attenuation of the anti-angiogenic effect of KLF4. Collectively, our present study provide the first evidence that miR-15a as a direct transcriptional target of KLF4 that mediates the anti-proliferative and anti-angiogenic actions of KLF4, which indicates that KLF4 upregulation of miR-15a may represent a therapeutic option to suppress proliferative vascular disorders.

1. Introduction

Angiogenesis is a biological process by which new blood vessels develop from pre-existing endothelial cells (ECs) in response to angiogenic stimuli. Following the EC basement membrane degradation by proteases, ECs migrate from the existing vessels towards the source of VEGF, and begin to proliferate and assemble into tubes with a parent lumen. After the formation of small blood vessels, proliferation and recruitment of smooth muscle cell (SMCs)-like pericytes to the immature vascular structures is required to stabilize these new vessels [1–3]. Therefore, the proliferations of ECs and SMCs have essential roles in establishing stable vascular structures.

The Krüppel-like factors (KLFs) are a family of evolutionarily conserved zinc finger-containing transcription factors, containing a DNA-binding domain consisting of three zinc fingers positioned at their carboxyl-terminal end, which enables them to bind to "CACCC" or "GT-box" sites [4]. Mounting evidence indicates that the KLF4 is highly expressed in ECs and VSMCs and acts as critical regulator of vascular homeostasis. Diverse vaso-protective stimuli, including laminar shear stress and simvastatin, induced the expression of KLF4 in ECs [5,6]. Moreover, KLF4 protects against atherothrombosis in mice via competition for the coactivator p300 [7]. Fang and Davies demonstrated that miRNA-92a regulates KLF4 in atherosusceptible endothelium [8]. In VSMCs, KLF4 is rapidly induced by TGF-b and PDGF-BB [9]. While previous studies provide compelling evidence for the importance of KLF4 in blood vessels, its pivotal role in vascular cell proliferation and angiogenesis, and thereby the potential mechanisms, is largely undefined.

MicroRNAs (miRNAs) represent a class of conserved non-coding small RNAs that repress gene expression post-transcriptionally by targeting the 3′-untranslated regions (3′-UTRs) of mRNAs [10]. The posttranscriptional titration of key angiogenic signaling nodes by miRNAs has gathered particular interest [11]. Several miRNAs were shown to stimulate (miR-221) or inhibit (miR-17–92 cluster, miR-100) angiogenesis and regulate vascular development (miR-126)

Abbreviations: HUVEC, human umbilical vein endothelial cell; VSMCs, vascular smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; BAECs, bovine aortic endothelial cells; ECs, endothelial cells; FBS, fetal bovine serum; KLF4, krüppel-like factor 4; miRNA, microRNAs; 3′-UTRs, 3′-untranslated regions; HE, hematoxylin eosin; VE-Cad, VE-cadherin; RT-PCR, reverse transcription polymerase chain reaction; NC, negative control; ChIP, chromatin immunoprecipitation.

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.07.017

[12–15]. Nonetheless, the crucial role of miR-15a in the KLF4-regulated proliferative and angiogenic processes is largely unknown.

In the present study, we have demonstrated for the first time that KLF4 can strongly inhibit the proliferation of ECs and VSMCs, and suppress the capillary-like tube formation. Importantly, we have found that miR-15a is a novel target gene of KLF4 transactivation, directly contributing to the anti-proliferative and anti-angiogenic behaviors of KLF4. This study provides insight into the therapeutic potential of KLF4 in proliferative vascular disorders and pathological angiogenesis.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured as previously described [16]. The cells were maintained in M199 supplemented with 20% FBS (Hyclone), 20 mM HEPES, 1 ng/ml of recombinant human αFGF (Sigma) and 90 μg/ml of heparin at 37 °C in a humidified 5% CO_2 atmosphere. In all experiments, HUVECs within five passages were used. Human aortic vascular smooth muscle cells (VSMCs) were from Invitrogen (Carlsbad, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS. Bovine aortic endothelial cells (BAECs) were harvested from bovine aorta and maintained in DMEM with 10% FBS.

2.2. Adenoviral vectors and infection

A recombinant adenovirus (Ad) expressing full-length KLF4 was prepared as described previously [17]. The cDNA encoding the full-length coding regions of human KLF4 was subcloned into the shuttle vector pAdlox, a tet-off expression cassette, and recombined with an E1- and E3-deleted Ψ5 viral DNA in CRE8 cells. Confluent HUVECs were infected with purified KLF4 adenovirus at a multiplicity of infection of 20.

2.3. Cell cycle analysis

The cell cycle analysis was performed as described previously [18]. At 80% confluence, ECs and VSMCs were synchronized by serum starvation in 1% FBS-containing M199 medium for 12 h and treated with KLF4 for 12 h followed by stimulation with M199 containing 20% FBS for 12 h. Adherent cells were harvested and fixed in 70% ethanol. Staining for DNA content was performed with 50 μg/ml propidium iodide (Sigma) and 100 μg/ml RNase A for 30 min. The cell cycle distribution was analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

2.4. TaqMan® miRNA assay for identification of miR-15a

Total RNA, including miRNA, was isolated with use of TRIzol reagent (Invitrogen) and treated with RNase-free DNase I. Amplification and detection of mature miR-15a was performed using the TaqMan® MiRNA Assay Kit (Applied Biosystems) in a DNA Engine Opticon real time system as previously described [18].

2.5. Western blot

Nuclear protein was extracted from cells as described previously [18]. For western blotting, 20 μg of the nuclear protein was separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Briefly, the PVDF membrane was incubated with a primary antibody against KLF4 (Santa Cruz) and cyclin D1 (Santa Cruz) or histone H3, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody. The immunoblots were visualized with an ECL chemiluminescence system and detected by using the ChemiDoc XRS system (Bio-Rad).

2.6. Pre-miR-15a or miR-15a inhibitor transfection

The HUVECs were transfected with 30 nmol/L Pre-miR-15a or a negative control (Applied Biosystems) using siPORT™ NeoFX™ Transfection Agent (Applied Biosystems) for 48 h. The anti-miR-15a oligonucleotide or a negative control (Applied Biosystems) was transfected at a final concentration of 40 nmol/L for 48 h.

2.7. MiRNA-reporter, transfection and reporter assay

The sense and antisense strands of the 3′UTR of human cyclin D1 containing the miR-15a binding sites were synthesized, annealed and cloned into the SpeI/HindIII sites of the pMIR-REPORT luciferase vector (Ambion). The seed mutations for target site 1, 2, or both were generated using the Quickchange II site-directed mutagenesis kit (Stratagene). The luciferase and β-galactosidase activities were measured as described previously [18].

2.8. Chromatin immunoprecipitation

After the HUVECs were infected with the KLF4 adenovirus for 24 h, the cells were crosslinked with 1% formaldehyde for 30 min and quenched prior to sonication as previously described [19]. The input fraction corresponded to 10% of the chromatin solution. The sheared chromatin was immunoprecipitated with an anti-KLF4 antibody or IgG at 4 °C. Then, 40 μl of protein A/G Sepharose beads (Promega) and 2 μl of 10 mg/ml herring sperm DNA (Sigma) were added. The primers used to amplify the areas containing the KLF4 binding sites were as follows: ChIP 1 (−1084 to −884 bp), 5′-GCCTGTAATTTTAGGACTT-3′ (forward) and 5′-TAGCCTCCCA AGTAGCTGG-3′ (reverse); ChIP 2 (−1727 to −1559 bp), 5′-GTACAT-CACCTTACTATTCT-3′ (forward) and 5′-TACCAGGTAAGGAAGAA-ACT-3′ (reverse).

2.9. Plasmid, transfection and reporter assay

The genomic fragment corresponding to −1639 to +61 bp relative to the TSS of the miR-15a gene was amplified using PCR from the genomic DNA and cloned into the MluI/BglII site of the pGL3 luciferase vector (Promega) to generate miR-15a-luc 1.7 kb Wt. The primer sets were as follows: forward, 5′-TTGTCTCTACAA-TAAAATAAA-3′; reverse, 5′-AATATGGCCTGCACCTTTTCAA-3′. The sequences of the mutations for KLF4 binding site 1 contained the miR-15a 1.7 kb site 1 *mut*. 5′-GT*TT*T*T*-3′ (the italic underlined nucleotides are mutated). BAECs were transfected with miR-15a 1.7 kb Wt. versus miR-15a 1.7 kb site 1 *mut*. The luciferase and β-galactosidase activities were measured as described previously [19].

2.10. Tube formation assay

Matrigel™ (BD Biosciences) was thawed and allowed to polymerize at 37 °C for 30 min. Following treatment with KLF4 adenovirus for 24 h, HUVECs (1×10^5 cells) were suspended in M199 medium and plated on the top of the gel, and they were incubated at 37 °C with 5% CO_2. Tube formations were analyzed on an inverted microscope to count the number of tube-like structures in three representative fields in each well.

2.11. In vivo vasculogenesis and oligonucleotide administration

Male athymic nu/nu mice (6–8 weeks old) were used for *in vivo* angiogenesis assays. Following pretreatment with KLF4 adenovirus, HUVECs were resuspended in 100 μl Matrigel™ and implanted on the flank of the nu/nu mice ($n = 5$) by subcutaneous injection. To inhibit miR-15a, a chemically modified and cholesterol-conjugated antisense oligonucleotide (antagomir) specific for miR-15a and a non-specific negative control (NC) were synthesized (RiboBio, China). The sequence of the antagomir against miR-15a is: 5′-mU(s)mA(s)mGmCmAmGmCmAmCmAmUmAmAmUmGmGmUmUmU(s)mG(s)mU(s)mG(s)-Chol-3′, where m is a 2′-OMe-modified nucleotide, (s) is a phosphorothioate linkage and Chol is a cholesterol group linked through a hydroxyprolinol linkage. After the implantation of the Matrigel plugs, the nude mice in each treatment group underwent tail vein injections of 80 mg/kg body weight miR-15a antagomir at days 1, 3 and 5. The Matrigel plugs were harvested at day 7 for histological analysis by staining with hematoxylin and eosin (H&E) or with VE-cadherin and human nucleus antibodies. The images were analyzed using an Olympus CKX41 microscope.

2.12. Statistical analysis

The results are expressed as the means ± SEM. Student's *t* test or one-way ANOVA followed by Newman–Keuls test was performed for statistical analysis. *P* values <0.05 were considered to be statistically significant.

3. Results

3.1. KLF4 inhibits capillary tube formation

To assess whether KLF4 interferes with the angiogenic process, we performed a Matrigel capillary tube formation assay. In the presence of KLF4, we observed that the KLF4-transduced HUVECs exhibited poor capillary-like tube formation (Fig. 1A), and the numbers of branch points decreased by 74.1% in comparison with the mock group (Fig. 1B). Furthermore, we assessed the effect of KLF4 on the angiogenic process *in vivo* using Matrigel plugs subcutaneously implanted in nude mice. H&E staining revealed less luminal structure in the implants with KLF4-treated ECs than in the untreated control or mock group (Fig. 1C, upper panel), as quantified in Fig. 1D. Immunostaining showed that many cells located in the luminal structure were positive for VE-cadherin and human nuclei (Fig. 1C, middle panel), which confirms that the ECs injected into nude mice were directly involved in vasculogenesis *in vivo* (Fig. 1C, lower panel). Together, these findings demonstrate that KLF4 dramatically impaired the potential of ECs to form capillary-like structures.

3.2. KLF4 blocks the G1/S transition of the cell cycle and cyclin D1 expression through the induction of miR-15a

Because the proliferations of ECs and VSMCs are essential for the development of functional neovessels [1–3], we first examined the effect of KLF4 on cell cycle progression in ECs and VSMCs. The

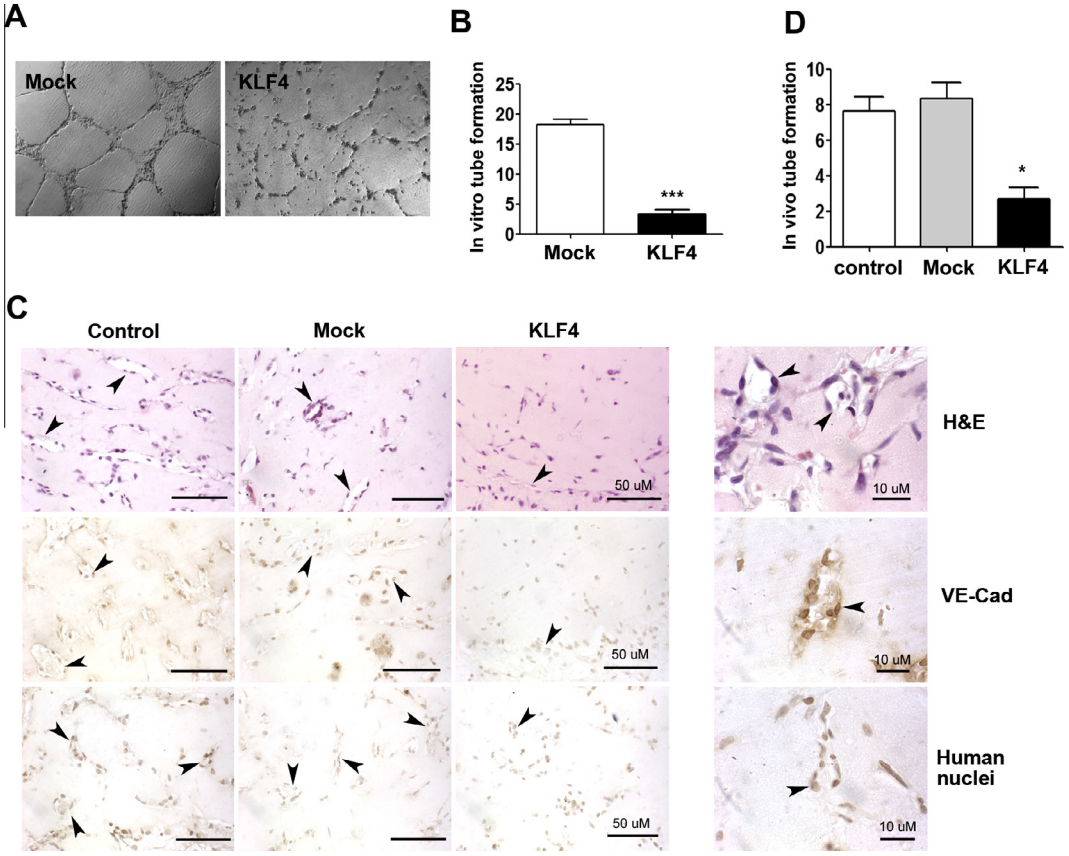

Fig. 1. KLF4 inhibits angiogenesis of ECs both *in vitro* and *in vivo*. (A) Representative microscopic view of tube formation in HUVECs without (mock) or with KLF4 treatment. (B) The tube formation was determined by counting the numbers of branch points in three randomly chosen fields (*$P < 0.05$). (C) *In vivo* KLF4 overexpression suppresses Matrigel plug angiogenesis. The cross-sections of the implants submitted to H&E, VE-cadherin and human nuclei staining (400×, scale bar = 50 μM). A representative image of microvessels under high-power magnification (1000×, scale bar = 10). The arrows indicate vessel-like structures. (D) The microvessel density in three different fields for each Matrigel plug was quantified by counting the luminal structures ($n = 5$. *$P < 0.05$).

cell cycle distribution showed that the KLF4-overexpressing ECs and VSMCs had a lower proportion of cells in S phase and a higher proportion in G0/G1 phase (Supplementary Fig. S1A and B), indicating the occurrence of G0/G1 cell cycle arrest. Furthermore, we compared the expression levels of cyclin D1, a critical regulator of the G1 to S phase transition. The cyclin D1 protein levels were significantly decreased in KLF4-transfected ECs and VSMCs (Supplementary Fig. S1C and D).

The discovery of miRNAs has shed light on how noncoding RNAs can regulate proliferation and angiogenesis. To gain insight into the mechanisms by which KLF4 may regulate the proliferations of ECs and VSMCs, as well as the angiogenesis, we first defined the specific miRNA gene. In our study, the miR-15a level was significantly increased by KLF4 in ECs and VSMCs (Fig. 2A). Conversely, KLF4 knockdown reduced the expression of miR-15a (Fig. 2B). Moreover, pretreatment with the miR-15a inhibitor effectively attenuated KLF4-induced cell cycle arrest in ECs and VSMCs, wherein larger cell populations were found to progress to the S phase in the presence of KLF4 (Fig. 2C and D), which suggests that the KLF4 upregulation of miR-15a contributed to the cell cycle arrest effect of KLF4 in ECs and VSMCs. Furthermore, silencing of miR-15a reversed the inhibitory effects of KLF4 on cyclin D1 expression in ECs and VSMCs (Fig. 2E and F), thus confirming that the KLF4 inhibition of cyclin D1 in ECs and VSMCs is under the control of miR-15a. Taken together, these results highlighted the critical role of miR-15a in the KLF4-elicited cyclin D1 suppression and G0/G1 cell cycle arrest in ECs and VSMCs.

Sequence alignment of human miR-15a with the 3′-UTR of cyclin D1 identified two conserved miR-15a binding sites (Supplementary Fig. S2A). We constructed a luciferase reporter containing the cyclin D1 3′-UTR fragment. The luciferase activity could be significantly repressed after co-transfection with pre-miR-15a (Supplementary Fig. S2B). Furthermore, mutation of the binding site 2, but not site 1 in the 3′-UTR of cyclin D1 abolished the repressive effect of miR-15a. Mutating both of the target sites simultaneously also relieved the repressive effect of miR-15a (Supplementary Fig. S2B). Accordingly, transfection of pre-miR-15a led to an obvious decrease in cyclin D1 levels (Supplementary Fig. S2C), whereas a miR-15a inhibitor effectively increased the cyclin D1 levels (Supplementary Fig. S2D). These results suggest that miR-15a decreased cyclin D1 translation by directly acting on target site 2.

3.3. KLF4 binds to and activates the miR-15a promoter

Sequence analysis of the 5′-flanking regions of human miR-15a revealed two putative binding sites for KLF4 at the locations of −1598 to −1593 bp and −975 to −970 bp upstream of the transcription start site (TSS) (Fig. 3A). To test whether KLF4 binds to these regions, a chromatin immunoprecipitation (ChIP) assay was performed. As shown in Fig. 3B, KLF4 detectably bound to the elements located between −1598 and −1593 bp. An isotypic IgG antibody was used as a negative control. These data strongly suggest that in the context of chromatin, binding site 1 between nucleotides −1598 and −1593 bp appears to be functional. To ascertain the functionality of binding site 1 in the miR-15a promoter, we cloned the 1700-bp (−1639 to +61 bp) promoter of human miR-15a into a luciferase reporter vector and transfected the wide type construct (miR-15a 1.7 kb Wt) into BAECs. As demonstrated in Fig. 3C, KLF4 efficiently induced a 2.2-fold increase in the luciferase

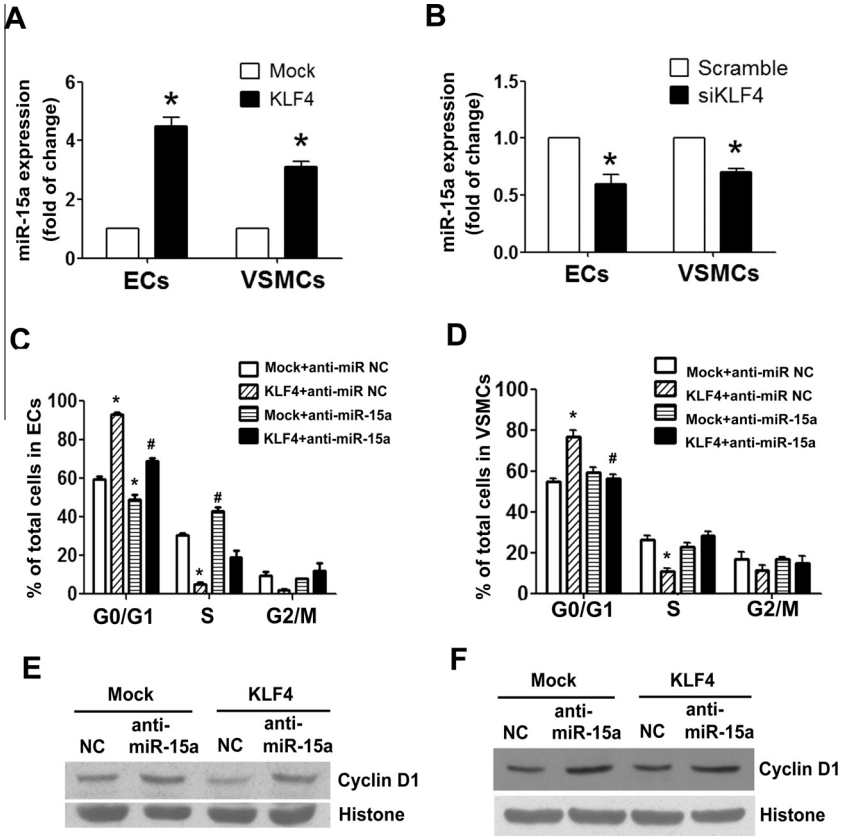

Fig. 2. KLF4 blocks the G1/S transition of the cell cycle and cyclin D1 expression through the induction of miR-15a. (A) miR-15a expression in ECs and VSMCs after transfection of KLF4 for 24 h. (B) miR-15a expression in ECs and VSMCs after knockdown of KLF4 ($^{*}P < 0.05$). (C) and (D) Flow cytometry showed that a miR-15a inhibitor attenuated the KLF4-induced cell cycle arrest in ECs and VSMCs ($^{*}P < 0.05$ *vs.* mock plus anti-miR negative control, $^{\#}P < 0.05$ *vs.* KLF4 plus anti-miR negative control). (E) and (F) Silencing miR-15a attenuated the inhibitory effect of KLF4 on cyclin D1 expression in ECs and VSMCs.

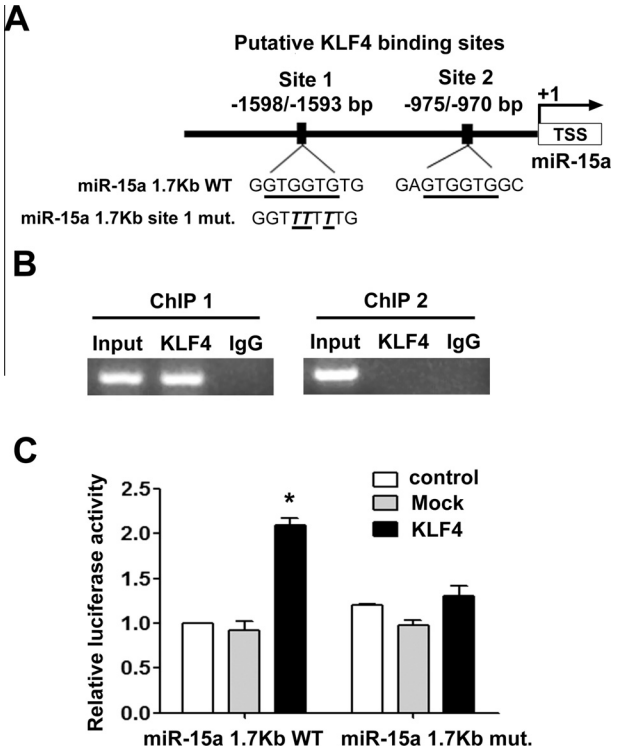

Fig. 3. Functional analysis of KLF4 binding sites located in the human miR-15a promoter. (A) Schematic representation of the two putative KLF4 binding sites in the promoter of miR-15a. (B) ChIP assay using an anti-KLF4 antibody (KLF4) or IgG. The immunoprecipitated DNA fragments and input were detected using PCR with specific primers flanking the putative KLF4 binding sites. (C) Luciferase reporter assays were performed by transfecting BAECs with a miR-15a 1.7 kb wild type promoter (miR-15a 1.7 kb Wt) versus a site 1 mutated miR-15a promoter (miR-15a 1.7 kb site 1 *mut.*). The data were from three independent experiments, each performed in triplicate ($^*P < 0.05$ *vs.* control).

activity, and site-directed mutagenesis showed that the binding site 1 was required for the induction of miR-15a by KLF4. Collectively, these results indicate that miR-15a was a direct target gene of KLF4 in ECs.

3.4. Blockade of miR-15a attenuates the anti-angiogenic effect of KLF4

To test the hypothesis that miR-15 may also mediate the anti-angiogenic activity of KLF4, we next examined the potential effect of silencing miR-15a on the anti-angiogenic activity of KLF4. As expected, in the presence of KLF4, the anti-miR-15a-pretreated ECs exhibited more tube formation (Fig. 4A), as quantified in Fig. 4B. An *in vivo* tube formation assay in the nude mice, to define the involvement of miR-15a revealed that there was less luminal structure in the implants with KLF4-treated ECs than the mock cells (Fig. 4C, upper panel). Notably, the number of luminal structure in the KLF4-treated ECs was dramatically increased in the miR-15a antagomir group (Fig. 4C, upper panel), as quantified in Fig. 4D. Similarly, VE-cadherin and human nuclei immunostaining confirmed that the transplanted human ECs were directly involved in vasculogenesis *in vivo* (Fig. 4C, middle panel & lower panel). Together, these data strengthened our hypothesis that the anti-angiogenic behavior of KLF4 might, at least in part, be mediated by miR-15a.

4. Discussion

In this study, we report a novel finding that KLF4 suppresses the proliferation and angiogenesis of ECs and VSMCs *via* the induction of miR-15a. Our findings are as follows: (1) KLF4 increases the expression of miR-15a in ECs and VSMCs, thus repressing cyclin D1 expression and, hence, leading to cell cycle arrest; (2) miR-15a is a direct target gene of KLF4; and (3) miR-15a mediates the anti-proliferative and anti-angiogenic effects of KLF4 in ECs and VSMCs.

Previous studies have shown that KLF4 is a master regulator of vascular biology. Nonetheless, its pivotal roles in regulating vascular proliferation, angiogenesis and the underlying molecular mechanism have not been addressed. The miRNA-mediated gene silencing mechanism described in the present study significantly advances our knowledge of how KLF4 fine-tunes gene expression in ECs and VSMCs and thereby regulates the proliferative and angiogenic function at the posttranscriptional level. Specifically, miR-15a emerged as a potentially important mediator. In the present study, we describe for the first time that the functional inhibition of miR-15a attenuates the anti-proliferative and anti-angiogenic effects of KLF4 in ECs and VSMCs. It is noteworthy that KLF4 transcriptionally induced miR-15a. Mechanistically, the results of a promoter reporter assay and a ChIP assay establish miR-15a as a novel target gene for KLF4. Therefore, our results raise the possibility that KLF4 could be exploited to augment miR-15a levels and thereby confer favorable anti-proliferative and anti-angiogenic effects to the vasculature. This miRNA machinery may particularly explain how KLF4 can restrict the expression of pre-existing transcripts to modulate the proliferative behavior of the vasculature.

As an endogenous gene, miR-15a is a member of the miR-15-16 cluster. The miR-15a/16 cluster is implicated in several cancers [20,21]. However, no evidence currently links miR-15a to the anti-proliferative and anti-angiogenic effects of KLF4. Our results suggest that the functional inhibition of miR-15a largely alleviated the suppressive effect of KLF4 on the proliferations of ECs and VSMCs, as well as the angiogenesis, implying that KLF4 exerts its anti-proliferative and anti-angiogenic role through the upregulation of miR-15a.

A critical step in blood vessel growth involves the local proliferations of ECs and VSMCs [1–3]. Abnormal VSMC proliferation and activation are associated with various vascular disorders such as angiogenesis, atherosclerosis and transplantation-associated vasculopathy. In fact, VSMCs are needed for the angiogenesis and vascular integrity [22,23]. In the present study, we observed that KLF4 effectively downregulates the expression of cyclin D1 in ECs and VSMCs and blocks the cell cycle transition from G1 to S phase, indicating the impairment on angiogenesis by KLF4 is predominantly attributable to the KLF4-elicited cyclin D1 suppression and G0/G1 cell cycle arrest in ECs and VSMCs. In agreement with these findings, a variety of anti-angiogenic molecules have been reported to inhibit cyclin D1 expression and cell cycle progression. A previous study showed that endostatin leads to G1 arrest by downregulating cyclin D1 [24]. MiR-19b-1 inhibits angiogenesis by repressing the expression of cyclin D1 and blocking cell cycle progression [12]. Specifically, one evolutionarily conserved miR-15a binding site in the cyclin D1 3′UTR was functionally validated in the present study. Therefore, blocking endogenous miR-15a resulted in EC and VSMC rescues from KLF4-elicited cyclin D1 suppression and cell cycle arrest. In other words, in the absence of miR-15a, the level of cyclin D1 is increased in turn and ultimately counteracts the cell cycle repression caused by KLF4.

Endothelial KLF4 has garnered attention as a guardian of vascular health. Our novel findings implicate KLF4 as a critical repressor of vascular cell proliferation and angiogenesis. The complex process of vessel growth requires the fine-tuned regulation of a set of miRNA genes. The mechanism of KLF4-repressed proliferation in ECs and VSMCs does not exclusively involve the elevation of miR-15a. Future efforts identifying alternative miRNA genes other

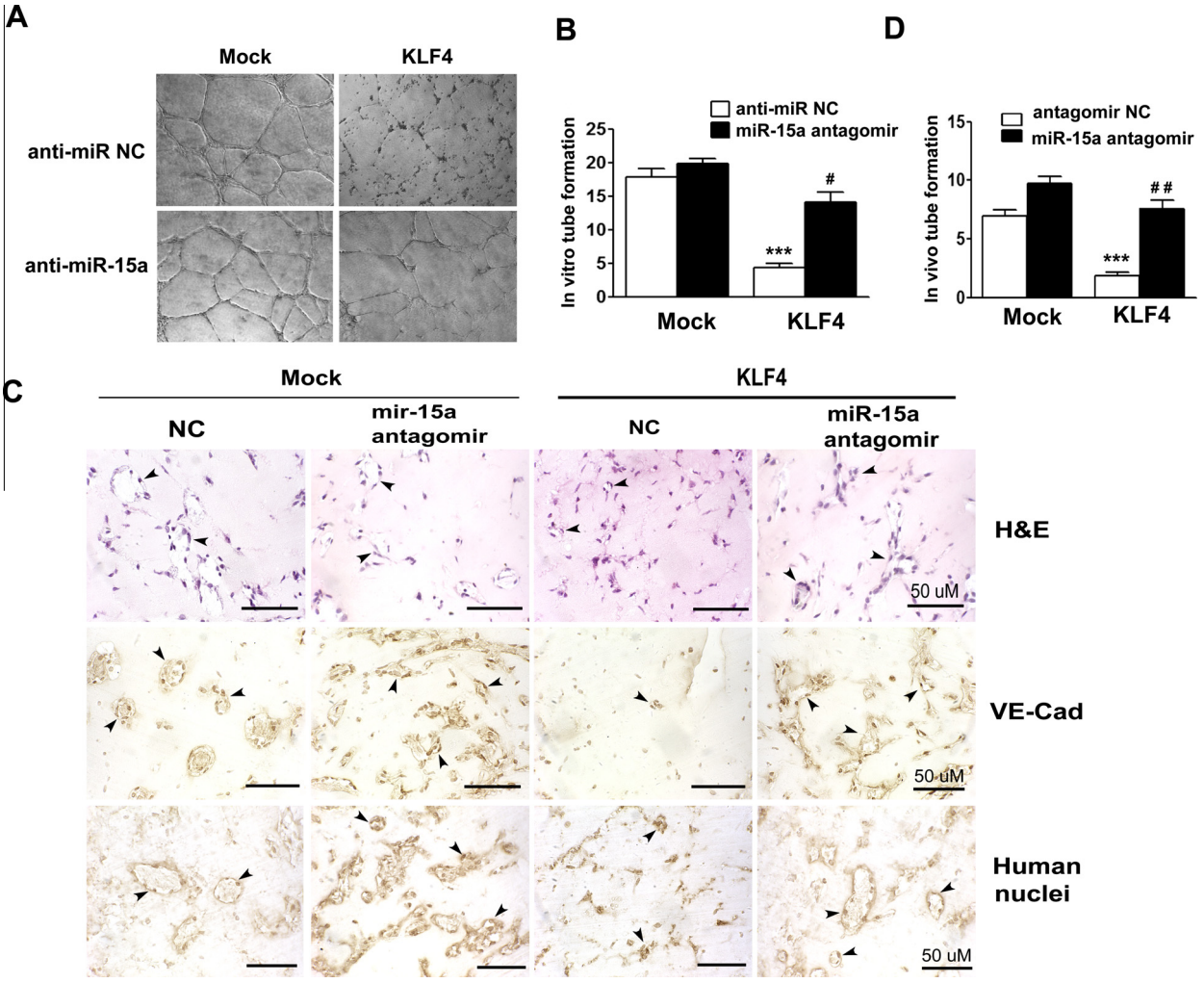

Fig. 4. MiR-15a mediates the anti-angiogenic effect of KLF4. (A) Representative microscopic view of tube formation in ECs without (mock) or with KLF4 treatment, following transfection with anti-miR-15a or negative control (NC) for 48 h. (B) The tube formation was determined by counting the numbers of branch points in three randomly chosen fields ($^{***}P < 0.001$ *vs.* mock, $^{\#}P < 0.05$ *vs.* KLF4 plus anti-miR NC). (C) *In vivo* miR-15a silencing attenuated the inhibitory effect of KLF4 on Matrigel plug angiogenesis (400×). (D) The microvessel numbers in three different fields for each Matrigel plug were quantified ($n = 5$. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* mock plus antagomir NC, $^{\#\#}P < 0.01$ *vs.* KLF4 plus antagomir NC).

than miR-15a are clearly required. Such efforts, along with our current findings, may provide important insights into the full spectrum of KLF4 function in vascular biology.

In conclusion, we have demonstrated for the first time that KLF4 inhibits the proliferation of ECs and VSMCs, as well as the angiogenesis through the induction of miR-15a. The discovery that KLF4 induction of miR-15a is required for the retardation of cell cycle progression in ECs and VSMCs, as well as the amelioration of angiogenesis suggests that strategies to enhance KLF4 expression may be efficacious in proliferative vascular disorders and pathological vascularization.

Acknowledgments

This work was supported by Grants from the National Natural Science Foundation of China (30700292, 30971160 and 31271493 to X.Q.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.017.

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