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MicroRNA-133a regulates insulin-like growth factor-1 receptor expression and vascular smooth muscle cell proliferation in murine atherosclerosis

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ABSTRACT

Objective: MicroRNA-133a (miR-133a) and insulin-like growth factor-1 (IGF-1) are two different molecules known to regulate cardiovascular cell proliferation. This study tested whether miR-133a affects expression of IGF-1 receptor (IGF-1R) and proliferation of IGF-1-stimulated vascular smooth muscle cells (VSMC) in a murine model of atherosclerosis.

Methods and results: Expression of IGF-1R was analyzed by immuno-fluorescence and immuno-blotting, and miR-133a by qRT-PCR in the aortas of wild-type C57BL/6J (WT) and apolipoprotein-E deficient (ApoE$^{-/-}$) mice. Compared to those in WT aortas, the IGF-1R and miR-133a levels were lower in ApoE$^{-/-}$ aortas. ApoE$^{-/-}$ VSMC grew slower than WT cells in the cultures with IGF-1-containing medium. MiR-133a-specific inhibitor decreased miR-133a, IGF-1R expression, IGF-1-stimulated VSMC growth in lipoprotein deficient media. By contrast, miR-133a precursor increased IGF-1R levels and promoted IGF-1-induced VSMC proliferation. In the luciferase-IGF-1R 3′UTR reporter system, the reporter luciferase activity was not inhibited in VSMC with miR-133a overexpression. IGF-1R mRNA half-life in ApoE$^{-/-}$ VSMC was shorter than that in WT VSMC. MiR-133a inhibitor reduced but precursor increased the mRNA half-life, although the effects appeared less striking in ApoE$^{-/-}$ VSMC than in WT cells.

Conclusion: MiR-133a serves as a stimulatory factor for IGF-1R expression through prolonging IGF-1R mRNA half-life. In atherosclerosis induced by ApoE deficiency, reduced miR-133a expression is associated with lower IGF-1R levels and suppressive VSMC growth. Administration of miR-133a precursor may potentiate IGF-1-stimulated VSMC survival and growth.

1. Introduction

During the development of atherosclerosis, vascular smooth muscle cells (VSMC) undergo a variety of pathological alternations. For instance, stimulated by mitogenic substances or growth factors, contractile VSMC may phenotypically transform into synthetic VSMC. In advanced plaques, exposed to atherogenic, cytotoxic factors, many of the transformed VSMC are induced to die by apoptosis or other types of cell death [1,2]. Clarification of the cellular and molecular mechanism underlying VSMC growth, survival and differentiation facilitates new strategies to prevent and treat atherosclerosis and other vascular disorders [3,4].

Insulin-like growth factor-1 (IGF-1) stimulates proliferation and inhibit apoptosis of VSMC. IGF-1 binds to its receptor or IGF-1 receptor (IGF-1R) with a high affinity, leading to activation of IGF-1R tyrosine kinase, which in turn delivers signals for survival and growth of various cell types, including VSMC [5,6]. However, whether IGF-1-induced VSMC growth in atherosclerosis is beneficial or harmful is still a debatable issue. IGF-1 may have a "double edge sword" effect on atherogenesis. Persistent exposure to IGF-1 may stimulate VSMC over-growth and migration, thus increasing intimal thickness and restenosis after angioplasty [7,8]. However, in advance plaques, the proliferative effect of IGF-1 reduces the plaque

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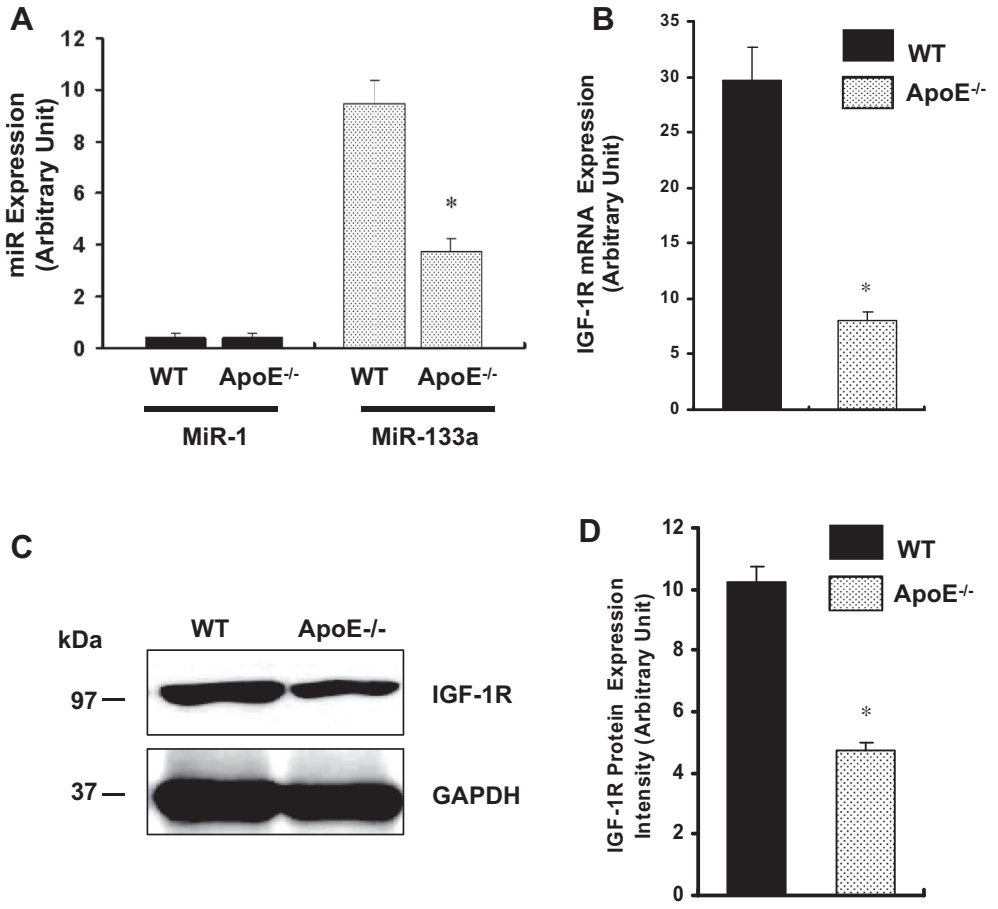

Fig. 1. Expression of miR-1, miR-133a and IGF-1R in WT and ApoE$^{-/-}$ aortic smooth muscle cells. (**A**) qRT-PCR analysis of miR-1 and miR-133a in total RNA extracted from VSMC of wild-type (WT) and ApoE$^{-/-}$ mice. (**B**) qRT-PCR analysis of IGF-1R mRNA in WT and ApoE$^{-/-}$ VSMC. (**C**) Western blot analysis of IGF-1R (upper panel) and GAPDH (lower panel) proteins in WT and ApoE$^{-/-}$ mice. (**D**) Densitometry of protein bands in blots. Data represent means ± SD. $^{*}p < 0.05$.

vulnerability to rupture as increased numbers of VSMC help stabilize the arterial wall with advanced atherosclerosis [9,10]. A recent study also suggests that low levels of circulating IGF-1 may increase the risk for ischemic heart disease [11].

MicroRNAs (miRs) represent a class of non-coding small RNA species that promote mRNA cleavage and/or translational repression through base-paring to the 3′untranslated region (UTR) of target mRNAs [12]. However, conflict reports have emerged concerning the role of miRs as an inhibitor [13] or stimulator [14] of gene expression. Recently, certain miRs have been shown to exert important biological impacts on cardiovascular cell functions [15,16]. Among them, miR-133 has been reported to participate in regulation of embryonic heart development, cardiohypertrophy, and muscle cell proliferation [17–19]. Two miR-133 isoforms, miR-133a and miR-133b, are expressed differentially in a variety of cell types. For instance, VSMC primarily express miR-133a but not miR-133b [20]. However, little is known about the role of miR-133a in regulation of growth factor-driven VSMC expansion during the development of atherosclerosis.

In this study, we tested the hypothesis that during the pathogenesis of atherosclerosis, miR-133a expression is suppressed, leading to attenuation of vascular cell growth stimulated by growth factors, such as IGF-1. Our results suggest that miR-133a expression declines in VSMC during the development of atherosclerosis, and subsequently affects IGF-1R expression and IGF-1-stimulated VSMC proliferation. Moreover, miR-133a regulators affect the half-life of IGF-1R mRNA. The data from the study provides novel insight into the epigenetic regulation of vascular cell growth in atherosclerosis.

2. Methods

Experimental design, materials and methods, and statistical analysis are described extensively in the Supplemental Material. Briefly, the aortic tissues of C57BL/6J (WT) and ApoE$^{-/-}$ mice (male, 6 months old) were collected for cell culture, extraction of RNA and proteins, and preparation of frozen sections. VSMC were cultured in DME medium supplemented with 10% lipoprotein deficient serum (LPDS). Synthetic miR-133a inhibitor (anti-mir-133a), precursor (pre-miR-133a) for mature mouse miR-133a and scrambled miRNA controls were commercially obtained from Amibon Company. Samples were processed for further molecular and histological analysis, including, Oil red O staining, immuno-fluorescence, cells growth analysis by EdU incorporation, qRT-PCR, Western blot and mRNA half-life determinations. All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at Houston.

3. Results

3.1. Differential expression of miR-1 and miR-133a and reduced expression of IGF-1R in ApoE$^{-/-}$ aortic smooth muscle cells

Located in the same gene cluster, both miR-1 and miR-133a participate in cardiovascular cell development [18,19]. To determine whether the two miRs exist in VSMC of atherosclerotic lesions, we examined miR-1 and miR-133a by PCR. We detected no or

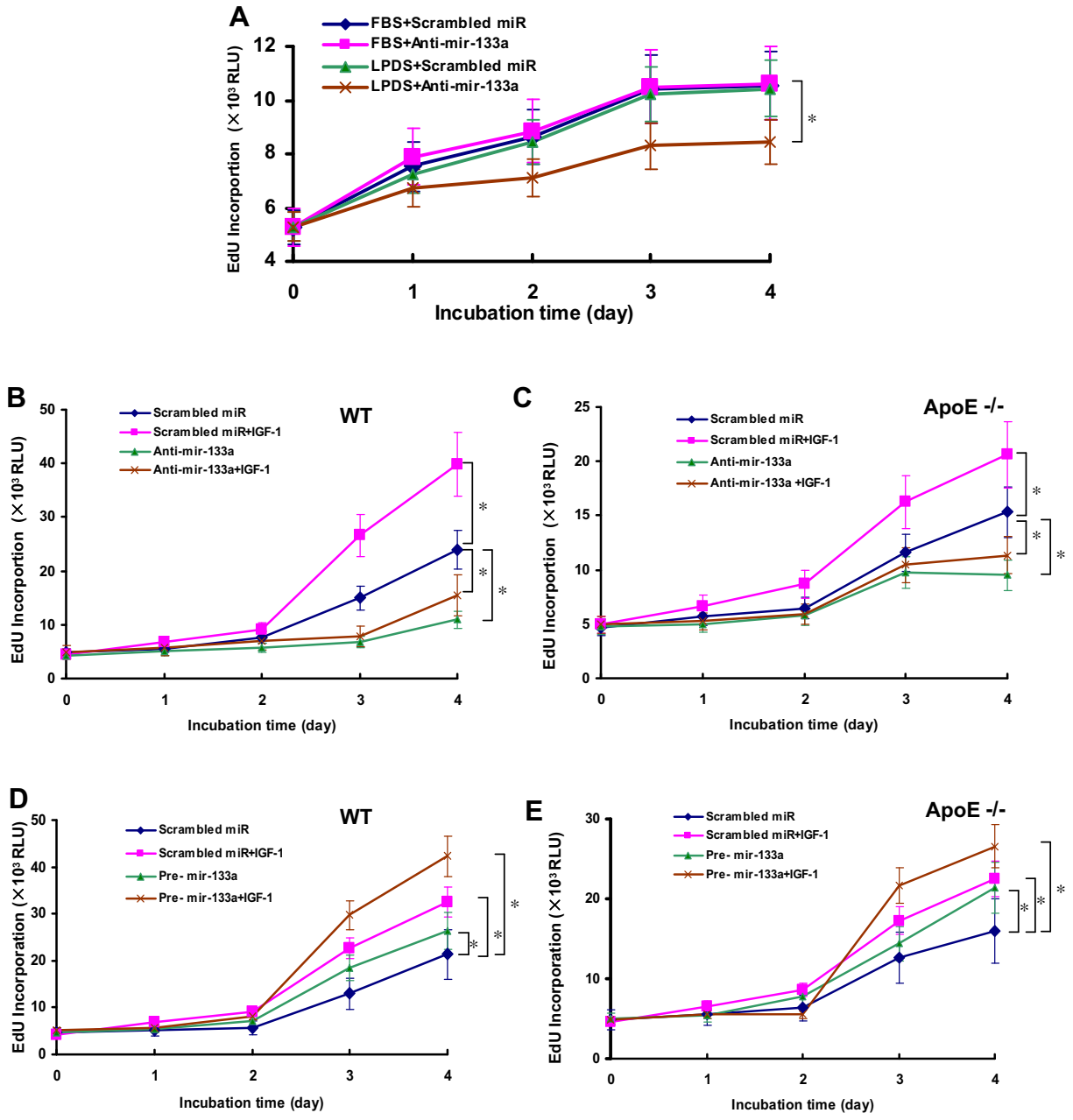

Fig. 2. MiR-133a regulation of IGF-1-stimulated growth of WT and ApoE$^{-/-}$ aortic smooth muscle cells. (**A**) Analysis of VSMC growth after treated with miR-133a inhibitors. EdU incorporation was analyzed in synchronized VSMC cultured in DME medium supplemented with 10% LPDS and treated with miR-133a inhibitor or precursor in the presence or absence of IGF-1 for different time points. (**B**) WT VSMC treated with miR-133a inhibitor and/or IGF-1. (**C**) ApoE$^{-/-}$ VSMC with miR-133a inhibitor and/or IGF-1. (**D**) WT VSMC with miR-133a precursor and/or IGF-1. (**E**) ApoE$^{-/-}$ VSMC with miR-133a precursor and/or IGF-1. Data represent means ± SD. *$p < 0.05$.

little miR-1 but relatively abundant miR-133a in VSMC (Fig. 1A). In comparison, ApoE$^{-/-}$ VSMC expressed lower levels of miR-133a than WT VSMC ($p < 0.05$). Thus, both WT and ApoE$^{-/-}$ VSMC selectively expressed miR-133a, albeit WT VSMC possessed higher levels of miR-133a expression than ApoE$^{-/-}$ VSMC.

In the same VSMC cultures, IGF-1R mRNA expression was analyzed by qRT-PCR. Significantly reduced expression of IGF-1R mRNA ($p < 0.05$) was found in ApoE$^{-/-}$ cells (Fig. 1B). Western blot analysis with VSMC total proteins also revealed lower IGF-1R protein expression in ApoE$^{-/-}$ cells. However, there was no difference in the band intensity of "house-keeping" gene product GAPDH between WT and ApoE$^{-/-}$ cells (Fig. 1C), suggesting the selectivity of the miR-133a effect on IGF-1R expression. The ratio of IGF-1R vs. GAPDH band intensity in WT cells was much higher than that in ApoE$^{-/-}$ cells (Fig. 1D). Similarly, IGF-1R expression was reduced in ApoE$^{-/-}$ mice aortas with atherosclerosis (Supplemental Fig. 2 A and C–F).

3.2. *Differential expression of miR-133a and IGF-1R in aortas of WT and ApoE$^{-/-}$ mice*

As expected, ApoE$^{-/-}$ mice fed normal chow developed fibrous plaques in the aortic wall, as visualized by Oil Red O staining. No atherosclerotic lesions existed in sex- and age-matched WT mice fed on the same diet (Supplemental Fig. 1A and B). The patterns of IGF-1R and α-SM-actin immunostains were, however, different

between WT and ApoE$^{-/-}$ mice. IGF-1R immunostains were uniformly co-distributed in the aortic tissue of WT mice, whereas, IGF-1R immunostains were unevenly localized in those of ApoE$^{-/-}$ mice with atherosclerosis. Globally, IGF-1R immunostains appeared weaker in ApoE$^{-/-}$ aorta, and it was mainly seen in the region with vascular cells expressing α-SM-actin (Supplemental Fig. 1C and D).

MiR-133a has been reported to participate in regulation of VSMC growth and contractile-synthetic switch [20]. To assess the age impact on miR-133a and IGF-1R expression, qRT-PCR analysis was conducted using total RNA extracted from the aortas of WT and ApoE$^{-/-}$ mice at different ages. Both IGF-1R mRNA and miR-133a expression was reduced in ApoE$^{-/-}$ aortic tissues, compared to those in WT aortic tissue (Supplemental Fig. 2A and B). In ApoE$^{-/-}$ aorta, the reduced miR-133a expression was apparently age-dependent. MiR-133a expression was much lower in old (12-month-old) than that in the 3-month-old mice. Interestingly, in WT mice, miR-133a expression underwent little change with aging (Supplemental Fig. 2B). Similarly, Western blot images and densitometry analysis results showed an age-dependent reduction in IGF-1R protein expression in the aortas of ApoE$^{-/-}$ but not WT mice (Supplemental Fig. 2 C–F).

3.3. Difference in IGF-1-stimulated proliferation between VSMCs from WT and ApoE$^{-/-}$ mice

To determine whether miR-133a regulates VSMC proliferation, EdU incorporation assays were performed in VSMC grown in lipoprotein-containing or -deficient media.

VSMC cultured in media containing 10% fetal bovine serum (FBS) or lipoprotein deficient serum (LPDS) displayed a discrepancy in terms of EdU incorporation in the presence or absence of miR-133 inhibitor. When exposed to miR-133a inhibitor, substantially reduced EdU incorporation occurred in VSMC cultured in LPDS media, and to a much less extent, in VSMC grown with the regular media (Fig. 2A). Because miR-133a inhibitor affects VSMC proliferation predominantly in LPDS media, the assays for IGF-1 stimulation of VSMC proliferation and other biological activities in the following experiments were performed mainly in the cultures with LPDS media.

IGF-1 is known to stimulate VSMC proliferation during atherogenesis [10]. In the cultures with IGF-1, indeed, EdU incorporation in both WT and ApoE$^{-/-}$ VSMC was higher than that in the cells without IGF-1. Compared to WT cells, ApoE$^{-/-}$ VSMC incorporated much less EdU (Fig. 2B–E). The IGF-1-stimulatory effect on VSMC proliferation was diminished in the presence of miR-133a inhibitor (Fig. 2B and C). ApoE$^{-/-}$ VSMC had a weaker growth response to the stimulation with IGF-1, and also the cells were less sensitive to the treatment of miR-133a inhibitor as determined by EdU incorporation.

To further confirm the regulatory role of miR-133a, VSMC were incubated with exogenous miR-133a precursor. Treatment with miR-133a precursor increased EdU incorporation in WT and ApoE$^{-/-}$ VSMC (Fig. 2D and E). Grown in the IGF-1-containing media, ApoE$^{-/-}$ VSMC incorporated more EdU when exposed to the miR-133a precursor. Thus, IGF-1-stimulated VSMC EdU incorporation was compromised in ApoE$^{-/-}$ VSMC and treatment with miR-133a precursor might enhance EdU incorporation.

3.4. MiR-133a regulates expression of α-SM-actin in VSMC

In atherosclerotic lesions, VSMC may transform from a contractile phenotype into a synthetic phenotype. To determine whether miR-133a is involved in the VSMC phenotypic switch, we analyzed expression of α-SM-actin, a characteristic contractile protein, commonly used as the marker of VSMC differentiation.

Western blot analysis showed a decline in expression of α-SM-actin protein in WT VSMCs after treatment with miR-133a inhibitor, as compared with that in scrambled control-transfected cells. ApoE$^{-/-}$ VSMC also showed reduced α-SM-actin but at a much greater level as compared to the WT cells (Fig. 3A and B). WT and ApoE$^{-/-}$ VSMC showed abundant long stress fibers, and a fine cortical actin network in the cell periphery (Fig. 3C and E). However, the α-SM-actin network was markedly diminished by miR-133a inhibitor (Fig. 3D and F). Using the image analysis software with the non-immune IgG-stained cells as the threshold, α-SM-actin positive cells were found significantly reduced in miR-133a inhibitor treated VSMC (Fig. 3G). Thus, the VSMC switch from contractile phenotype to synthetic phenotype might be regulated by miR-133a.

3.5. MiR-133a inhibitor reduces but precursor increases IGF-1R expression in VSMC

The above finding that miR-133a inhibitor and precursor changed EdU incorporation and α-SM-actin expression in VSMC points to the possibility that miR-133a regulates IGF-1R expression. To test this possibility, Western blot analysis was conducted in total proteins extracted from VSMC treated with or without miR-133a inhibitor or precursor. Compared with scrambled control treated cells, IGF-1R expression was markedly reduced in VSMC treated with miR-133a inhibitor (Fig. 4A and C), which significantly diminished miR-133a contents in the cells (Fig. 4E). Moreover, incubation with miR-133a precursor (50 nM) significantly increased expression of IGF-1R in VSMC (Fig. 4B and D). As expected, qRT-PCR confirmed that the cells treated with exogenous miR-133a precursor contained high levels of miR-133a when compared with scrambled control group (Fig. 4F). Taken together, the miR-133a precursor treatment stimulated rather than inhibited IGF-1R expression in VSMC.

3.6. MiR-133a does not block IGF-1R mRNA 3′UTR function in VSMC

To further investigate whether miR-133a targets IGF-1R mRNA 3′untranslated region (UTR), we established an IGF-1R mRNA 3′UTR-regulated luciferase reporter system. A cDNA fragment coding for IGF-1R mRNA 3′UTR with miR-133a targeting site was inserted into psiCHECK2 reporter vector in downstream of the *Renilla luciferase* reporter gene (Fig. 5A, Luciferase-IGF-1R 3′UTR). In addition, a positive control luciferase reporter plasmid was constructed with antisense miR-133a (Fig. 5A, Luciferase-miR-133a AS) replacement of the IGF-1R 3′UTR sequence. When co-transfected with pLVX-miR-133a expression vector and Luciferase-miR-133a AS, VSMC expressed the sense miR-133a selectively targeted to the miR-133a antisense sequence that localized downstream of the luciferase gene, leading to reduced expression of luciferase enzymatic activities (Fig. 5B). However, overexpression of miR-133a in VSMC did not decrease the luciferase activities in the cells co-transfected with pLVX-miR-133a expression vector and Luciferase-IGF-1R-3'UTR (Fig. 5B). Thus, miR-133a did not appear to inhibit the luciferase/IGF-1R 3′UTR reporter activities in VSMC.

3.7. MiR-133a treatment increases IGF-1R mRNA stability in VSMC

IGF-1R mRNA stability was assessed in VSMC incubated with miR-133a inhibitor and precursor in the presence of actinomycin D (Act-D), an RNA polymerase inhibitor that blocks *de novo* mRNA synthesis but not degradation. Addition of Act-D led to a time-dependent decline in IGF-1R mRNA in both WT and ApoE$^{-/-}$ VSMC (Fig. 5C and D). However, a marked difference in IGF-1R mRNA stability existed between WT and ApoE$^{-/-}$ cells. In the

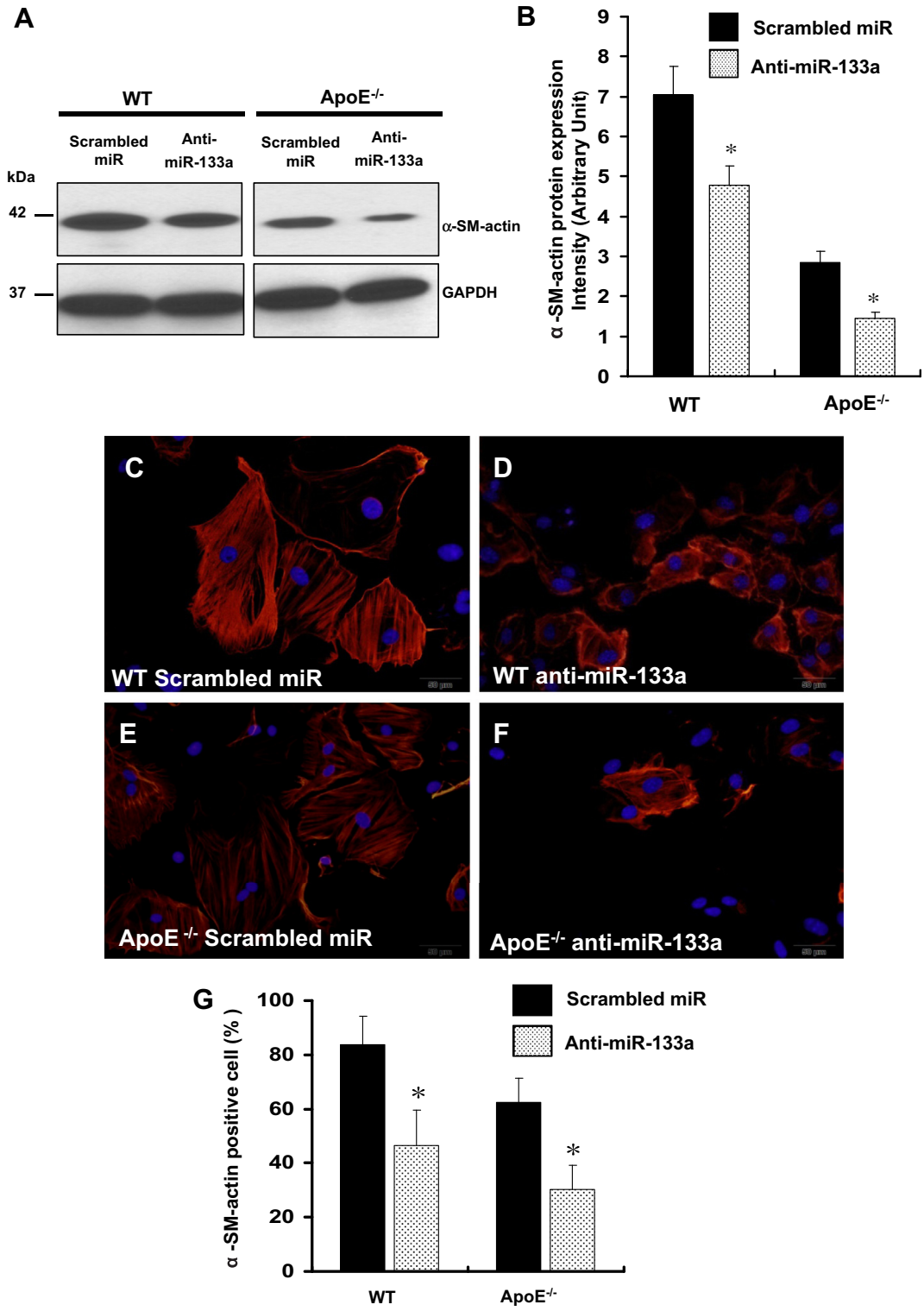

Fig. 3. MiR-133a regulation of α-SM-actin expression in aortic smooth muscle cells isolated from WT and ApoE$^{-/-}$ mice. (**A**) α-SM-actin (upper panel) and GAPDH (lower panel) Western blot. (**B**) Densitometry of α-SM-actin protein bands in the blots normalized by that of GAPDH. (**C**) WT VSMC treated with scrambled miRNA control. (**D**) WT VSMC treated with miR-133a inhibitor. (**E**) ApoE$^{-/-}$ VSMC treated with scrambled miRNA control. (**F**) ApoE$^{-/-}$ VSMC treated with miR-133a inhibitor. (**G**) Quantification of α-SM-actin positive cells in cultures treated with scrambled controls or miR-133a inhibitor. The positive cells were identified by using an image analysis computer software with the non-immune IgG-stain fluorescence as the threshold. Data represent means ± SD. $^{*}p < 0.05$. Images taken using 20× objective.

presence of Act-D, the IGF-1R mRNA half-life was estimated 4.2 h in WT VSMC, vs. 2.2 h in ApoE$^{-/-}$ cells, suggesting that IGF-1 mRNA degraded much faster in ApoE$^{-/-}$ cells.

Moreover, WT and ApoE$^{-/-}$ VSMC showed different IGF-1R mRNA stability in the presence of miR-133a inhibitor or precursor (Fig. 5C and D). In the presence of Act-D, treatment with miR-133a inhibitor shortened the IGF-1R mRNA half-life to 2.6 h in WT cells and 1.5 h in ApoE$^{-/-}$ cells. By contrast, under the same culture condition, treatment with miR-133a precursor resulted in prolongation of IGF-1R mRNA half-life to 6.7 h in WT cells and 6 h in

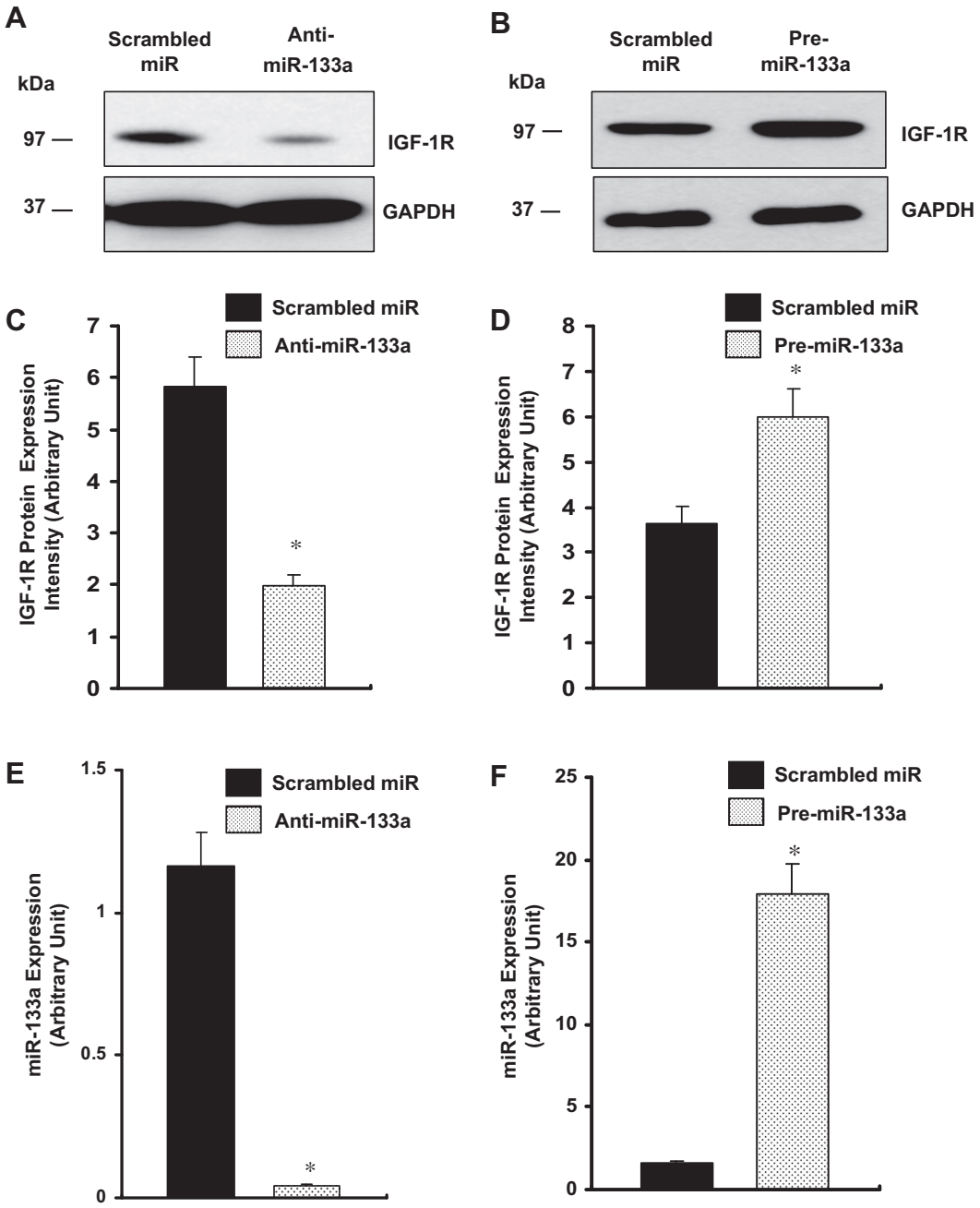

Fig. 4. Impacts of miR-133a regulators on expression of IGF-1R protein and miR-133a in aortic smooth muscle cells. (**A** and **B**) Western blot analysis of IGF-1R and GAPDH. (**C** and **D**) IGF-1R expression quantified by densitometry. (**E** and **F**) miR-133a expression detected by qRT-PCR. Data represent means ± SD. $^{*}p < 0.05$.

ApoE$^{-/-}$ cells. Thus, IGF-1R mRNA half-life in ApoE$^{-/-}$ VSMC was much shorter than that in WT VSMC, and treatment with miR-133a precursor increased, whereas miR-133a inhibitor reduced IGF-1R mRNA half-life in the cells (Fig. 5C and D).

4. Discussion

There has been increasing experimental and clinical evidence supporting the notion that VSMC phenotypical switch and altered responses to cytokines and growth factors characterize the cellular pathobiology of atherosclerotic plaque development. The association of miR-133a with IGF-1R expression in VSMC has now been documented by this and other research teams. Because the IGF-1/IGF-1R signaling pathway contributes to vascular cell growth and differentiation, miR-133a regulation of IGF-1R expression and IGF-1 dependent growth response in VSMC is of significance in vascular biology and atherosclerosis. In line with recent work by other investigations [21,22] the data presented in this study reveal the presence of a miR-growth factor regulatory network that is critical for VSMC proliferation in atherosclerosis.

Both miR-1 and miR-133 are important components of the miR-1 axis, which plays essential roles in cardiac and skeletal muscle development, physiology, and disease [17–19]. Interestingly, our results show that miR-1 and miR-133a were differentially expressed in VSMC from atherosclerosis prone mice. While miR-1 is almost undetectable, miR-133a is abundant in the aorta of WT mice, and appears to a lower degree in that of ApoE$^{-/-}$ mice. Our data further demonstrate that VSMC from the ApoE$^{-/-}$ mice have

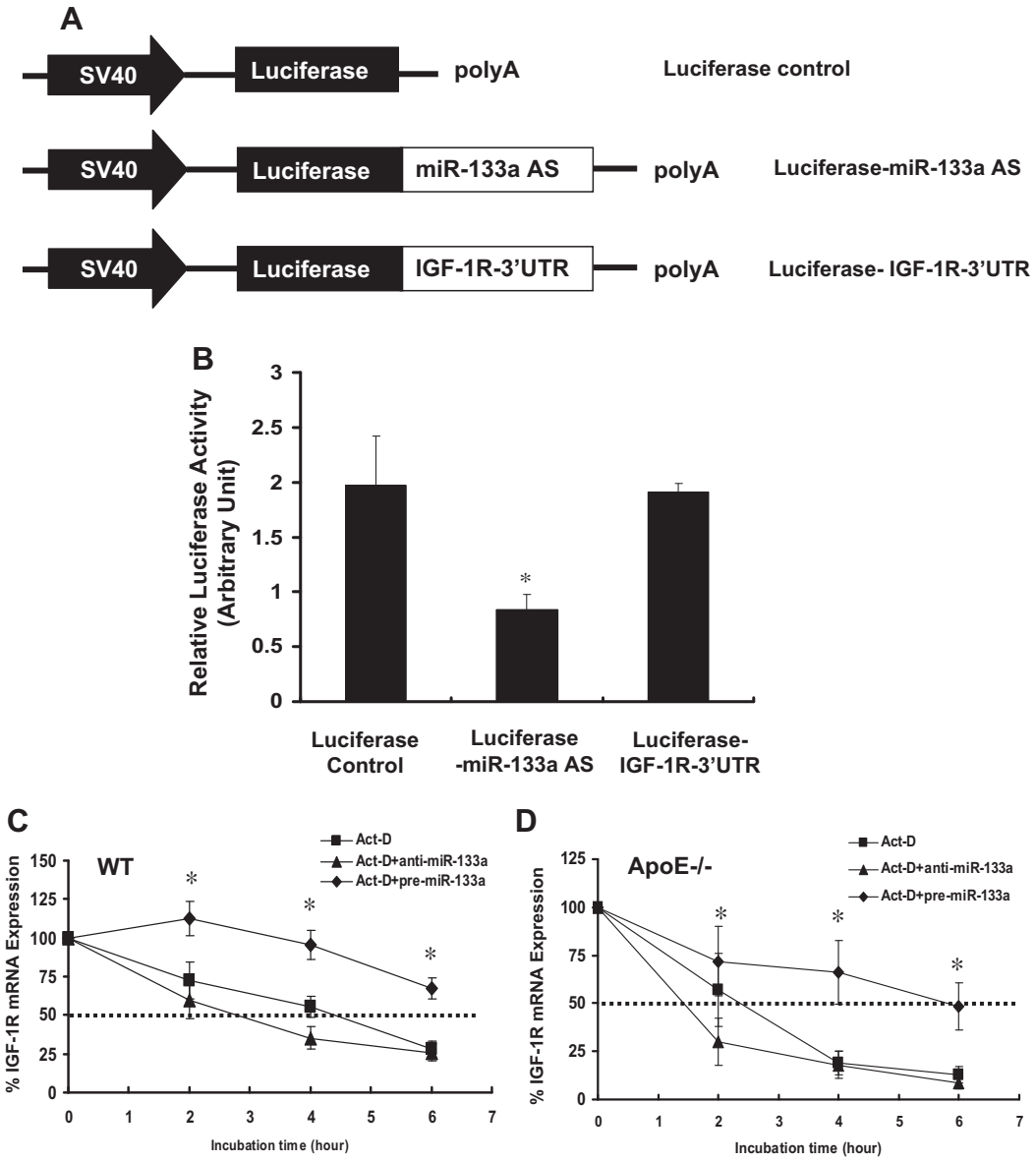

Fig. 5. Luciferase reporter assays and IGF-1R mRNA half-life detection. (**A**) Schematic representation of psiCHECK2 luciferase reporter constructs. The IGF-1R mRNA 3′UTR sequence containing miR-133a targeting site (IGF-1R-3′UTR) and miR-133a antisense (miR-133a AS) were inserted into the psiCHECK2 reporter vector at the site downstream of the luciferase reporter gene. (**B**) Interaction between miR-133a and IGF-1R 3′UTR sequence were analyzed by luciferase reporter assays. VSMC cells were transfected with psiCHECK2 luciferase reporter vectors containing miR-133a targeting site downstream of the *Renilla luciferase* gene (50 ng), and the internal *Firefly luciferase* gene was used to normalize for transfection efficiency. A pLVX-miR-133a expression vector was co-transfected (150 ng). Dual-luciferase assays were performed 48 h after transfection. (**C** and **D**) MiR-133a precursors increase, while miR-133a inhibitors reduce, the half-life of IGF-1R mRNA in aortic smooth muscle cells. Total RNA was extracted from synchronized VSMC. The mRNA stability was denoted with the IGF-1R mRNA decay curve after 5 μg/mL Act-D treatment. Synchronized VSMC from WT and ApoE$^{-/-}$ mice were treated with miR-133a inhibitor or precursor in the presence of Act-D at different time points. At the end of culture, cellular RNA was extracted. IGF-1R mRNA was measured at each time point using qRT-PCR. Data represent means ± SD. $^{*}p < 0.05$.

relatively low levels of miR-133a and IGF-1R, suggesting the vulnerability of those cells to pro-apoptotic, cytostatic insults in the microenvironment of atherosclerotic lesions. Our findings are consistent with previous report that miR-133a rather than miR-1 is the predominant miR that modulates VSMC proliferation [20].

The low levels of miR-133a in ApoE$^{-/-}$ VSMC suggest that the apolipoprotein may contribute to the miR expression and function. In the presence of lipoproteins, specific inhibitor for miR-133a acts less effectively on IGF-1 expression and VSMC growth, evidencing that miR-133a function is under the influence by the lipoproteins. At this moment, however, we do not fully understand the molecular basis of the lipid-dependency for miR-133a action. One of the hypotheses is that in the presence of excess amounts of lipids, as a hydrophilic agent, less miR-133a molecules transport across the lipid bilayer of plasma membrane. We are currently performing additional experiments and seek more evidence to support this hypothesis.

In general, miRs are considered as an inhibitory factor on their target mRNAs, and thereby block the translation of mRNA [23]. In contrast to the traditional view, miR-133a functions as a stimulatory factor for IGF-1R expression and VSMC growth. Torella et al. have recently reported that miR-133 inhibitor administration does not affect rat VSMC proliferation [20], and miR-133 overexpression triggers the cell phenotypical transformation and vascular muscle layer remodeling. Our results provide further experimental evidence that miR-133a may act as an enhancer of IGF-1R expression

and IGF-1-driven VSMC growth. IGF-1R expression in VSMC is highly regulated by a variety of environmental factors, including mechanical force [24], oxidative stress [25], and even other types of growth factors [26]. It would be interesting to investigate if the environmental factors affect miR-133a, and subsequently IGF-1R expression.

IGF-1 activates IGF-1R receptor tyrosine kinase that in turn transduces a mitogenic signal to promote VSMC proliferation [27]. It has been reported that increased IGF-1R expression inhibits oxLDL-induced VSMC apoptosis [28]. It is expected that VSMC may be prone to apoptosis when expression of IGF-1R, an inhibitor of apoptosis, is compromised as the consequence of low miR-133a expression in VSMC of atherosclerotic aortas. Because VSMC maintains the stability of atherosclerotic plaques, the miR-133a/IGF-1R promotion of VSMC proliferation and differentiation may help prevent plaque rupture. This notion is supported by previous reports that stimulation of VSMC IGF-1 signaling may improve atherosclerotic plaque stability. [9,10]

MiR-133 but not miR-1 has been reported to regulate the VSMC phenotypic switch in the gain- and loss- of-function models [20]. In this study, we showed that miR-133a, but not miR-1, is highly expressed in VSMC. In our study, using non-viral, synthetic miR-133a inhibitor or precursor, we further demonstrated that miR-133a inhibits VSMC proliferation driven by the growth factor IGF-1, whereas increasing miR-133a stimulates the cell growth. Our current study shows that knockdown of miR-133a may reduce expression of α-SM-actin, supporting the view that miR-133 is involved in smooth muscle development. In addition, to determine the IGF-1R expression in VSMC cultured with LPDS, we have isolated and cultured rat VSMC, then treated them with miR-133a inhibitor and precursor. Our data show that in rat VSMC, miR-133a can also stimulate IGF-1R expression (Supplemental Fig. 3). The discrepancy between our current data and others may reflect the effects of a variety of factors, including the cells culture, methods for the delivery of miR-133 regulators, and experimental models. Recently, the miR-143/miR-145 gene cluster has been shown to regulate VSMC development [29]. It is possible that miR-133 interacts with other miR molecules in regulation of VSMC growth and differentiation stimulated with IGF-1 during the development of atherosclerosis.

Our results suggest that miR133s stabilization of IGF-1R mRNA may enhance IGF-1R expression in VSMC. We found no evidence that miR-133a targets the 3′UTR of IGF-1R mRNA and promotes the mRNA degradation. Instead, miR-133a stabilizes IGF-1R mRNA and prolongs its half-life in VSMC. The comparison between the 3′UTR sequence of IGF-1R mRNA and that of miR-133a shows their matching segments. However, our reporter experiments on VSMC co-transfected with miR-133 and luciferase-IGF-1R 3′UTR show no reporter activity in the cells. Therefore, our results raise an interesting question as to whether certain miR molecules, such as miR-133a, may function as an mRNA stabilizer that prolongs the half-life of their targeted mRNA.

A recent study in mouse and human myoblasts has demonstrated that RNAs can compete for their miR targets, including the miR-1/miR-133 axis [30]. The competing endogenous RNAs (ceRNAs) play a role in the distribution of miR molecules on their targets and thereby impose an additional level of post-transcriptional regulation. For instance, a ceRNA acts as a decoy abolishing miR repressing activity on both MAML1 and Mef2C 3′UTR by binding miR-133 and miR-135 [30]. Our experimental evidence suggests that VSMC may contain such a specific long non-coding RNA, which acts as a ceRNA capable of interacting with miR-133 in targeting IGF-1R mRNA and subsequently regulating the vascular cell growth. Additionally, we have identified a key motif (GACCA) in the IGF-1R protein coding sequence (CDS) of mRNA. Whether miR-133a binding with this motif can increase IGF-1R mRNA stability or stimulate other unknown indirect regulatory pathways to produce a miRNA stimulatory effect is currently under investigation.

The cells from atherosclerotic lesions have different patterns of growth and differentiation. MiR-133 is expected to play a different role in different types of cells, such as the C2C12 myoblasts cell line [13] or balloon-injured vascular cells [20]. At different stages of atherosclerosis, the vascular wall may undergo different pathological changes and express different gene products. In this study, expression of miR-133a and IGF-1R was studied in both early and advanced atherosclerotic lesions. IGF-1R is suppressed in advanced atherosclerotic plaques or atheroma. Advanced plaque tissues comprise multiple cell types, which may express different levels of IGF-1R, reflected by the heterogeneous immunostains. In the aortic tissue of ApoE$^{-/-}$ mice, weak IGF-1R immunostains mainly localized in certain areas, where α-SM-actin positive VSMC were located. This observation suggests that increased expression of miR-133a and IGF-1R may occur in those stable plaques that usually contain more smooth muscle cells. Nevertheless, further studies are needed to clarify the association of miR-133a and IGF-1R with the development of stable or unstable atheroma, and regulation of vascular cell function during therapeutic procedures.

In conclusion, this study provides experimental evidence indicating that VSMC from advanced atherosclerotic lesions of ApoE$^{-/-}$ mice possess lower levels of miR-133a/IGF-1R expression followed by attenuation of IGF-1-stimulated proliferation. The half-life of IGF-1R mRNA is shortened in ApoE$^{-/-}$ VSMC, and miR-133a does not target IGF-1R mRNA 3′UTR and promote the mRNA degradation. The miR-133a-IGF-1R pathway offers a new revenue for designing a therapeutic approach for atherosclerosis.

Conflict of interest

No conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.11.029.

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