MiR-652-3p inhibition enhances endothelial repair and reduces atherosclerosis by promoting Cyclin D2 expression

Rongzhong Huang a,1, Zicheng Hu c,1, Yu Cao a, Hongrong Li a, Hong Zhang b, Wenhua Su b, Yu Xu e, Liwen Liang b, N.D. Melgiri d, Lihong Jiang a,⁎

a Department of Cardiothoracic Surgery, The First People’s Hospital of Yunnan Province, Kunming, Yunnan Province, China
b Department of Cardiology, The First People’s Hospital of Yunnan Province, Kunming, Yunnan Province, China
c Department of Neurology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, China
d Impactys Foundation for Biomedical Research, San Diego, CA, USA
e Statistical Laboratory, Chongxu Institute of Lifescience, Chongqing, China

Abstract

Background: Atherosclerosis is a hyperlipidemia-induced condition affecting the arterial wall that damages healthy endothelial cell (EC) function, leading to enhanced risk of atherothrombotic events. Certain microRNAs regulate EC dysfunction in response to hyperlipidemia and may be suitable therapeutic targets to combat atherosclerosis.

Methods: miRNA expression in human ECs was analyzed under various conditions to identify key microRNAs. High-cholesterol diet (HCD)-fed Mir652 -/−/ Apoe −/− (Mir652 −/−/ ) mice and matching Mir652 +/+/ Apoe −/−/ (Mir652 +/+ ) mice were subjected to carotid injury to analyze the effects of miR-652 knockdown on endothelial repair. In silico analysis followed by in vitro and in vivo experiments were applied to identify miR-652’s target gene Ccnd2 and investigate the pair’s effects on ECs. miR-652-3p and miR-652-3p antagonist therapies were tested in Mir652 +/+ mice under normal and HCD diet to assess their effect on endothelial repair.

Findings: miR-652-3p, which is upregulated in human and murine atherosclerotic plaques, suppresses expression of the endothelial repair gene Ccnd2, thereby enhancing atherosclerotic lesion formation. Post-denudation recovery of ECs was promoted in Mir652 −/−/ mice due to enhanced EC proliferation attributable to de-repression of miR-652-3p’s (but not miR-652-5p’s) regulation of Ccnd2 expression. Under hyperlipidemic conditions at non-predilection sites, miR-652-3p produces anti-proliferative effects in ECs, such that Mir652 −/−/ mice display reduced atherosclerotic progression. In contrast, neither miR-652-3p nor Ccnd2 displayed significant effects on the endothelium at predilection sites or under disturbed flow conditions. Administration of a miR-652-3p antagonist rescued the proliferation of ECs in vivo, thereby limiting atherosclerotic development.

Interpretation: miR-652-3p blockade may be a potential therapeutic strategy against atherosclerosis.

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1. Introduction

Atherosclerotic diseases are one of the most prominent causes of morbidity and mortality worldwide [1]. Inflammation leads to atherosclerotic lesion formation through a host of cell types [2]. A defect in the resolution of vascular inflammation may lead to advanced atherosclerotic lesions with overt clinical manifestations [3].

miRNAs are small non-coding RNAs that post-transcriptionally regulate gene expression [4]. Binding of miRNAs to target messenger RNAs (mRNAs) may lead to mRNA degradation, gene silencing, or translational repression [4]. Individual miRNAs are potent regulators of biological activity, as they can negatively regulate the expression of several hundred target miRNAs and profoundly affect multiple biological networks [5].
2. Results

2.1. miR-652 abundantly expressed in human ECs, upregulated in response to moxLDL

Reduced EC expression of the miR-processing endonuclease Dicer suppresses EC proliferation and migration [14,15]. Hartmann et al. previously identified four miRNAs (miR-103, miR-301b, miR-433, and miR-652) that are significantly downregulated in endothelial Dicer-silenced Apoe−/− mice after three months of high-fat feeding; these same four miRNAs are also downregulated in Dicer-silenced human aortic ECs [16]. This previous evidence suggests that these four miRNAs may play a role in EC proliferation and atherosclerosis [16].

Therefore, here we initially analyzed the expression of these four miRNAs in cultured HUVECs under various conditions to identify ones that may be suitable targets or further investigation. Of the four candidate miRNAs, miR-652 was the second-most highly abundant miRNA in non-stimulated HUVECs after miR-103, whose role in atherosclerosis has been previously investigated (Fig. 1a) [16]. miR-652-3p is significantly enriched in the miRNA-induced silencing complex (RISC) of HUVECs as determined by AGO2-IP (Fig. 1b), suggesting a putative role for miR-652-3p in regulating EC function [16]. There was no significant difference in miR-652-3p expression in HUVECs in the presence of pro-inflammatory TNF-α or the NF-κB inhibitor BAY11-7085 (Fig. 1c, d), suggesting that HUVEC miR-652-3p expression does not significantly change in response to classical pro-inflammatory or anti-inflammatory stimuli [17]. However, miR-652-3p expression was significantly increased after treatment with normal low-density lipoprotein (nLDL) and even more so after treatment with mildly-oxidized low-density lipoprotein (moxLDL) (Fig. 1e).

To specify the subcellular localization of miR-652-3p within HUVECs, the cells were transfected with a Cy3-labeled miR-652-3p. After 24 h, Cy3-miR-652-3p fluorescence was detected within the HUVEC cytoplasm (Supp. Fig. 1). This preliminary evidence suggests that cytoplasmic miR-652-3p may be linked to LDL-induced EC dysfunction and requires further study.

2.2. miR-652-3p is upregulated in human and murine atherosclerotic plaques

As our evidence suggests that miR-652-3p may be linked to LDL-induced EC dysfunction, we next analyzed miR-652-3p expression in carotid atherosclerotic lesions. We discovered ex vivo evidence of prominent EC miR-652-3p expression in human atherosclerotic plaques using in situ PCR combined with von Willebrand factor (vWF) staining (Fig. 1f). We also discovered significantly elevated intimal EC has-miR-652-3p expression in human carotid lesions relative to post-mortem normal human carotid tissue (Fig. 1g). Furthermore, we found significantly elevated mmu-miR-652-3p expression in murine carotid lesions from high cholesterol diet (HCD)-fed Mir652−/+Apoe−/− mice 28 days post-wire injury relative to contralateral normal carotid tissue (Fig. 1h). This evidence suggests that endothelial miR-652-3p upregulation may play a pro-atherogenic role in humans and mice.

2.3. Endothelial miR-652 knockdown promotes endothelial repair in injured arteries

Endothelial denudation of carotid arteries in HCD-fed Mir652−/+Apoe−/− (Mir652−/−) mice and matching Mir652+/+Apoe−/− (Mir652+/+) mice (12–14 months old) were used to investigate the role of miR-652 in endothelial repair. Lesion formation was much smaller in HCD-fed Mir652−/− than in matching Mir652+/+ mice (Fig. 2a). Lesional accumulation of SMC (Fig. 2b) and collagen (Fig. 2c) was not significantly different in Mir652−/− and Mir652+/+ mice. However, we observed a significant decrease of Mac2+ area in Mir652−/− mice compared to Mir652+/+ mice (Fig. 2d). Specifically, although the
The number of lesional Mac2+ macrophages was not significantly different (Fig. 2e), we observed a significant decrease in their size in \( \text{Mir652}^{-/-} \) mice compared to \( \text{Mir652}^{+/+} \) mice (Fig. 2e). Notably, endothelial repair after carotid injury was significantly greater in \( \text{Mir652}^{-/-} \) mice compared to \( \text{Mir652}^{+/+} \) mice (Fig. 2f). Moreover, proliferation of carotid ECs, as assessed by immunostaining for vWF/Ki67 (Fig. 2g) or CD31/PCNA (Fig. 2h) 28-day post injury, was significantly greater in \( \text{Mir652}^{-/-} \) mice relative to \( \text{Mir652}^{+/+} \) mice.

Given that bone marrow (BM)-derived cells also express miR-652 [18], we next utilized a murine BM chimera model to elucidate the roles of endothelial-resident miR-652 versus BM-derived miR-652 in endothelial repair. We found that knockdown of endothelial-resident miR-652 significantly reduced lesion size and improved EC coverage 28-days post injury (Fig. 2i, j). However, knockdown of BM-derived miR-652 did not have statistically significant effects on lesion size or EC coverage in recipient mice (Fig. 2i, j). These results indicate that endothelial-resident miR-652 knockdown (not BM-derived miR-652 knockdown) impairs endothelial recovery.

### 2.4. miR-652-3p negatively regulates the endothelial repair gene Ccnd2

Normal endothelial repair is dependent upon pro-proliferative genes that stimulate EC recovery following injury [19,20]. In order to identify putative miR-652-3p targets that may serve to stimulate EC recovery post-injury, we conducted a bioinformatics analysis of a proliferation-associated gene dataset (\( n = 3025 \) genes; GeneCards) integrated with the miRNA target predictions for miR-652-3p (\( n = 39 \) putative target genes; RNA22 version 2.0 and DIANA-TarBase version 8). This bioinformatics analysis identified seven proliferation-associated putative target genes of miR-652-3p: CAV1, CCND2, CSNK1A1, G3BP1, PRKCI, SFPQ, and SOX4. Of these seven candidate genes, only CCND2 has been shown to be a key promoter of EC proliferation and has also been shown to be downregulated in atherosclerotic plaques [21]. Therefore, CCND2 was selected for further investigation.

Bioinformatics algorithms predicted two miR-652-3p binding sites (5682–5701 bp and 5996–6014 bp) on the human \( \text{Ccnd2} \) 3′UTR and one binding site (1038–1059 bp) on the murine \( \text{Ccnd2} \) coding sequence (CDS, Fig. 3a). Several lines of evidence support miR-652-3p's targeting of \( \text{Ccnd2} \). First, the expression level of carotid \( \text{Ccnd2} \) mRNA in \( \text{Mir652}^{-/-} \) mice was significantly greater compared to \( \text{Mir652}^{+/+} \) mice 28 days post-injury (Fig. 3b). Second, miR-652-3p significantly reduced luciferase activity in HEK293 cells carrying the wild-type CDS of murine \( \text{Ccnd2} \) in a dose-dependent manner, an effect abolished by the corresponding mutated CDS (CDSmut) (Fig. 3c). Third, HUVECs treated with a miR-652-3p mimic revealed significant downregulation of \( \text{Ccnd2} \) mRNA in \( \text{Mir652}^{-/-} \) mice 28 days post-injury (Fig. 3b). Second, miR-652-3p significantly reduced luciferase activity in HEK293 cells carrying the wild-type CDS of murine \( \text{Ccnd2} \) in a dose-dependent manner, an effect abolished by the corresponding mutated CDS (CDSmut) (Fig. 3c). Third, HUVECs treated with a miR-652-3p mimic revealed significant downregulation of \( \text{Ccnd2} \) expression in a time-dependent manner, while those treated with a locked nucleic acid (LNA) inhibitor of miR-652-3p (LNA-652-3p) displayed significant upregulation of \( \text{Ccnd2} \) expression (Fig. 3d).

Flow cytometry was then applied to study the effects of miR-652-3p and \( \text{Ccnd2} \) on HUVEC proliferation under lipidi apparatus-induced...
shear flow conditions. HUVEC proliferation was significantly enhanced with LNA-652-3p (but not LNA-652-5p), an effect that was abolished with CCND2-silencing (siCCND2) (Fig. 3e). HUVEC proliferation was significantly attenuated with a miR-652-3p mimic (but not the miR-652-5p mimic), an effect that was abolished with CCND2 overexpression (Fig. 3f). Following miR-652-3p mimic exposure, HUVECs with the miR-652-3p recognition element (CCND2\textsuperscript{+3pMRE}) showed less proliferation than those without the miR-652-3p recognition element (CCND2\textsuperscript{Δ3pMRE}) (Fig. 3g). Given the relatively high constitutive levels of miR-652 in HUVECs, we expected that HUVECs transfected with CCND2\textsuperscript{Δ3pMRE} would display higher proliferation levels under control conditions. Therefore, we assessed the relative expression of endogenous versus transfected CCND2 protein and found transfected CCND2 protein levels to be significantly lower than the endogenous CCND2 protein levels (Supp. Fig. 2). This may explain why CCND2\textsuperscript{Δ3pMRE} had no significant effect upon HUVEC proliferation under control conditions.

Mir652\textsuperscript{−/−} mice were then employed to the effects of Ccnd2 expression on miR-652-knockout endothelium in vivo. Treatment of carotid arteries of Mir652\textsuperscript{−/−} mice with Ccnd2-specific siRNA (siCcnd2) starting on day 14 post-injury resulted in decreased Ccnd2 mRNA and protein expression (Fig. 3h, i), enhanced lesion area (Fig. 3j), reduced EC recovery (Fig. 3k), and reduced EC proliferation (Fig. 3l) at day 28 post-injury.

2.5. Engineered miR-652-3p antagomir promotes endothelial repair in vivo and ex vivo

The roles of the two miR-652 strands (miR-652-3p and −652-5p) were comparatively investigated by studying the effects of their antagonists on denuded arteries from HCD-fed Mir652\textsuperscript{−/−} mice 28-days post-carotid injury. As expected, the respective antagonists reduced miR-652-3p and miR-652-5p expression (Fig. 4a). However, only the miR-652-3p antagonist decreased lesion formation (Fig. 4b),...
miR-652-3p negatively regulates the endothelial repair gene Cond2.  

(a) Bioinformatics algorithms predicted two miR-652 binding spots in the 3′ UTR of human CCND2 and one binding spot in the CDS of mouse Ccnd2. (b) Intimal EC expression of Ccnd2 mRNA at 28 days post-injury. (c) The effect of miR-652-3p on luciferase activity of psiCHECK-2 Cond2 promoter constructs. Empty psiCHECK-2 (psiCHECK), WT Ccnd2 CDS (wild-type murine Ccnd2), and Ccnd2 CDSmut (mutated murine Ccnd2) constructs were co-transfected with scrambled pre-miRNA (30 nM) or synthetic miR-652-3p (0 nM, 15 nM, or 30 nM). (d) Western blot analysis of CCND2 expression in HUVECs. Band intensities were normalized with respect to β-actin band intensity. *P < .05 vs. Ctrl, †P < .05 vs. LNA ctrl. (e-g) Flow cytometric HUVEC proliferation assays under shear flow conditions. (e) HUVECs were treated with LNA-652-5p and/or LNA-652-3p, or siCCND2 (CCND2-specific siRNA). (f) HUVECs were treated with 652-5p mimic and/or 652-3p mimic (miR-652-5p mimic and miR-652-3p mimic, respectively), with (+) or without (−) overexpression of Ccnd2. (g) HUVECs, with (CCND2^3pMRE) or lacking (CCND2^Δ5pMRE) the miR-652-3p recognition element, were treated by a miR-652-3p mimic. (h-l) Carotid-injured Mir652^−/− mice were perivascularly administered a single 4-nmol dose of scrambled control or a Ccnd2-specific siRNA on days 14 and 21 post-carotid injury. On day 28 post-injury, mice were sacrificed, and intimal EC expression of (h) Ccnd2 mRNA and (i) Ccnd2 protein, (j) plaque area, as well as (k) endothelial recovery and (l) proliferation were analyzed. Scale bar = 100 μm. n = 12–18 mice per experimental group. Data reported as means ± SEMs. *P < .05 vs. first group, †P < .05 vs. second group.
Fig. 4. Promotion of endothelial repair by miR-652-3p antagomir. Carotid-injured high cholesterol diet-fed Mir652+/- mice were perivascularly administered a single 160-μg dose of control (non-specific antagomir), miR-652-5p antagomir, or miR-652-3p antagomir on days 7, 14, and 21 post-carotid injury. On day 28 post-injury, mice were sacrificed, and (a) intimal EC expression of miR-652-5p and miR-652-3p, (b) plaque area (scale bar = 200 μm), (c) Mac2+ macrophage area, (d) number and (e) size of Mac2+ macrophages, (f) endothelial coverage area, and (g) EC proliferation, as well as (h) Ccne, Ccna and Cdk2 transcript expression, and (i) Ccnd2 transcript expression and (j) Ccnd2 protein expression in intimal ECs were analyzed. (k-m) Carotid-injured high cholesterol diet-fed Mir652+/- mice were perivascularly administered a single 5-nmol dose of carrier-packaged miR-652-3p mimic or scrambled control mimic on days 7, 14, and 21 post-carotid injury. On day 28 post-injury, mice were sacrificed, and (k) lesion formation (scale bar = 100 μm), (l) endothelial coverage, and (m) EC proliferation were analyzed. n = 12–18 mice per experimental group. Data reported as means ± SEMs. *P<.05 vs. first group, †P<.05 vs. second group.
reduced lesional Mac2+ macrophage area (Fig. 4c), number (Fig. 4d), and size (Fig. 4e), while increasing EC coverage (Fig. 4f) and EC proliferation (Fig. 4g). With respect to effects on Ccnd2, only the miR-652-3p antagonist enhanced Ccne, Ccna, and Cdk2 transcript expression (Fig. 4h), enhanced Ccnd2 transcript expression (Fig. 4i), and enhanced Ccnd2 protein expression in ECs (Fig. 4j).

To further investigate the effects of miR-652-3p ex vivo, the carotid arteries from HCD-fed Mir652+/− mice after injury was subjected to perivascular treatment with a miR-652-3p mimic for 28 days. The miR-652-3p mimic enhanced lesion formation (Fig. 4k), decreased luminal EC coverage (Fig. 4l), and reduced EC proliferation (Fig. 4m). Taken together, these results demonstrate that the miR-652-3p antagonist therapy promotes endothelial repair.

2.6. miR-652-3p antagonist therapy improves EC proliferation under hyperlipidemic conditions

To assess the effects of miR-652 under hyperlipidemic conditions in vivo, we investigated the effects of genetic miR-652 knockdown under HCD conditions using Mir652+/− and Mir652−/− mice. After a 12-week HCD regimen, Mir652+/− mice displayed reduced lesion formation (Fig. 5a) and aortic roots (Fig. 5b) as well as enhanced EC proliferation in aortic root sections (Fig. 5c).

Having shown the positive effects of genetic miR-652-3p knockdown under hyperlipidemic conditions in vivo, we next investigated the effects of miR-652-3p antagonist administration in Mir652+/− mice under HCD and normal diet conditions. HCD-fed Mir652+/− mice were systemically administered a miR-652-3p antagonist for the last four weeks of a 12-week HCD regimen. We validated miR-652-3p knockdown and associated Ccnd2 upregulation in HCD-fed murine aortas after four weeks of miR-652-3p antagonist therapy (Supp. Fig. 3). Mir652+/− mice systemically administered a miR-652-3p antagonist showed decreased lesion formation in the thoracoabdominal aortas (Fig. 5d) and aortic roots (Fig. 5e). We also assessed the effects of miR-652-3p antagonist therapy on EC proliferation at predilection (P) and non-predilection (NP) sites in aortic arches of normal diet-fed and HCD-fed Mir652+/− mice. Notably, we found that miR-652-3p antagonist therapy improved EC proliferation only at NP sites (Fig. 5f).

We next analyzed CCND2 expression levels in human carotid lesions (using post-mortem normal human carotid arterial specimens as controls) and discovered significantly decreased CCND2 mRNA and protein expression in human carotid lesions (Fig. 5g–i). We next analyzed correlations of miR-652-3p and CCND2 in human carotid lesions (Fig. 5j). Relative expression levels of miR-652-3p in human carotid lesions were negatively correlated with endothelial CCND2 abundance (Fig. 5k) and EC proliferation (Fig. 5l) but positively correlated with the macrophage percentage (Fig. 5m). Based on this evidence, we propose a model of faulty endothelial regeneration in atherosclerosis, where miR-652-3p-mediated Ccnd2 downregulation counteracts the pro-proliferative response to hyperlipidemic EC injury.

2.7. Neither miR-652-3p nor Ccnd2 affect ECs under disturbed flow conditions

We finally analyzed the effects of miR-652 and Ccnd2 under disturbed flow conditions using Mir652+/− and Mir652−/− mice following partial carotid ligation of the left carotid (LC) artery, with the unperforated right carotid (RC) artery used as a control. We found no significant changes in miR-652-3p, miR-652-5p, or Ccnd2 levels when comparing LC to RC arteries within one week post-ligation (Supp. Fig. 4a), indicating that disturbed flow does not significantly affect miR-652 levels or Ccnd2 gene expression. Mir652−/− mice displayed higher initial EC Ccnd2 protein expression than Mir652+/− regardless of disturbed flow conditions (Supp. Fig. 4b). Notably, control RC arteries of HCD-fed Mir652−/− mice displayed decreased lesion formation (Supp. Fig. 4c) and higher EC proliferation (Supp. Fig. 4d) relative to matching Mir652+/− mice, effects not observed in disturbed-flow LC arteries. Similarly, control RC arteries of HCD-fed Mir652+/− mice after a four-week treatment with a mir-652-3p antagonist displayed decreased lesion size (Supp. Fig. 4e) and greater EC proliferation (Supp. Fig. 4f), effects not observed in disturbed-flow LC arteries. In addition, control RC arteries of HCD-fed Mir652+/− mice after siCcnd2 administration displayed larger lesion sizes (Supp. Fig. 4g) and decreased EC proliferation (Supp. Fig. 4h), effects not observed in disturbed-flow LC arteries. In aggregate, these results support that disturbed flow conditions do not affect miR-652-3p or Ccnd2 expression and that neither miR-652-3p nor Ccnd2 affect EC characteristics under disturbed flow conditions.

3. Discussion

Endothelial inflammation is a key indicator of atherosclerotic progression [22]. Elucidating the critical roles of miRNAs in regulating inflammation, proliferation, and regeneration of ECs is a key area of research [23]. Thus, discovering the cellular and molecular mechanisms through which miRNAs regulate these EC processes is likely to aid in the design and development of novel therapeutic agents useful in managing atherosclerotic diseases.

Endothelial miRNAs are recognized to be involved in modulating vascular inflammation [24]. In human ECs, the most abundant miRNA miR-103 has been shown to induce inflammation [16]. Our results show that miRNA-652 is the second most abundant miRNA expressed in HUVECs following miR-103 (Fig. 1a). Previous reports have identified intracellular miRNAs that make use of RNA-binding proteins, such as AGO2, to protect themselves against degradation [25,26]. Our results show that miR-652 is significantly enriched in the AGO2-IP fraction from human ECs, suggesting that the observed abundance of miR-652 within the cytoplasm of human ECs may be due to an AGO2-mediated anti-degradation mechanism (Fig. 1, Supp. Fig. 1).

Oxidized LDLs (oxoLDL and moxoLDL), which are products of oxidative modification of circulating LDL molecules (particularly under hyperlipidemic conditions [27,28]), enhance endothelial activation and monocyte adhesion at an early stage of atherosclerotic plaque development [29,30]. Here, we found that HUVEC expression of miR-652 was significantly enhanced as a result of nLDL exposure, with a more profound effect observed after moxoLDL exposure (Fig. 1). The observed increase in the expression of miR-652 after nLDL exposure may result from the presence of some products of oxidative modification present in the nLDL preparation [31]. Naturally, the levels of these oxidation products would be much higher in the moxoLDL preparation. This initial evidence suggested that hyperlipidemia/oxoLDL exposure promotes miR-652 expression in the human endothelium, making miR-652 an exciting target for further investigation.

Dysfunction in EC repair is critical to the pathogenesis of atherosclerosis [32]. We show that, in our murine model of atherosclerosis, the absence of miR-652 promotes endothelial repair (Fig. 2). We further show that atherosclerotic lesion formation is much smaller in HCD-fed Mir652−/− mice relative to matching Mir652+/− mice (Fig. 2). This is consistent with the accelerated endothelial repair following carotid denudation in the absence of miR-652 (Fig. 2). In addition, changes in macrophage phenotype are of paramount importance for the progression of atherosclerotic diseases [33]. Specifically, increases in macrophage size indicate enhanced pro-atherogenic foam cell formation within atherosclerotic plaques [34]. Although the number of lesional Mac2+ macrophages after injury was not affected by miR-652 knockout, macrophage size was smaller in Mir652−/− mice compared to Mir652+/− mice (Fig. 2). This evidence suggests the involvement of miR-652 in promoting endothelial dysfunction and increasing foam cell formation in atherosclerosis.

Bioinformatics is an established tool for identifying miRNA-target gene couples in silico [35]. Here, our bioinformatics algorithms predicted two miR-652 binding sites in the human CND2 3’UTR and one
Fig. 5. Rescue of EC proliferation during hyperlipidemia by miR-652-3p antagomir. (a–c) Non-injured Mir652+/+ and Mir652−/− mice were placed on 12-week high cholesterol feeding regimen. Then, (a) en face preparations of the aortic lesion area and (b) aortic root lesion area (scale bar = 200 μm), as well as (c) aortic root EC proliferation were analyzed. (d, e) Non-injured Mir652+/+ mice were systemically administered a miR-652-3p antagomir or negative control (100 μg/dose, i.v.) every 3 days over the last four weeks of the 12-week high cholesterol feeding regimen to analyze lesion areas in the (d) thoracoabdominal aorta and (e) aortic root. (f) Non-injured Mir652+/+ mice consuming either regular chow or high cholesterol feed were systemically administered a miR-652-3p antagomir or negative control (100 μg/dose, i.v.) every 3 days over the last four weeks of the 12-week feeding regimen. EC proliferation was assayed via CD31/EdU immunostaining in en face prepared aortic arch at predilection (P) sites and non-predilection (NP) sites. *P < .05 vs. predilection (P) normal diet, †P < .05 vs. non-predilection (NP) normal diet. Analysis of (g) CCND2 transcript expression and (h, i) CCND2 protein expression in intimal ECs from human carotid lesions (n = 30 samples), with post-mortem normal human carotid intimal ECs used as controls (n = 20 samples). (j) Immunofluorescent staining images of vWF and CCND2 in human carotid lesions (scale bar = 200 μm). High miR-652-3p expression was defined as greater than or equal to the median value, while low miR-652-3p expression was defined as lower than the median value. Correlations were observed between intimal EC miR-652-3p expression and (k) intimal EC CCND2 abundance, (l) EC proliferation, and (m) lesion macrophages (n = 30 samples), n = 12–18 mice per experimental group. Data reported as means ± SEMs. *P < .05 vs. first group, †P < .05 vs. second group.
binding site in the CDS of mouse Ccnd2 (Fig. 3). This target gene was of particular interest, as upregulated activity/expression of the Ccnd2 gene (which encodes the cell cycle checkpoint protein Cyclin D2 [36]) is critically involved in promoting EC proliferation [37,38]. Consistent with our in vitro findings, we found that carotid Ccnd2 mRNA levels in Mir652−/+ mice were significantly greater than those in Mir652+/+ mice (Fig. 3). Using several experimental approaches, we also revealed that miR-652-3p negatively regulates Ccnd2 expression (Fig. 3). Consistent with previous work [37,38], we also found that enhanced Ccnd2 expression promotes EC proliferation and endothelial repair (Fig. 3).

When investigating the roles of miRNA in disease processes, it is crucial that the -3p and -5p strands be analytically distinguished due to the significant differences in sequences and target transcripts that may be present. For example, in vitro studies in the lung cancer cell line A549 showed that miR-652-5p displays an anti-proliferative effect, while the miR-652-3p strand produces the completely opposite effect [38,39]. Therefore, here we distinguished the effects of miR-652-5p and miR-652-3p in endothelial repair using a wide variety of experimental approaches and demonstrated that the miR-652-3p antagonist enhances EC proliferation and endothelial repair ex vivo (Fig. 4) and in vivo (Fig. 5). Furthermore, we show that miR-652-3p levels in human carotid artery lesions are negatively associated with EC CCND2 expression, negatively associated with EC proliferation, but positively correlated with the macrophage percentage (Fig. 5). This combined evidence from our murine model supports the application of miR-652-3p antagonist therapy in preventing atherosclerotic progression.

ECTs are best adapted to the high pulsatile shear conditions in unbranched vessel segments (termed non-predilection sites), with high pulsatile shear producing a healthy, quiescent EC phenotype [40–42]. At predilection sites of vessel branching, disturbed blood flow results in lower pulsatile shear levels and higher oscillatory shear levels, which increases pro-inflammatory activity and EC proliferation [40,42–44]. Moreover, the miRNA profile in laminar flow and disturbed flow-exposed ECs is significantly different, with disturbed flow supporting pro-atherogenic miRNA profiles [45]. Therefore, here we analyzed the effects of miR-652-3p or Ccnd2 on lesion size and EC proliferation under disturbed flow conditions using a murine partial carotid ligation model. We demonstrated that disturbed flow does not significantly affect miR-652 levels or Ccnd2 gene expression and that miR-652-3p or Ccnd2 do not affect lesion size or EC proliferation under disturbed flow conditions (Supp. Fig. 4). This is consistent with previous research showing that disturbed flow does not impact miR-652-3p or Ccnd2 expression in ECs [45,46]. We speculate that the proliferative EC phenotype present under disturbed flow, but not under laminar flow conditions, may not be susceptible to regulation by the miR-652-3p–Ccnd2 axis due to upregulation of other key pro-proliferative cyclins, such as CCND1 [46]. Targeting the miR-652-3p–Ccnd2 axis still remains a promising strategy for controlling atherosclerotic progression at non-predilection sites.

The foregoing findings raised an additional question of why miR-652-3p was dysregulated under hyperlipidemic stress conditions but not under disturbed flow conditions. It is known that hyperlipidemic stress influences distinct molecular pathways from those influenced by disturbed flow conditions in ECs [47]. According to Schobert et al.’s two-hit model of atherosclerosis, disturbed flow at susceptible arterial sites initially induces pathways primarily associated with low-grade, chronic EC apoptosis and activation [47]. Then, hyperlipidemia promotes alternative pathways primarily associated with EC inflammation and inhibition of EC proliferation, thereby further hampering EC regeneration and promoting atherosclerosis [47]. This two-hit model is consistent with our current findings, as hyperlipidemia (but not disturbed flow) promotes inhibition of EC proliferation via the miR-652-3p–Ccnd2 axis.

Based on our experimental findings, we advance a model of impaired endothelial regeneration during atherosclerosis, where the increase in EC miR-652-3p expression downregulates Ccnd2, thereby negatively regulating cell proliferation under hyperlipidemic stress (Fig. 5). On this basis, our results suggest that miR-652 blockade in the endothelium may offer a potentially novel strategy to combat atherosclerosis.

4. Materials and methods

The methods are fully detailed in the Supplementary Information.

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Competing interests

None.

Author contributions

Conceived and designed the study: LHJ and RZH. Performed the experimental procedures: ZCH, YC, HRL, HZ WHS, and LWL. Analyzed the data: YX and RZH. Drafted the manuscript: RZH and NDM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.01.032.

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