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MicroRNA-146a Induces Lineage Negative Bone Marrow Cell Apoptosis and Senescence by Targeting Plk2 Expression

Shanming Deng, MD, PhD[1], **Huilan Wang, MD**[1], **Chunling Jia, MD, PhD**[1], **Xianming Chu, MD, PhD**[1,2], **Qi Ma, Ph.D**[1], **Jianqin Wei, MD**[1], **Emily Chen, B.S.**[1], **Wei Zhu, PhD**[1], **Conrad J Macon, MD**[1], **Dushyantha T Jayaweera, M.D**[1], **Derek M Dykxhoorn, Ph.D.**[3], and **Chunming Dong, MD**[1]

[1]Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL 33101

[2]Department of Cardiology, the Affiliated Hospital of Qingdao University, Qingdao, 266100, China

[3]John T. Macdonald Foundation Department of Human Genetics, Miller School of Medicine, University of Miami, Miami, FL 33136

Abstract

Objective—Lineage negative bone marrow cells (lin$^-$ BMCs) are enriched in endothelial progenitor cells (EPCs) and mediate vascular repair. Aging-associated senescence and apoptosis result in reduced number and functionality of lin$^-$ BMCs, impairing their pro-repair capacity. The molecular mechanisms underlying lin$^-$ BMC senescence and apoptosis are poorly understood. MicroRNAs (miRNAs) regulate many important biological processes. The identification of miRNA-mRNA networks that modulate the health and functionality of lin$^-$ BMCs is a critical step in understanding the process of vascular repair. The aim of this study was to characterize the role of miR-146a—Plk2 network in regulating lin$^-$ BMC senescence, apoptosis, and their angiogenic capability.

Approach and Results—Transcriptome analysis in lin$^-$ BMCs isolated from young and aged wild type (wt) and apoE$^{-/-}$ mice showed a significant age-associated increase in miR-146a expression. *In silico* analysis, expression study and Luciferase reporter assay established Plk2 as a direct target of miR-146a. MiR-146a overexpression in young lin$^-$ BMCs inhibited Plk2 expression, resulting in increased senescence and apoptosis, via p16^{Ink4a}/p19^{Arf} and p53, respectively, as well as impaired angiogenic capacity *in vitro* and *in vivo*. Conversely, suppression of miR-146a in aged lin$^-$ BMCs increased Plk2 expression, rejuvenated lin$^-$ BMCs, resulting in decreased senescence and apoptosis, leading to improved angiogenesis.

Conclusions—1) miR-146a regulates lin$^-$ BMC senescence and apoptosis by suppressing Plk2 expression that, in turn, activates p16^{Ink4a}/p19^{Arf} and p53; and **2)** modulation of miR-146a or its

Correspondence: Chunming Dong, MD, FACC, 1501 NW 10th Ave. BRB, Room 812, Interdisciplinary Stem Cell Institute, Department of Medicine, University of Miami, Miami, FL 33136, Phone: (305) 243-4706, Fax: (305) 243-6847, cdong3@med.miami.edu.

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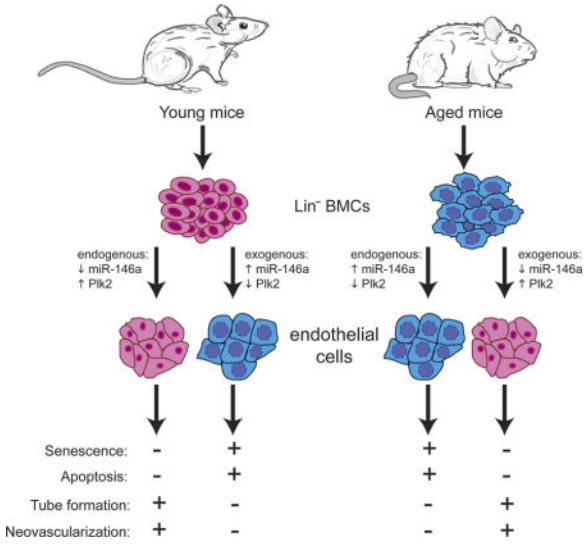
DISCLOSURES

none

target Plk2 may represent a potential therapeutic intervention to improve lin⁻ BMC-mediated angiogenesis and vascular repair.

Graphical abstract



Keywords

lin⁻ BMCs; angiogenesis; apoptosis; senescence; microRNAs; Plk2

INTRODUCTION

MicroRNAs (miRNAs) are small, non-coding RNA gene products of approximately 21 nucleotides that downregulate gene expression by binding to the 3' untranslated regions (3' UTR) of specific target mRNAs leading to mRNA degradation and/or translation inhibition[1]. MiRNAs have been implicated in regulating diverse cellular processes, including embryonic development, cell differentiation, proliferation, senescence and apoptosis[2]. Some miRNAs have been reported to play key roles in aging and atherosclerosis[3]. Furthermore, miRNAs may play a role in regulating angiogenesis. Indeed, we reported previously that miR-10A* and miR-21 induce lineage negative bone marrow cell (lin⁻ BMC)/endothelial progenitor cell (EPC) senescence by inhibiting Hmga2 expression and activating $p16^{Ink4a}$/$p19^{Arf}$ expression, resulting in decreased self-renewal potential and angiogenesis[4]. A growing number of reports have been focused on miRNAs as important modulators of apoptosis and inflammation. In particular, miR-146a has been shown to be involved in type 2 diabetes (T2DM), coronary artery diseases (CAD), and peripartum cardiomyopathy (PPCM)[5–8]. However, the role of miR-146a in regulating lin⁻ BMC/EPC senescence and apoptosis, as well as, their aging related functional impairments remains unclear.

Polo-like kinases (Plks) are a highly conserved family of serine-threonine kinases that regulate crucial cell processes, including cell cycle progression and mitosis. To date, five mammalian Plk family members (Plk1-5) have been identified[9]. Plk2 is activated near the G1/S phase transition and regulates the duplication of centrosomes in a tissue-specific

manner in neurons, heart, and lungs. Plk2 and Plk3 have emerged as important mediators of stress response resulting from DNA damage or exposure to reactive oxygen species (ROS)[10]. Accumulating evidence supports a mutual crosstalk between members of the Plk family and the tumor suppressor p53 under stress-inducing conditions in cancer cells[11]. Plk2 knockout mouse embryos show a delay in skeletal development and retarded growth[12]. Moreover, Plk2 is transcriptionally induced by p53 and it's silencing in the presence of cytotoxic drugs, such as paclitaxel, results in increased apoptosis[13]. The putative role of Plk2 in regulating lin^- BMC/EPC senescence and apoptosis and the miRNAs involved in these processes remains to be elucidated.

In this study, we report that miR-146a is significantly upregulated in aged lin^- BMCs. We identified Plk2 as a downstream target of miR-146a and established the role of the miR-146a—Plk2 network in regulating lin^- BMC senescence and apoptosis. Specifically, overexpression of miR-146a suppressed Plk2 expression resulting in increased lin^- BMC senescence and apoptosis—effects that were mediated by $p16^{Ink4a}$/$p19^{Arf}$ and p53, respectively. In contrast, down-regulation of miR-146a expression led to upregulation of Plk2 expression, reduction in apoptosis, and functional rejuvenation of aged lin^- BMCs. Furthermore, we demonstrate that dysregulation of miR-146a contributes to the impaired angiogenesis in aged mice by downregulating Plk2 resulting in lin^- BMC senescence and apoptosis. These findings provide therapeutic targets that may allow for the rejuvenation of lin^- BMCs/EPCs, improving their angiogenic and vascular repair capacity.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

MiR-146a Is Upregulated in Aged Lin^- BMCs

MiRNAs have been shown to play important roles in a wide range of cellular processess, including development, differentiation, growth and metabolism[14–16]. In our previous studies, we have shown by transcriptome analyses that there are significant changes in miRNA and gene expression in young compared to aged lin^- BMCs from both $apoE^{-/-}$ and wt mice[4]. Similar to our previously published results and that of others[4,17], the identity of these lin^- BMCs was confirmed by culturing the isolated cells on endothelial basal medium-2 supplemented with vascular endothelial growth factor (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor, ascorbic acid, hydrocortisone and 15% fetal bovine serum. The lin^- BMCs cultured in this manner were found to express the endothelial markers CD31, CD34, CD144, VEGF, and VWR, as well as, uptake of acetylated LDL (Dil- Ac-LDL) and binding of Ulex Lectin, confirming their identity (Figure SI). Examination of the miRNA profiles in these cells found 38 miRNAs that had consistent changes in expression patterns in lin^- BMCs from young (3-week old $apoE^{-/-}$ and wt) versus aged $apoE^{-/-}$ (1 year-old) and wt (2.5 year-old) mice (14 miRNAs were elevated and 24 miRNAs were decreased in aged mice). Among these changes, miR-146a was found to be significantly overexpressed in aged lin^- BMCs using the Sanger 13 microRNA Array analyses (Figure 1A).

MiR-146a Regulates Plk2 Expression

The miRNA expression analysis was complemented by genome-wide analysis of mRNA expression using the MEEBO (Mouse Exonic-Evidence Based-Oligonucleotide) Microarray chip. We found 1,135 genes to be differentially expressed in young compared to aged lin^- BMCs, irrespective of apoE expression. Of the 1,135 genes that passed the permutation analysis, about half of the genes showed a decreased expression in aged mice. In silico analysis of miRNA targets was performed using the miRNA databases—microRNA.org and Targetscan—to identify potential miR-146a targets. Comparison of the potential miR-146a targets with the differentially expressed genes by microarray analysis implicated polo-like kinase 2 [Plk2 or serum inducible kinase (SNK)] as a potential miR-146a target gene whose expression was also inversely correlated with that of miR-146a in lin^- BMCs (Figure 1B). Interestingly, CD34+ cells purified from the bone marrow of aged and young mice showed the same pattern of Plk2 and miR-146a expression (Figure SII). Our miRNA target analysis revealed two potential miR-146a binding sites in the 3'UTR of Plk2 (Figure SIII). To determine whether miR-146a directly targeted Plk2 through the miRNA binding sites in the 3′UTR, luciferase reporter constructs containing either the wild-type Plk2 3′UTR (WT3'UTR) or the 3′UTR bearing point mutations that disrupt the two putative miR-146a binding sites (M3'UTR) were developed. Transient co-transfection of miR-146a and the Plk2 WT 3'UTR-containing reporter construct in HEK293T cells showed significantly reduced luciferase activity compared to cells treated with a control, scrambled miRNA (miR-Ctr) (Figure 1C). In contrast, miR-146a had no effect on the luciferase expression from the reporter construct containing the mutated 3'UTR (M3'UTR) (Figure 1C). These data indicate that Plk2 is a direct target of miR-146a that interacts through two miR-146a binding sites in the 3'UTR of Plk2.

To determine whether miR-146a could regulate endogenous Plk2 expression in lin^- BMCs, cells from young mice (3 weeks old), which normally express high levels of Plk2 and low levels of miR-146a were transduced with lentivirus encoding miR-146a or the non-specific miR-Ctr. Overexpression of miR-146a repressed Plk2 mRNA and protein expression compared with miR-Ctr treated cells. Furthermore, the introduction of transgenic Plk2 containing a miR-146a-resistant, mutated 3'UTR (M3'UTR) into miR-146a expressing young lin-BMC was able to restore Plk2 expression in the cells compared to treatment with Plk2 bearing an intact 3'UTR (WT3'UTR) (Figure 1 D & F). Conversely, transduction of aged lin^- BMCs (from 2.5 years old wt mice) with the lentiviral vector expressing a miR-146a antagonist (miRZip-146a) reduced miR-146a expression and resulted in a concomitant increase in Plk2 expression (Figure 1E & G). Collectively, these data show that miR-146a directly regulates Plk2 expression in lin^- BMCs. Interestingly, we saw no difference in the pattern of miR-146a and Plk2 expression between the $ApoE^{-/-}$ and wild type mice (Figure 1 A and B). In addition, similar to the results in the wild type mice, the transduction of miR-146a into lin^- BMCs from young Apo $E^{-/-}$ mice led to a significant decrease in Plk2 expression and, reciprocally, the inhibition of miR-146a expression in aged lin- BMCS from $ApoE^{-/-}$ mice resulted in elevated levels of Plk2 expression (Figure SIV). These results suggest that this miR-146a-Plk2 regulatory network functions independently of ApoE status. Therefore, we focused the remainder of the analysis of the functional

consequences of modulating this regulatory network only in lin⁻ BMCs from wild type mice.

MiR-146a Regulates Lin⁻ BMC Senescence

Cellular senescence plays an important role in the complex process of biological aging of tissues, organs, and organisms and is driven by many factors, including oxidative stress, inflammation, and DNA damage and repair responses. Previous studies have shown that Plk2 regulates cell cycle progression and the cellular response to DNA damage[11,18]. Based on these studies and our data showing that Plk2 is a direct target of miR-146a, we hypothesized that this miRNA might drive lin⁻ BMC senescence by regulating Plk2 expression. To that end, young lin⁻ BMCs were infected with lentivirus coding for miR-146a or miR-Ctr. As expected, overexpression of miR-146a decreased Plk2 expression in young lin⁻ BMCs at both the mRNA and protein levels (Figure 2A & B). The decrease in Plk2 expression was associated with increased expression of cell cycle inhibitors, $p16^{Ink4a}$ and $p19^{Arf}$ (Figure 2 A & B). The upregulation of $p16^{Ink4a}$ and $p19^{Arf}$ have been shown to be responsible, at least in part, for stem cell senescence[19]. The examination of senescence-associated beta-galactosidase (SA-β-gal)—a marker for cell senescence – showed that the introduction of miR-146a into young lin-BMCs resulted in elevated levels of SA-β-gal compared to the control miRNA-treated cells (Figure 2 E & F). Conversely, the targeted inhibition of miR-146a expression (miRZip-146a) in aged lin⁻ BMCs resulted in increased Plk2 expression (Figure 2A & B), decreased $p16^{Ink4a}$ and $p19^{Arf}$ expression (Figure 2A & B), and reduced SA-β-gal activity (Figure 2E & F) compared to miR-Ctr-treated cells. Targeted inhibition of miR-146a expression (miRZip-146a) in aged lin⁻ BMCs significantly enhanced the self-renewal potential of lin⁻ BMCs, as demonstrated by the capability of miRZip-146a-treated lin⁻ BMCs to expand into primary (1°) colonies and the number of progeny cells in these 1° colonies that could form secondary (2°) colonies on soft agar compared to miR-Ctr-treated cells (Figure SV). These data indicate that miR-146a regulates lin⁻ BMC senescence.

Plk2 Knockdown Mediates the Effects of MiR-146a in Lin⁻ BMC Senescence

An individual miRNA can potentially regulate a large number of gene targets. Therefore, we sought to determine if the effect of miR-146a overexpression on the health and viability of lin⁻ BMCs was specifically mediated by Plk2. Treatment of the young lin⁻ BMCs with Plk2 siRNA resulted in a significant reduction in Plk2 expression at both the mRNA and protein levels (Figure 2C & D). Similar to the treatment with miR-146a, siRNA-mediated silencing of Plk2 resulted in increased expression of the cellular senescence markers $p16^{Ink4a}$ and $p19^{Arf}$ (Figure 2C & D) and SA-β-gal (Figure 2E and F). Remarkably, the treatment of young lin⁻ BMCs overexpressing miR-146a with Plk2 cDNA lacking the 3'UTR (Plk2-3'del), but not Plk2 bearing the intact 3'UTR (wt Plk2), was able to rescue the miR-146a- driven cellular senescence phenotype (Figure SVI). Since the wt Plk2 construct contains the 3'UTR, the inability of wt Plk2 to rescue the miR-146a effects is most likely a result of inhibition of Plk2 expression by the overexpressed miR-146a. Taken together, these results demonstrate that Plk2 mediates the effects of miR-146a on lin⁻ BMC senescence.

MiR-146a and Plk2 Regulate Lin⁻ BMC Apoptosis

The senescence of lin⁻ BMCs could result in alterations in both the functionality and viability of the cells. To determine if miR-146a induces cell apoptosis, young lin⁻ BMCs were infected with lentivirus overexpressing miR-146a or miR-Ctr. Overexpression of miR-146a resulted in a significant increase in cell apoptosis as measured by TUNEL staining (Figure 3A and quantified in Figure SVII) and Caspase 3 nuclear translocation (Figure SVIII) compared to miR-Ctr treatment. In contrast, inhibition of miR-146a by miRZip-146a in aged lin⁻ BMCs protected lin⁻ BMCs from apoptosis as measured by a decrease in the number of TUNEL-positive cells (Figure 3A) and reduced Caspase 3 nuclear translocation (Figure SVII). Further confirming that miR-146a exerts its pro-apoptotic effect through the regulation of Plk2 expression, the siRNA-mediated silencing of Plk2 caused an increase of apoptosis in young lin⁻ BMCs similar to that seen with miR-146a overexpression (Figure 3A & S7). Similarly, the overexpression of miR-146a in young lin⁻ BMCs derived from $ApoE^{-/-}$ mice lead to increased levels of apoptosis compared to miR-Ctr treated cells (Figure SIX and SX). Reciprocally, the suppression of miR-146 in aged lin⁻ BMCs from $ApoE^{-/-}$ resulted in decreased apoptosis (Figure SIX and SX).

P53 is a known proapoptotic factor that activates other cell death factors, including Cytochrome C, Bad and caspase 3. To dissect the molecular mechanisms whereby miR-146a and Plk2 regulate lin⁻ BMC apoptosis, we examined p53 and Bad expression, caspase-3 cleavage, and Cytochome C release from the mitochondria. miR-146a overexpression in young lin⁻ BMCs resulted in elevated p53 and Bad expression levels, as well as, increased caspase-3 cleavage and Cytochrome C release (Figure 3B). These miR-146a overexpression-induced molecular and cellular phenotypes were inhibited by simultaneous expression of Plk2-3'del, but not wt Plk2, and siRNA-mediated p53 silencing (Figure 3B). Plk2 silencing in young lin⁻ BMCs recapitulated the effects of miR-146a on the proapoptotic factors and cell apoptosis, particularly p53 upregulation and caspase 3 cleavage (Figure 3C). The induction of p53 expression by Plk2 silencing is remarkable as it has been shown that Plk2 is a target gene for p53 and its expression can be transcriptionally induced by p53. Indeed, p53 silencing abolished the effects of Plk2 repression on the expression of proapoptotic factors (Figure 3B) and cell death in young lin⁻ BMCs (Figure 4A & B). It also resulted in decreased p53 expression and caspase-3 cleavage in aged lin⁻ BMCs (Figure 4C). Our current findings suggest that a positive feedback loop may exist between p53 and Plk2 supression since decreased p53 levels lead to reduced plk2 expression, which may help sustain the proapoptotic effects of Plk2 suppression. Conversely, if Plk2 is upregulated, p53 expression is further inhibited, sustaining the survival effects of Plk2 overexpression. Together, these data indicate that p53 works downstream of miR-146a and Plk2 to affect lin⁻ BMC apoptosis. It is notable that the miR-146a—Plk2—p53 apoptotic pathway operates independently of cell senescence since targeted silencing of $p16^{Ink4a}/p19^{Arf}$ had no impact on p53 expression or cell apoptosis (Figure 4A & B).

MiR-146a Impairs Cell Migration and Angiogenesis in lin⁻ BMCs in vitro

Since miR-146a and Plk2 regulate lin⁻ BMC senescence and apoptosis, we reasoned that this pathway might affect the functionality of these cells, including cell migration and vascular tube formation, both of which play crucial roles in the angiogenic process. Wound

healing assays were used to determine the effects of miR-146a on cell migration. Overexpression of miR-146a in young lin⁻ BMCs reduced the ability of these cells to close the scratch wound compared to miR-Ctr treated cells (Figure 5A & B). Conversely, silencing of miR-146a expression in aged lin⁻ BMC using miRZip-146a restored the ability of the cells to migrate, at a level comparable to that of young lin⁻ BMCs. This effect of miR-146a on cell migration was likely mediated through Plk2 since the siRNA-mediated silencing of Plk2 phenocopied the impairment in wound healing shown with miR-146 overexpression in young lin⁻ BMCs (Figure 5A and B). Furthermore, overexpression of the miRNA-resistant Plk2-3'del rescued the effects of miR-146a overexpression on cell migration in the young cells (Figure SXI).

Next, we examined whether miR-146a overexpression affected the ability of young lin⁻ BMCs to form vascular-like network in vitro as measured by the Matrigel tube formation assay. Young lin⁻ BMCs infected with lentiviral vectors overexpressing miR-146a or miR-Ctr were loaded onto Matrigel. After a 10-hour incubation, miR-146a overexpressing cells showed a significant impairment in capillary tube formation compared with miR-Ctr transfected cells (Figure 5C and D). Conversely, inhibition of miR-146a by miRZip-146a in aged lin⁻ BMCs rescued tube formation (Figure 5C and D). Furthermore, Plk2 silencing impaired capillary tube formation compared to control treatment in young lin⁻ BMCs (Figure 5C and D). Consistently, overexpression of the miRNA-resistant Plk2-3'del, but not wild type Plk-2, in young lin⁻ BMCs overexpressing miR-146a abolished the miR-146a-mediated impairment of vascular tube formation (Figure SXII). Collectively, these data indicate that miR-146a and Plk2 regulate lin⁻ BMC migration and vascular tube formation, perhaps via their effects on cell senescence and apoptosis of these cells.

MiR-146a and Plk2 Regulate Angiogenesis in vivo

Our in vitro data indicate that the modulation of the miR-146a—Plk2 pathway regulates the health (cellular senescence and apoptosis) and the functionality (cell migration and vascular tube formation) of lin⁻ BMCs. We then determined if this pathway affected the angiogenic capability of lin⁻ BMCs in vivo. We first performed matrigel plug assay to determine the potential of lin⁻ BMCs to form new blood vessels (neovascularization) in vivo. Untreated lin⁻ BMCs or lin⁻ BMCs with altered expression of components of the miR-146a-Plk2 regulatory network were embedded in matrigel and injected subcutaneously into C57BL/J mice using our published protocol[4]. MiR-146a overexpression or Plk2 suppression in young lin⁻ BMCs resulted in a marked reduction in the level of neovascularization measured 11 days post injection compared to control treatment (Figure 6A–C). In contrast, matrigel plugs containing aged lin⁻ BMCs overexpressing miRZip-146a showed a significant improvement in neovascularization (Figure 6A and D). Furthermore, overexpression of the miRNA-resistant Plk2-3'del, but not wild type Plk-2, in these aged cells abolished miR-146a overexpression-mediated impairment of neovascularization (Figure 6B).

To further determine the role of miR-146a and Plk2 on the angiogenic capability of lin⁻ BMCs in vivo, we injected genetically modified young or aged lin⁻ BMCs intramuscularly in the hindlimbs of FVB/NJ mice whose femoral arteries were ligated on one side (hindlimb ischemic model) and measured blood flow by serial laser Doppler perfusion imaging and

angiogenesis by immunofluorescence microscopy. Similar to the in vivo matrigel plug assay, young lin$^-$ BMCs with miR-146a overexpression or Plk2 silencing showed a marked reduction in the ratio of blood flow (ischemic/non-ischemic leg) and in the degree of angiogenesis compared to control treatment (Figure 7A and B). Figure SXIII shows the quantification of blood vessel number. Conversely, inhibition of miR-146a expression (miRZip-146a) or overexpression of miR-146a-resistant Plk2-3'del in aged lin$^-$ BMCs significantly improved blood flow and angiogenesis to a level similar to the control treated young cells. Collectively, these findings indicate that miR-146a inhibits the angiogenic capability of lin$^-$ BMCs by negatively regulating Plk2 expression. These effects of modulating the miR-146a-Plk2 pathway components were replicated in a second strain of mice (FVB/RacCA mice) (Figure SXIV)

miR-146a is secreted and able to induce apoptosis in young lin- BMCs

While most miRNAs function in the cell in which they are expressed, there is mounting evidence that miRNAs may be actively secreted in membrane bound vesicles (for example, exosomes) that can than be delivered to other cells affecting the functionality of these recipient cells[20]. This release from one cell and uptake by a recipient cell provides an effective means of cell-cell communication[21]. For example, Valadi et al (2007) showed that exosomes released from mouse mast cells contained mRNAs and miRNAs that, upon delivery to recipient cells, affected the functionality of the recipient cells[22]. We examined if miR-146a could be released from the transduced lin$^-$ BMCs into the culture supernatant and alter the functionality of naïve recipient lin$^-$ BMCs. To that end, we purified exosomes from the culture supernatant of young lin-BMCS that had been stably transduced with a lentiviral vector expressing miR-146a. Quantitative real-time PCR (qRT-PCR) analysis showed that elevated levels of miR-146a were present in the supernatant of miR-146a-transduced young lin$^-$ BMCs but not miR-Ctr-transduced cells (Figure SXVA). Interestingly, the treatment of naïve young lin$^-$ BMCs with the supernatant from the miR146a-expressing cells but not the control miRNA expressing cells resulted in an induction of apoptosis in the recipient cells as measured by elevated levels of nuclear translocation of caspase 3 (Figure SXVB) and increased TUNEL staining (Figure SXVB). These results suggest that miR-146a can be released in the culture supernatant, presumably in exosomes, and act in a paracrine fashion to affect the health and viability of surrounding target cells. This release of the miR-146a may effectively spread its proapoptotic effect.

DISCUSSION

Experimental and clinical evidence supports the notion that adult stem/progenitor cells derived from aged bone marrow (BM) are deficient in tissue repair/regeneration. These cells show impaired proliferation and differentiation capabilities and increased apoptosis rates as they age[23]. Yet, the mechanisms that underlie the increased functional deterioration with aging remain incompletely understood. Increasing evidence supports the role of miRNAs as key regulators for cellular senescence and apoptosis in different cell types[24,25]. Indeed, using high content gene and miRNA expression analysis, we were able to identify sets of genes and miRNAs that were differentially expressed in lin$^-$ BMCs as a function of aging in wt and apoE$^{-/-}$ mice. Among these aging-associated changes, we have characterized that the

miR-10A*/miR-21—Hmag2—p16^{Ink4a}/p19^{Arf} predominantly regulates lin$^-$ BMC self-renewal[4]. As a continuation of our ongoing efforts to thoroughly study the impact of aging on lin$^-$ BMCs, we report here that the miR-146a—Plk2 axis serves as another mechanism to engage p16^{Ink4a}/p19^{Arf} expression—causing lin$^-$ BMC senescence. Furthermore, miR-146a—Plk2 can also activate p53 expression, leading the senescent cells to undergo apoptosis, which is conceivably the ultimate fate of these cells. These findings not only extend our previous discoveries, but also provide novel mechanistic insights as to how aging in individuals/animals leads to decreased levels and functionality of stem/progenitor cell populations — the formation of youth.

MiR-146a appeared to be high on our list of candidate miRNAs that showed differential expression changes between young and aged lin$^-$ BMCs based on our prioritization approach that included both the difference of expression and the biological relevance of the miRNA and its potential target genes. MiR-146a expression was upregulated in not only aged lin$^-$ BMCs relative to their young counterparts, but also ECs derived from young lin$^-$ BMCs cultured to passage 26, as compared with the same cells at passage 6 (data not shown). This is consistent with a previous report, demonstrating up-regulation of miR-146a in senescent ECs[26] and fibroblasts[6,27]. Our in silico analysis identified Plk2 as a potential target gene for miR-146a. The expression of miR-146a correlated inversely with Plk2 expression in young and aged lin$^-$ BMCs. Using luciferase reporter assays with wt Plk2 3'UTR and mutant Plk2 3'UTR with site-directed mutagenesis targeting the specific miR-146a binding sites in the Plk2 3'UTR, we confirmed that Plk2 was the direct target of miR-146a. Ectopic expression of miR-146a could repress endogenous expression of Plk2 in young lin$^-$ BMCs cells. Importantly, co-expressing Plk2 bearing a 3'UTR deletion and is, therefore, resistant to miRNA degradation could reverse the effects of ectopically expressed miR-146a in young lin$^-$ BMCs. These findings demonstrate that Plk2 is a direct target gene and mediates the effects of miR-146a in lin$^-$ BMCs.

Informatic algorithms suggest that, in addition to miR-146a, several other miRNAs, including miR-219, miR-27a/b, miR-146b and miR-214 may target Plk2 mRNA. However, these miRNAs did not show any meaningful changes in their expression in young versus aged lin$^-$ BMCs, suggesting that they may not play a significant role in regulating Plk2 protein levels in lin$^-$ BMCs during aging. MiR-146a is a pleiotropic miRNA that has been shown to serve as a guardian of the quality and longevity of hematopoietic stem cells (HSCs). Indeed, deletion of miR-146a causes excessive myeloproliferation, leading to HSC exhaustion[28]. Furthermore, increased levels of miR-146a are observed in the plasma of patients with type 2 diabetes mellitus (T2DM)[5], coronary artery disease (CAD)[7] and peripartum cardiomyopathy (PPCM)[8]. Intriguingly, miR-146a may play a pathogenetic role in PPCM by attenuating angiogenesis[8]. These findings are consistent with our observation that miR-146a is associated with decreased lin$^-$ BMC/EPC proliferation and impaired angiogenesis. Together, these studies show that miR-146a may serve as a biomarker for EPC/EC aging and a therapeutic target for cardiovascular disease and CAD risk equivalents, such as T2DM.

Each miRNA can regulate multiple target genes. The same miRNA may have different regulatory mechanisms for different cell types due to their diverse cellular

microenvironments[29]. Indeed, the effects of miR-146a in HSCs, ECs and PPCM are mediated by different signaling proteins, including TRAF6 (HSC), Erbb4 (PPCM), Notch1 (PPCM), Irk1(PPCM), NFκB, and IL-6. Furthermore, miR-146a induces apoptosis in hepatic stellate cells by targeting SMAD and in cardiomyocytes by targeting Erbb4[30–33]. Notably, none of these gene targets showed any significant changes in young versus aged lin$^-$ BMCs in our gene and miRNA expression profiling, indicating that these targets are unlikely to mediate the effects of miR-16a in the aging process of lin$^-$ BMCs. Instead, using both genomic profiling and in-depth functional studies, we have demonstrated that miR-146a directly targets Plk2, which in turn activates p16^{Ink4a}/p19^{Arf} and p53 pathways, regulating lin$^-$ BMC senescence and apoptosis, respectively.

Plk2 is most abundantly expressed in testis and fetal tissues suggesting that Plk2 plays an important role in cells undergoing rapid cell division. Plk2 knockout embryos show retarded growth and delayed skeletal development late in gestation indicating that Plk2 is involved in embryonic development[12]. Plk2 serves as a mediator for cell survival in the context of mitochondrial dysfunction and in cholangiocarcinoma[34]. In addition, silencing of Plk2 by RNAi leads to cancer cell apoptosis in the presence of Taxol[35]. In this study, we report important phenotypic similarity between enforced miR-146a expression and Plk2 knockdown in lin$^-$ BMCs. Importantly, the effects of miR-146a overexpression in lin$^-$ BMC senescence and apoptosis are rescued by the overexpression of Plk2 ORF clone which lacks the 3'UTR, indicating that Plk2 is a central mediator for the effects of miR-146a in lin$^-$ BMCs. The inability for wt Plk2 bearing an intact 3'UTR to rescue the miR-146a effects is most likely due to the inability to overcome miR-146a repression or, potentially, through regulation by other as of yet unidentified miRNAs.

The INK4A locus encodes two independent but overlapping genes, p16^{Ink4a} and p19^{Arf}. Inhibition of these two genes results in increased cell lifespan. In contrast, activation of this locus is associated with cell senescence[36]. In our previous work, we have demonstrated that miR-10A* and miR-21 suppress Hmga2 expression, resulting in activation of p16^{Ink4a} and p19^{Arf} expression and lin$^-$ BMC senescence[4]. In this study, we provide evidence that miR-146a—Plk2 serves as another mechanism in activating p16^{Ink4a} and p19^{Arf} expression. Thus, these two miRNA pathways converge on p16^{Ink4a} and p19^{Arf} and work together to regulate lin$^-$ BMC senescence.

Senescence is a cellular response to damage and stress. The senescence response prevents cancer by suppressing the proliferation of cells with a compromised genome and contributes to optimal repair in normal tissues. Persistent senescent cells are also thought to drive aging and age-associated pathologies through their secretion of inflammatory factors that modify the tissue microenvironment and alter the function of nearby normal or transformed cells[37]. Thus, it is necessary for senescent cells to undergo apoptosis to prevent age-associated diseases. The ability of p53 to eliminate excess, damaged or senescent cells by apoptosis is vital for the proper regulation of cell proliferation in multi-cellular organisms. P53 is activated by external and internal stress signals that promote its nuclear accumulation in an active form. In turn, p53 induces either viable cell growth arrest or apoptosis[38]. Missense mutations of p53 contribute to the maintenance, spreading and increased resistance to anticancer drugs of tumors, as the mutant p53 are incapable of recognizing wt p53 DNA

binding sites[39]. Plk2 has been shown to sustain mutant p53 activity through an autoregulatory feedback loop[40]. Specifically, Plk2 binds and phosphorylates mutant p53 protein, increasing its oncogenic activity and its efficacy to transcriptionally induce Plk2 production, thereby generating an oncogenic positive feedback loop. Furthermore, mutant p53 proteins upregulated Plk2 gene expression in response to DNA damaging agents leading to an aberrant increase of cell proliferation[38]. In this study, we provide evidence for p53 involvement in miR-146a-mediated apoptosis of the senescent lin$^-$ BMCs. Remarkably, we demonstrated a positive feedback loop between wt p53 and Plk2 suppression, as indicated by Plk2 silencing-induced p53 upregulation, which may help drive the proapoptotic effects of Plk2 deficiency. Thus, the feedback loops between p53 and Plk2 appear to be a two-way street. On one hand, upregulation of Plk2 increases tumor cell proliferation by binding and phosphorylating mutant p53; on the other hand, Plk2 deficiency promotes senescent cell apoptosis by inducing wt p53 expression. Our data also indicate that the miR-146a—Plk2 signaling in aged lin$^-$ BMCs diverges into p16^{Ink4a} and p19^{Arf}–mediated cell senescence and p-53—mediated cell apoptosis, with the latter being crucial in eliminating the senescent lin$^-$ BMCs, which may underlie the decreased circulating EPC levels observed in aged individuals and those with CAD. As with miR-146a-induced cell senescence, other putative miR-146 targets may also be involved in mediating senescent lin$^-$ BMC apoptosis. For example, miR-146a has previously been reported to target FADD to modulate activation-induced cell death in Jurkat T Cells[41]. Neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) emerged as a new putative direct target of miR-146a conserved in humans, and mice. Overexpression of miR-146a repressed NRAS mRNA and protein levels in HUVECs resulting in increased apoptosis[8]. The microRNA TargetScan software analysis also revealed other target genes, including BRCA1, Myo1b, Klf4, Zxdb, and CXCR4. However, none of these target genes showed significant changes in expression in lin$^-$ BMCs with aging, and were thus not selected for further study.

Recent studies have shown that miRNAs can be incorporated into exosomes which can be released from cells and be taken up by recipient cells. This mechanism can serve as a means of cell-cell communication and change the functionality of the recipient cells. The overexpression of miR-146a in young lin$^-$ BMCs led to the uptake of miR-146a into exosomes, which were released into the culture superntatant. When naïve lin$^-$ BMCs from young mice was cultured in the presence of the miR-146a-containing supernatant, these cells showed an elevated level of apoptosis. This provides a mechanism by which the pro-apoptotic signal can be transmitted to surrounding cells increasing the pathogenicity of the miRNA. Recent studies have shown that cardiovascular pathologies, such as, myocardial infarction, coronary artery disease, and heart failure are responsive to exosome-mediated delivery of bioactive molecules (eg. proteins, mRNA and miRNAs). For example, Rautou et al (2011) suggested that exosomes released from atherosclerotic plaques led to the stimulation of endothelial cell proliferation and inflammatory cell recruitment resulting in accelerated plaque development[42]. Exosomes derived from endothelial cells were enriched with miR-126, which drove re-endothelization[43]. In addition, exosomes derived from cardiomyocytes of a rat type 2 diabetes model were enriched in miR-320, which inhibited endothelial cell proliferation and impaired angiogenesis[44]. The release of exosomes containing miR-21 from cardiac fibroblasts induced a hypertrophic phenotype in

cardiomyocytes[45]. Importantly, it has been shown that the 16 kDa N-terminal prolactin fragment (16K PRL) induced the release of exosomes carrying miR-146a from endothelial cells driving peripartum cardiomyopathy[46]. Our finding that miR-146a release from lin$^-$ BMCs further supports a role for this molecule in the induction of cardiovascular disease through the induction of senescence and apoptosis and may help to explain the poor treatment efficacy of aged lin$^-$ BMCs for CVD.

Importantly, the miR-146a—Plk2 pathway has direct implications in lin$^-$ BMC functionality and angiogenesis. Senescent lin$^-$ BMCs and young cells overexpressing miR-146a or with Plk2 silencing show decreased endothelial forming capabilities in vitro and reduced angiogenesis in vivo. Therefore, these findings may not only be helpful in developing approaches to enhance angiogenesis for cardiovascular repair and wound healing, but may also be beneficial in finding new ways to inhibit angiogenesis in the case of cancer treatment.

In summary, our findings reveal the existence of the miR-146a—Plk2 pathway in lin$^-$ BMCs. MiR-146a increases with aging, which negatively regulates Plk2. We can speculate that miR-146a—Plk2 signaling may work together with miR-10A*/miR-21—Hmga2 pathway to withdraw lin$^-$ BMCs from normal cycling via p16^{Ink4a}/p19^{Arf} activation as these cells age. Either simultaneously or subsequently, the miR-146a—Plk2 pathway will activate p53, subjecting these cells to apoptosis. Together, these molecular events result in decreased circulating EPC levels and impaired angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Lin- BMC	lineage negative bone marrow cell
EPC	endothelial progenitor cell
miRNA	microRNA
UTR	untranslated region
Plk2	Polo-like kinase 2
wt	wild type

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Highlights

- miR-146a expression is elevated in aged compared to young lin$^-$ BMCs.
- miR-146a directly targets Plk2.
- The overexpression of miR-146a increases cellular senescence and apoptosis in young lin$^-$ BMCs leading to impaired vascular tube formation *in vitro* and neovascularization *in vivo*.
- Silencing of miR-146a in aged lin$^-$ BMCs rejuvenated aged lin$^-$ BMCs as demonstrated by enhanced neovascularizton in a hindlimb ischemia mouse model.

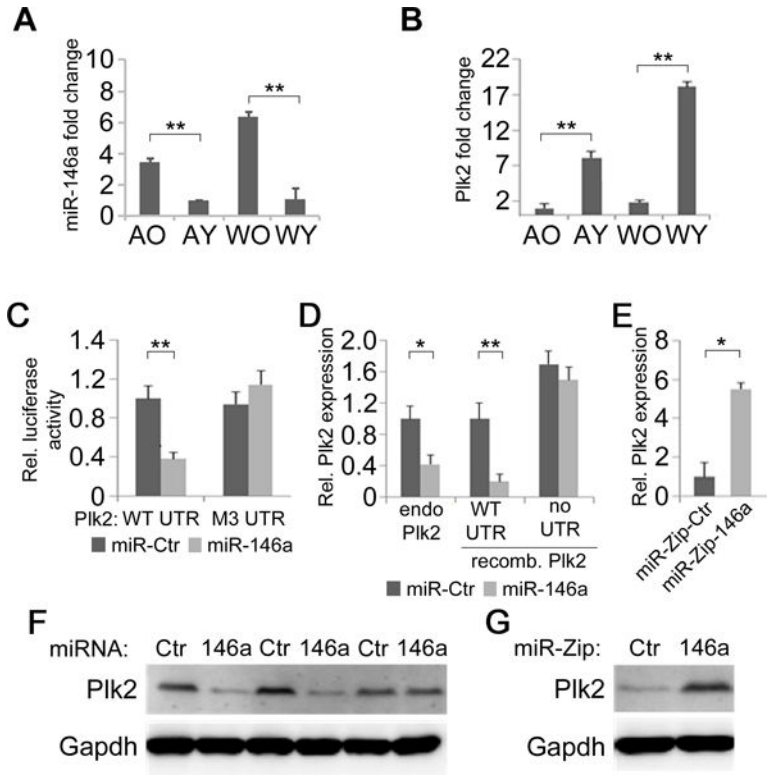

Figure 1. Plk2 is a direct target of miR-146a in lin⁻ BMCs

Differential expression of miR-146a (A) and its target gene Plk2 (B) was detected by microRNA profiling in lin⁻ BMCs from old apoE$^{-/-}$ (AO) and wild type (WO) and young apoE$^{-/-}$ (AY) and wild type (WY) mice. Similar reciprocal expression changes are observed in young and aged lin⁻ BMCs between microarray and qRT-PCR analyses. Regulation of Plk2 expression by miR-146a was examined by luciferase reporter assays (C). HEK293T cells were co-transfected with precursors of miR-146a, or scrambled miR-control (miR-Ctr) and psiCHECK-2 vector containing Plk2 with WT 3'UTR (WT UTR) or the 3'UTR containing mutations to the miR-146a binding sites (M3 UTR) downstream of a renilla luciferase reporter. Luciferase activities were measured 48 h after transfection and the values represent the Renilla/firefly luciferase ratios (mean ± SD, n ≥ 6; $^{**}p < 0.01$, *p<0.05). Endogenous levels of Plk2 mRNA were measured by qRT-PCR in young wt lin⁻ BMCs (D) 48 h following treatment with lentivirus encoding miR-146a or miR-Ctr alone or co-transfected with the recombinant Plk2 gene containing either the wt 3'UTR (WT UTR) or without 3'UTR (No UTR). Endogenous levels of Plk2 mRNA were also measured by qRT-PCR in aged WT lin⁻ BMCs (E) infected with lentivirus encoding the antagonists for miR-146a (miRZip-146a) and miR-Ctr (miRZip-Ctr). Protein expression of Plk2 levels were measured by immunoblotting in young lin⁻ BMCs treated with miR-Ctr or miR-146a with or without concomitant transfection of Plk2 WT UTR or Plk2 no UTR (F) and in aged lin⁻ BMCs treated with miRZip-Ctr and miRZip-146a (G).

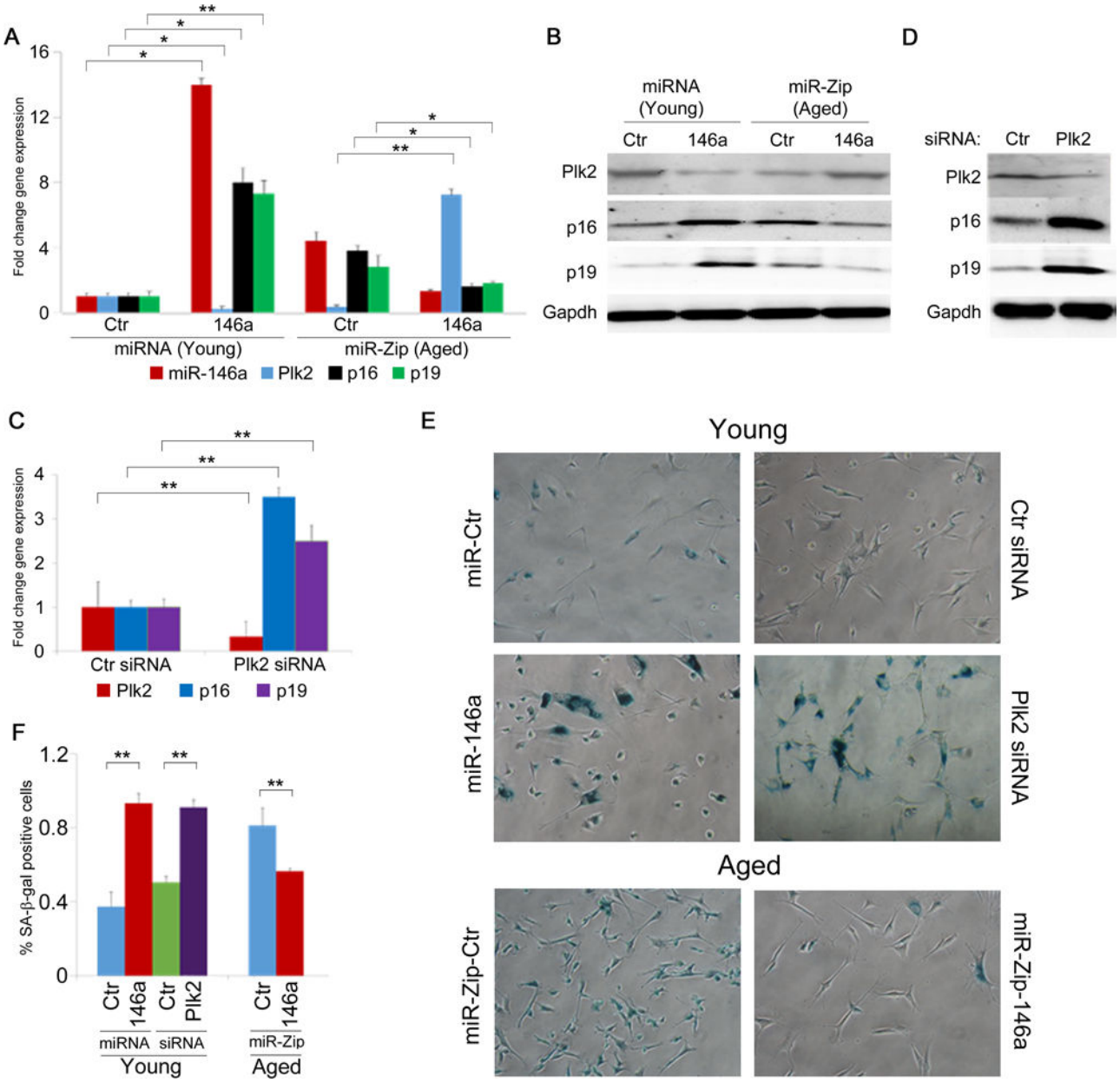

Figure 2. MiR-146a overexpression or inhibition regulates lin⁻ BMC senescence through Plk2

Young and aged wt lin⁻ BMCs were infected with lentivirally-encoded mature miR-146a or the miRNA antagonist miRZip-146a, respectively. The impact of modulating miR-146a on the expression levels of miR-146a, Plk2 and p16Ink4a/p19Arf were measured at RNA and protein levels using qRT-PCR (A) and immunoblotting (B), respectively. Expression of Plk2 and p16Ink4a/p19Arf was measured at RNA (C) and protein levels (D) in young lin- BMCs treated with siRNA for Plk2 (Plk2 siRNA). Plk2 silencing phenocopies the effects of miR-146a overexpression. The effects of miR-146a overexpression and inhibition, and Plk2 silencing on cellular senescence were analyzed by senescence-associated (SA)-β-gal expression (E and F). Data are presented as mean ± SD (n=3; ** $p < 0.05$, *$p<0.01$).

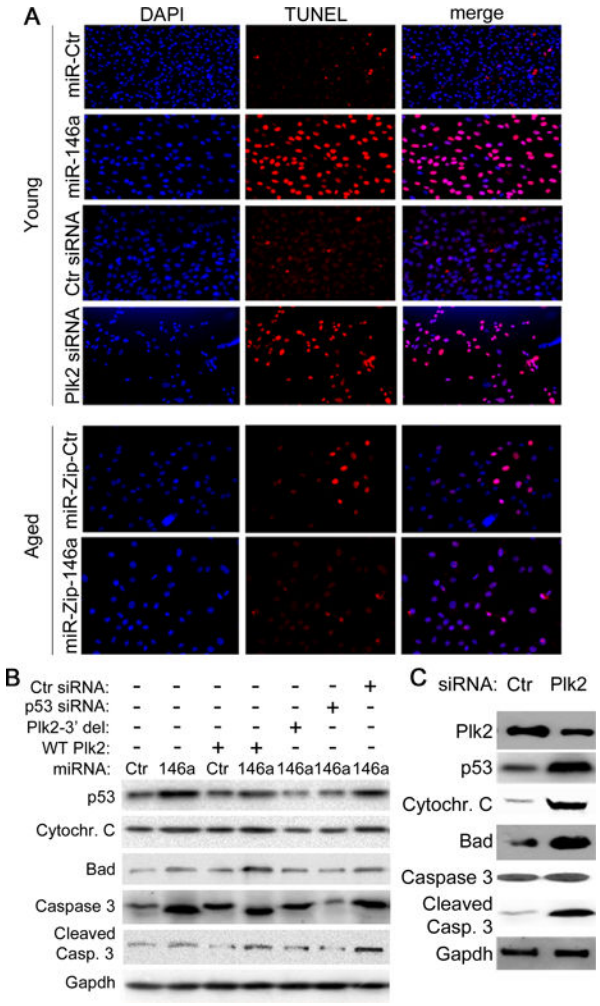

Figure 3. MiR-146a regulates lin⁻ BMC apoptosis

MiR-146a overexpression and Plk2 silencing in young lin⁻ BMCs increased apoptosis levels as measured by TUNEL staining. Conversely, suppression of miR-146a (miR-Zip-146a) in aged lin⁻ BMCs inhibited apoptosis as measured by TUNEL staining (A). The impact of modulating miR-146a and Plk2 expression on apoptotic factors, including p53, Cytochrome C, Bad and Caspase 3 were measured at the protein levels by immunoblotting (B&C). Remarkably, when young lin- BMCs were transfected with miR-146a together with Plk2 cDNA containing WT 3' UTR (WT Plk2) or Plk2 cDNA with 3' UTR deletion (Plk2-3'del), a miR-146a-resistant version of Plk2, the overexpression of Plk2-3'del, but not WT Plk2, counteracted miR-146a-induced upregulation of p53, silencing of p53 also blocked the effects of miR-146a on apoptosis (B). Plk2 silencing in young lin⁻ BMCs phenocopies the effects of miR-146a overexpression on the expression of apoptotic factors (C). Western blots are representative of experiments performed at least in triplicate.

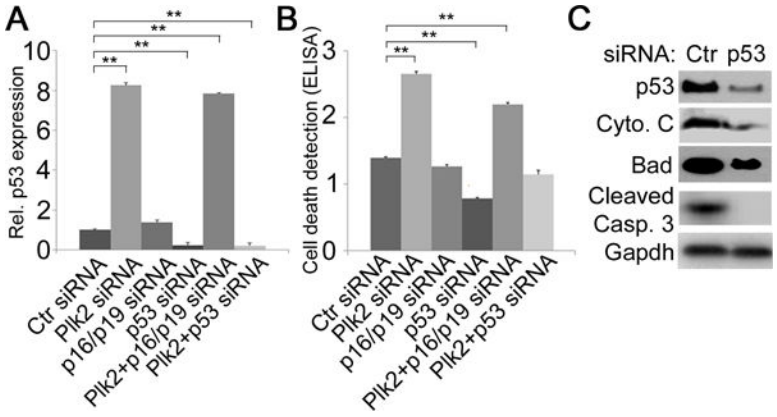

Figure 4. Silencing of Plk2 increases p53 expression levels with resultant apoptosis

SiRNA-mediated silencing of Plk2 leads to enhanced expression of p53 as measured by qRT-PCR (A), whereas p16Ink4a/p19Arf silencing with siRNA (p16/p19 siRNA) has no effects on p53 expression. Notably, when young lin$^-$ BMCs were co-transfected with Plk2 siRNA together with p16/p19 siRNA or siRNA for p53 (p53 siRNA), p53 siRNA treatment, but not p16/p19 siRNA, counteracted the effects of Plk2 siRNA-induced p53 expression (A) and apoptosis measured by cell death ELISA (B). Furthermore, p53 silencing (p53 siRNA) abolished the effects of endogenous miR-146 expression in aged lin$^-$ BMCs, resulting in decreased p53 and cleavage caspase 3 expression (C).

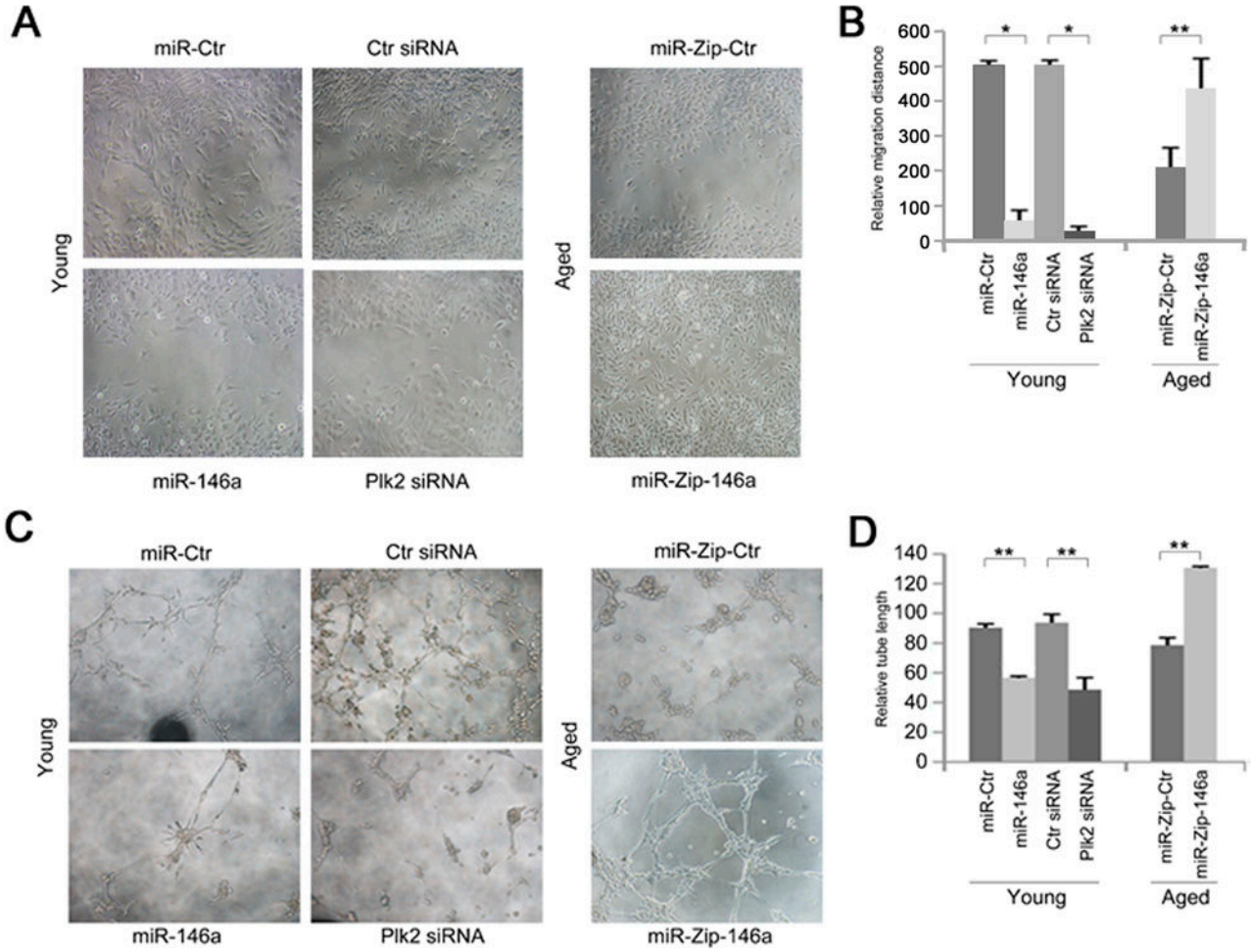

Figure 5. MiR-146a regulates wound healing and angiogenesis *in vitro*

Young wt lin- BMCs were infected with lentivirus encoding miR-Ctr, miR-146a, siRNA Control (Ctr siRNA), or Plk2 siRNA, and aged wt lin$^-$ BMCs were infected with lentivirus encoding miR-Zip-Ctr or miR-Zip-146a. Wound closure was assessed and quantified by the relative migration distance 20 hours after performing a “scratch” in confluent monolayer of cells (n=3; *p<0.01, **p<0.05) (A & B). The effects of miR-146a, Plk2 siRNA on young lin- BMCs and miR-Zip-146a on aged cells to form capillary tubes were determined by Matrigel assay 10 hours after seeding (C). The impaired wound closure and tube formation seen in the miR-146a treated young lin$^-$ BMC was recapitulated by siPlk2 transfection (A & C). The images are representative examples of at least three independent experiments. The relative migration distance of the cells in the wound healing assay and the relative tube length in the tube formation assay (averaged from 5 fields of view) are shown in (C) and (D), respectively. (**p < 0.05 relative to miR-Ctr, miR-Zip-Ctr or Ctr siRNA treatments).

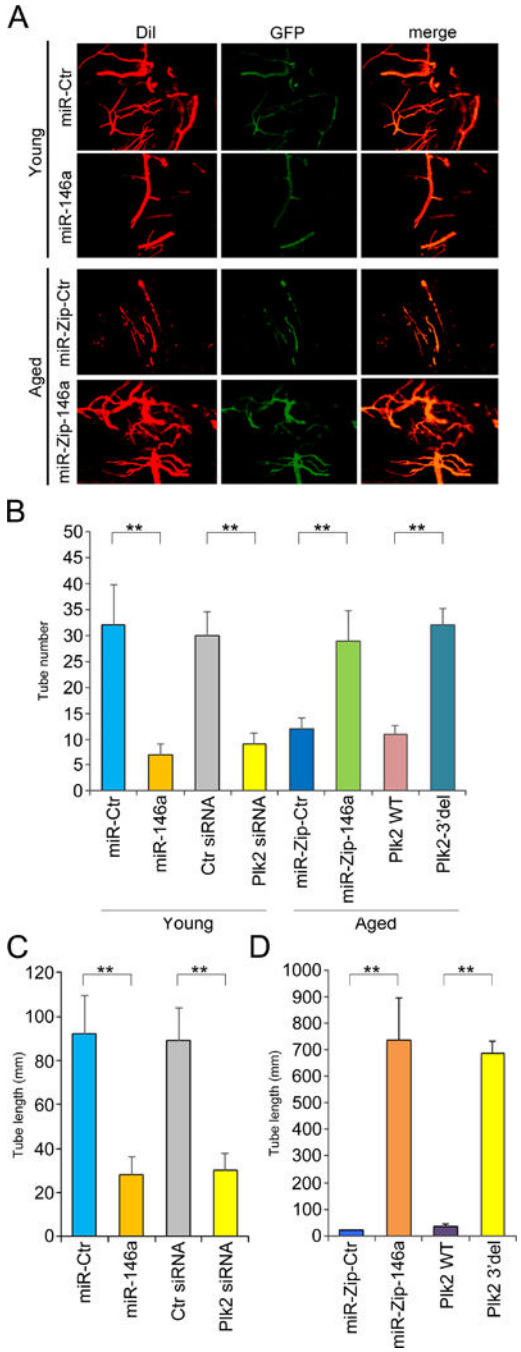

Figure 6. MiR-146a regulates angiogenesis *in vivo*

Matrigel (250 μl per plug) was mixed with 1.3 × 106 young lin⁻ BMCs transduced with lentivirus coding for miR-Ctr or miR-146a or transfected with Ctr siRNA or Plk2 siRNA and was injected subcutaneously into C57BL/6J mice (n = 3). Aged lin⁻ BMCs transduced with lentivirus coding for miR-Zip-Ctr, miR-Zip-146a, wild type Plk-2, or Plk2-3'del were also included. After 11 days, Matrigel plugs were removed and capillary tube formation was detected by confocal microscopy. Representative new vessel formation is shown in (A) with the DiI stained images, GFP from the transduced lin⁻ BMCS and the merges images (x100

magnification). Quantitative analysis of tube formation in Matrigel plugs for the number of tubes formed (B) and the length of tubes in young (C) and aged (D) lin^- BMCs is shown. Data represent means ± SD for the number of tubes and the length of tubes detected in Matrigel plugs for each group. **$p < 0.01$ vs. control group. The capillaries in green and red colors are newly formed vessels. Red fluorescence indicates blood vessels stained by DiL, green fluorescence indicates GFP-positive cells, double-labeling (yellow color) indicates capillaries derived from the injection of GFP-positive, lentivirus transduced lin- BMCs.

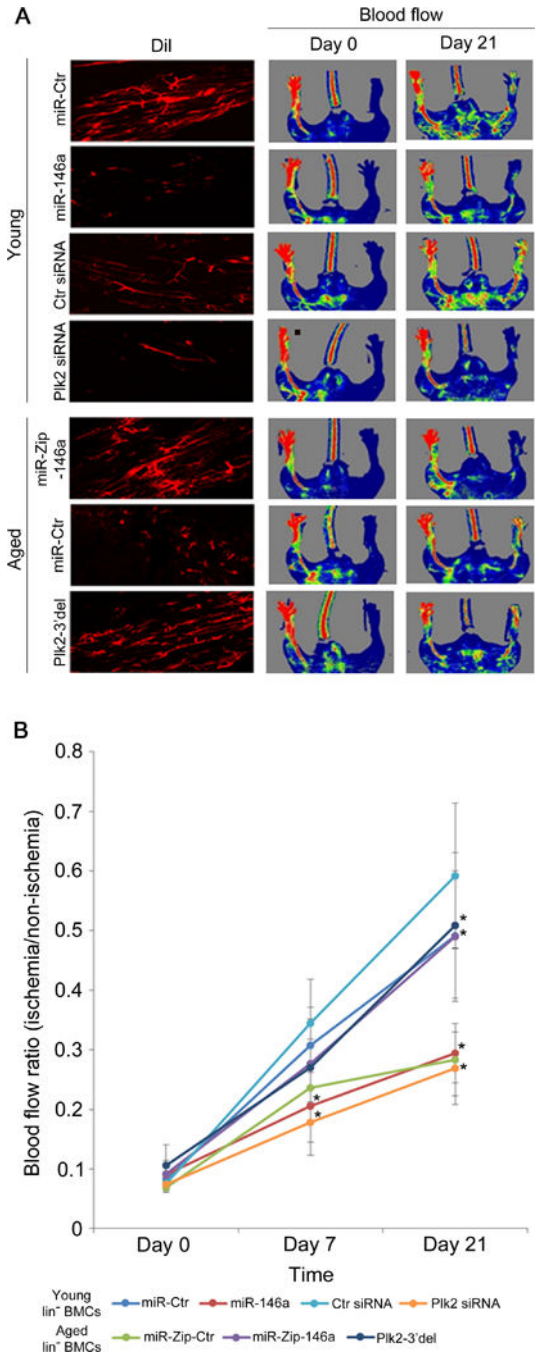

Figure 7. Silencing of miR-146a promotes angiogenesis in a hindlimb ischemia mouse model

MiR-146a overexpression or Plk2 silencing in young lin⁻ BMCs inhibited the neovascularization potential of these cells *in C57BL6/J mice* compared to control treatments as determined by Dil staining of tissue sections (**A**) and Laser Doppler perfusion scan (**A and B**) in the ischemic hindlimbs. In contrast, suppression of miR-146a or overexpression of Plk2-3'del in aged lin⁻ BMCs enhanced neovascularization in the ischemic hindlimbs (**A** and **B**). Representative photomicrographs of neovascularization in ischemic muscle sections for each group were obtained 21 days after femoral artery ligation (**A, left panel**).

Representative Laser Doppler perfusion imaging on day 0 and day 21 (**A, right panel**). B) Quantitation of Laser Doppler perfusion imaging at 0, 7, and 21 days after operation, and recovery of perfusion as expressed by the blood flow ratio (ischemic leg/non-ischemic leg) was measured (**B)**. Data represents average ± SD. (n=4, *p<0.05). The p-values were calculated for each comparison group: miR-Ctr vs MiR-146a in young lin- BMCs; Ctr siRNA vs Plk2 siRNA in young lin- BMCs; miR-Zip-Ctr vs miR-Zip-146a in aged lin- BMCs; miR-Zip-Ctr vs Plk2-3'del in aged lin- BMCs.