MicroRNA-10A* and MicroRNA-21 Modulate Endothelial Progenitor Cell Senescence Via Suppressing High-Mobility Group A2

Shoukang Zhu, Shanming Deng, Qi Ma, Taifang Zhang, Chunling Jia, Degen Zhuo, Falin Yang, Jianqin Wei, Liyong Wang, Derek M. Dykxhoorn, Joshua M. Hare, Pascal J. Goldschmidt-Clermont, Chunming Dong

Rationale: Endothelial progenitor cells (EPCs) contribute to the regeneration of endothelium. Aging-associated senescence results in reduced number and function of EPCs, potentially contributing to increased cardiac risk, reduced angiogenic capacity, and impaired cardiac repair effectiveness. The mechanisms underlying EPC senescence are unknown. Increasing evidence supports the role of microRNAs in regulating cellular senescence.

Objective: We aimed to determine whether microRNAs regulated EPC senescence and, if so, what the underlying mechanisms are.

Methods and Results: To map the microRNA/gene expression signatures of EPC senescence, we performed microRNA profiling and microarray analysis in lineage-negative bone marrow cells from young and aged wild-type and apolipoprotein E−deficient mice. We identified 2 microRNAs, microRNA-10A* (miR-10A*), and miR-21, and their common target gene Hmga2 as critical regulators for EPC senescence. Overexpression of miR-10A* and miR-21 in young EPCs suppressed Hmga2 expression, caused EPC senescence, as evidenced by senescence-associated β-galactosidase upregulation, decreased self-renewal potential, increased p16ink4a/p19arf expression, and resulted in impaired EPC angiogenesis in vitro and in vivo, resembling EPCs derived from aged mice. In contrast, suppression of miR-10A* and miR-21 in aged EPCs increased Hmga2 expression, rejuvenated EPCs, resulting in decreased senescence-associated β-galactosidase expression, increased self-renewal potential, decreased p16ink4a/p19arf expression, and improved EPC angiogenesis in vitro and in vivo. Importantly, these phenotypic changes were rescued by miRNA-resistant Hmga2 cDNA overexpression.

Conclusions: miR-10A* and miR-21 regulate EPC senescence via suppressing Hmga2 expression and modulation of microRNAs may represent a potential therapeutic intervention in improving EPC-mediated angiogenesis and vascular repair. (Circ Res. 2013;112:152-164.)

Key Words: aging | angiogenesis | cellular transplantation | endothelial progenitor cells | high-mobility group A microRNAs | senescence

Postnatal neovascularization involves endothelial progenitor cells (EPCs) derived from the bone marrow. EPCs play important roles in vascular homeostasis and compensatory vasculogenesis with their plasticity to differentiate into endothelial cells (ECs) and as a source of paracrine proangiogenic factors. EPCs are usually defined retrospectively by their ability to differentiate into ECs. Because diverse progenitor cell types have been shown to differentiate into ECs, there is no universally accepted molecular definition for EPCs. Several types of EPCs have been studied: (1) EPCs defined by cell surface markers, including CD34, CD133/CD34, CD133/vascular endothelial growth factor receptor-2 or CD133/CD34/vascular endothelial growth factor receptor-2; (2) early outgrowth EPCs, of myeloid origin, which stimulate angiogenic responses through paracrine secretion of angiogenic factors; and (3) late outgrowth EPCs, of nonhematopoietic origin, possessing the capacity of de novo vessel formation through direct incorporation into the growing vasculature. Regardless of what markers/techniques are used to define EPCs, it is conceivable that these cells are enriched in the lineage-negative bone marrow cells (lin−BMCS). Remarkably, multiple studies have shown that the number and angiogenic function of EPCs...
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MicroRNAs (miRNAs) are a class of small noncoding RNAs of ≈22 nucleotides. During miRNA biogenesis, 1 strand of the duplex, the guide strand, is preferentially incorporated by the argonaute proteins into the RNA-induced silencing complex, promoting degradation or inhibiting translation of transcripts with basepair complementarity.6 In contrast, the partner strand, the guide strand, is preferentially incorporated by the cells.4,5 Little is known, however, regarding the molecular mechanisms governing these senescent changes.

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Recent evidence has shown that miRNAs also may play a role in regulating angiogenesis and endothelial functions.6,12 For example, miR-27b, let-7f, miR-17–92 cluster, and miR-126 play a proangiogenic role, whereas miR-221/miR-222, miR-34, and miR-217 act as antiangiogenic miRNAs in ECs.10,11 miR-21 is dysregulated in many cancers.13 An increased expression of miR-21 in tumor cells is associated with a higher proliferation, invasion capacity, and increased angiogenesis.14 Interestingly, miR-21 in ECs exhibits antiangiogenic functions.15 The expression profile and functions of miRNAs in EPCs remain to be defined.

Hmga2 is a member of the high-mobility group A (Hmga) family that encodes a small, chromatin-associated protein that modifies transcription by altering chromatin structure.16 Recent studies have shown that Hmga2 is preferentially expressed in fetal and young adult neural stem cells (NSCs), but not in aged adult NSCs. Importantly, Hmga2 expression decreases with aging of embryonic stem cells, hematopoietic stem cells, and NSCs, and deletion of Hmga2 impairs NSC function.17,18 Furthermore, Hmga2 promotes NSC self-renewal activity by negatively regulating p16\(^{ink4a}\) and p19\(^{arf}\) expression.19–21 Disrupting the repression of Hmga2 enhances colony formation of NIH3T3 cells.21 Downregulation of Hmga2 by let-7, miR-23a, miR-26a, and miR-30a accelerates cellular senescence of human umbilical cord blood–derived multipotent stem cells.22 The putative role of Hmga2 in regulating EPC senescence and the miRNAs involved remain to be elucidated.

In this report, we show that miR-10A\(^*\) and miR-21 are progressively expressed in lin\(^−\) BMCs, that are enriched for EPCs, during aging. We provide evidence that miR-10A\(^*\) and miR-21 regulate Hmga2 expression. Importantly, the miR-10A\(^*\)/miR-21–Hmga2–P16\(^{ink4a}/\)P19\(^{arf}\) axis controls EPC senescence and angiogenesis.

**Methods**

**Animals**

Apolipoprotein E–deficient (apoE\(^−/−\)) and wild-type (WT) C57BL/6J mice and FVB/N mice were used.

**Isolation and Culture of Lin\(^−\) BMCs**

Mouse lin\(^−\) BMCs were isolated from the WT or apoE\(^−/−\) C57B/6 mice of different ages by magnetic separation using mouse Lineage Cell Depletion Kit.

**RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction**

Detailed Methods are available in the Online Data Supplement material.

**miRNA Microarray Hybridization and Data Analysis**

Low-molecular-weight RNA was sent to Ocean Ridge Biosciences for analysis.

**Hierarchical Clustering of MiRNA Array Data**

Data for the 282 mouse miRNA probes were clustered using Cluster 3.0 software.

**Gene Expression Microarray Hybridization and Data Analysis**

High-molecular-weight RNA fraction from each sample was used for hybridization to the mouse exonic evidence-based oligonucleotide microarrays containing 38 083 70-mer-oligonucleotide probes.

**miRNA and mRNA Inverse Correlation Analysis**

miRNA gene targets were determined using the online miRNA database. The MicroCosm Targets version 5 was used.

**miRNA Transfection**

To study the biological effects of miRNAs on target gene expression, we performed transfection of pre-miR mimic or anti-miR inhibitors.

**Lentivirus Production and EPC Cell Lines**

Lentiviral supernatants were produced using standard procedures.

**Self-Renewal Assay and Cell Proliferation**

Self-renewal potential was examined by colony formation assay.

**Western Blotting Analysis**

Cell extracts were separated by SDS-PAGE in reducing and denaturing conditions.
Scratch Wound Migration Assay
The scratch wound migration assay was used to assess the potential effects of miR-10A*, miR-21, and Hmga2 on EC migration and wound healing.

In Vitro Angiogenesis Assay
The in vitro angiogenic activity of lin− BMCs was determined by Matrigel tube formation assay.

In Vivo Angiogenesis Assay
The Matrigel plug assay and hindlimb ischemic mouse model were performed to study the effects of miR-10A*, miR-21, and their anti-miRs on angiogenesis in vivo.

Senescence-Associated β-Galactosidase Staining and P16ink4a/P19Arf Expression
Lin− BMC senescence was determined by in situ staining for senescence-associated β-galactosidase (SA-β-gal) and by Western blot and fluorescence-activated cell sorter analysis for p16ink4a/p19Arf expression.

Site-Directed Mutagenesis
Point mutations in the miR-10A* and miR-21 binding sites within the Hmga2 3′ untranslated region (UTR) were introduced with the QuikChange II XL site-directed mutagenesis kit (Stratagene).

Luciferase Reporter Assay
The effects of miRNAs on their target mRNA expression were examined by luciferase reporter assay in 293 T cells.

Statistical Analysis
Data were expressed as mean±SD. Student t test and analysis of vari- ants were used to assess differences, with P<0.05 considered to be significant.

Results
miR-10A* and miR-21 Are Upregulated in Aged EPCs
To identify miRNAs involved in EPC senescence, we performed miRNA profiling using the Sanger 13 miRNA. Array targeting 686 mouse miRNAs in lin− BMCs from young (3-week-old apoE−/− and WT) and aged (1-year-old apoE−/− and 2.5-year-old WT) mice, 2 extremes of the aging spectrum. After quality-control, background subtraction, transformation, and normalization of probe intensity, 282 miRNAs were found to be present in at least 1 group of lin− BMCs. Fifty-six miRNAs were differentially expressed in young vs aged cells at a false discovery rate of 0.05. Among them, 38 miRNAs had consistent changes in expression patterns in lin− BMCs from young vs aged apoE−/− and WT mice: 14 were elevated and 24 were downregulated in aged mice. Principal component analysis demonstrated that these differentially expressed miRNAs classified mice well by age regardless of the apoE genotype (Figure 1A), suggesting that the aging process impacts the expression of these miRNAs in lin− BMCs, independent of apoE expression. The top 20 differentially expressed miRNAs in young and aged mice with P<0.001 are listed in Online Table II. Two of the most robustly upregulated miRNAs in aged mice were miR-10A* and miR-21. To validate these findings, we performed reverse-transcription polymerase chain reaction (qRT-PCR) for these 2 miRNAs, which revealed fold changes comparable with those found by miRNA array (Figure 1B).

Hmga2 Is Downregulated in Aged EPCs
Using the same lin− BMC samples, we performed genomewide analysis of miRNA expression using mouse exonic evidence-based oligonucleotide microarray containing 38 467 probes for mouse genes and alternative transcripts. Approximately half of the genes targeted by the mouse exonic evidence-based oligonucleotide array were present in at least 1 group of lin− BMCs. Principal component analysis found 1135 genes to be differentially expressed in these young and aged lin− BMCs, irrespective of apoE expression. Of the 1135 genes that passed the permutation analysis, 67% showed an increased expres- sion in aged mice. Genes with greatest increases were those encoding immunoglobulin and inflammation. Genes that showed decreased expression included insulin-like growth factor 2 mRNA binding protein 3, secreted acidic cysteine rich glycoprotein, and procollagen type Iα2. The top 100 differently expressed miRNAs in young and aged mice with P<0.00001 are listed in Table III. Intriguingly, Hmga2, a gene that regulates stem cell senescence, showed the greatest decrease (86-fold) in aged lin− BMCs. This was further confirmed by qRT-PCR (Figure 1C).

miR-10A* and miR-21 Regulate Hmga2 Expression
To identify those genes whose expression might be regulated by miRNA during EPC aging among the differentially expressed genes, we first performed in silico search for candidate mRNA targets for the identified miRNAs using different miRNA target prediction algorithms such as MicroCosm, TargetScan, PicTar, and EMBL. Seventy-eight of the differentially expressed genes were predicted to be the targets of the 38 candidate miRNAs, and their expression levels also correlated inversely with the miRNA expression levels. Of these, 12 genes, including Hmga2, have been implicated in aging, cell senescence, cell differentiation, and proliferation in other tissues. Hmga2 expression was inversely correlated with the levels of its predicted miRNAs, miR-10A*, and miR-21. Furthermore, computational miRNA target analysis by TargetScan algorithm and RNAHybrid identified 3 and 2 conserved sites in the Hmga2 3′UTR that are complementary to the sequences of miR-10A* and miR-21, respectively, suggesting that Hmga2 may be a shared molecular target for miR-10A* and miR-21 (Online Figure I).

To determine whether miR-10A* and miR-21 directly targeted Hmga2 3′UTR, we constructed luciferase reporter constructs with WT Hmga2 3′UTR (WT3′UTR) and the 3′UTR with point mutations disrupting the 3 binding sites (M3′UTR1, M3′UTR2) for miR-10A* and the 2 binding sites (M3′UTR) for miR-21. In human embryonic kidney cell line 293T cells, cotransfection of miR-10A* or miR-21 and WT3′UTR repressed luciferase activity, whereas cotransfection of an unrelated miRNA had little effect (miR-199b). Cotransfection of miR-10A* or miR-21 and M3′UTR1/M3′UTR2 and M3′UTR, respectively, abolished the repression of luciferase activity (Figure 1D and 1E). These data indicate that miR-10A* and miR-21 directly interact with Hmga2 3′UTR, repressing Hmga2 expression.

To determine whether miR-10A* and miR-21 could negatively regulate endogenous Hmga2 expression in lin− BMCs, cells from young mice (3 weeks old) with a considerable level of Hmga2 expression were infected with lentivirus encoding miR-10A*, miR-21, a combination of the 2, or a scrambled miRNA control.
MiR-10A* and miR-21 repressed Hmga2 mRNA and protein expression as compared with miR-Ctr. The combined overexpression of miR-10A* and miR-21 had greater repressive effects. We also infected young lin− BMCs with lentivirus coding for WT Hmga2 or mutant Hmga2, where the 3 binding sites for miR-10A* and 2 binding sites for miR-21 in the 3′ UTR were mutated in the presence and absence of miR-10A*, miR-21, or miR-Ctr. The repressive effects of miR-10A* and miR-21 on the mutant Hmga2 expression in lin− BMCs were not observed, whereas considerable repression of exogenous WT Hmga2 was detected (Figure 2A–2C). Next, we analyzed lin− BMCs isolated from aged WT mice (2.5 years old) in which miR-10A* and miR-21 levels were high and Hmga2 expression was low. Lentiviral-mediated transduction of anti-miRs for miR-10A* and miR-21 led to a reduction of miR-10A* and miR-21 expression, resulting in an increased Hmga2 expression level (Figure 2D–2F). Collectively, these data indicate that miR-10A* and miR-21 causally regulate Hmga2 expression, particularly in lin− BMCs.

**miR-10A* and miR-21 Regulate EPC Senescence and Self-Renewal Potential**

Because Hmga2 promotes NSC self-renewal and hematopoietic progenitor cell clonal expansion, and because miR-10A* and
miR-21 suppress Hmga2 expression, we reasoned that these 2 miRNAs might impact on the senescence of lin− BMCs via regulating Hmga2 expression. Young lin− BMCs were infected with lentivirus encoding miR-10A*, miR-21, or miR-Ctr alone or in combination with WT Hmga2 3′ untranslated region (UTR) (WT 3′ UTR) or mutant Hmga2 3′ UTR (M3′ UTR). The Hmga2 mRNA and protein expression were detected by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (A) and Western blotting (B) and quantified by densitometry (C) after 48 hours and 72 hours, respectively. Aged WT lin− BMCs were infected with lentivirus encoding anti-miR-10A*, anti-miR-21, and miR-Ctr. The effects of miR-10A* and miR-21 on endogenous Hmga2 mRNA and protein expression were detected by qRT-PCR (D) and Western blotting (E and F). The blots are representatives of 3 independent experiments. Mutant 10A*-2 is mutant Hmga2 3’ UTR disrupting binding for miR-10A* at binding sites 860 and 1548; mutant miR-10A* is mutant Hmga2 3′ UTR for miR-10A* at binding sites 860 and 1504. qRT-PCR and densitometry data are presented as means±SD. *P<0.05, **P<0.01 vs corresponding controls (n≥3).

Figure 2. MicroRNA (miR)-10A* and miR-21 repress endogenous and exogenous Hmga2 expression in lineage-negative bone marrow cells (lin− BMCs). Young wild-type (WT) lin− BMCs were infected with lentivirus encoding miR-10A*, miR-21, or miR-Ctr alone or in combination with WT Hmga2 3′ untranslated region (UTR) (WT 3′ UTR) or mutant Hmga2 3′ UTR (M3′ UTR). The Hmga2 mRNA and protein expression were detected by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (A) and Western blotting (B) and quantified by densitometry (C) after 48 hours and 72 hours, respectively. Aged WT lin− BMCs were infected with lentivirus encoding anti-miR-10A*, anti-miR-21, and miR-Ctr. The effects of miR-10A* and miR-21 on endogenous Hmga2 mRNA and protein expression were detected by qRT-PCR (D) and Western blotting (E and F). The blots are representatives of 3 independent experiments. Mutant 10A*-2 is mutant Hmga2 3′ UTR disrupting binding for miR-10A* at binding sites 860 and 1548; mutant miR-10A* is mutant Hmga2 3′ UTR for miR-10A* at binding sites 860 and 1504. qRT-PCR and densitometry data are presented as means±SD. *P<0.05, **P<0.01 vs corresponding controls (n≥3).

miR-21 suppress Hmga2 expression, we reasoned that these 2 miRNAs might impact on the senescence of lin− BMCs via regulating Hmga2 expression. Young lin− BMCs were infected with lentivirus encoding miR-10A*, miR-21, a combination of the 2, or miR-Ctr. As expected, transduction of miR-10A* and miR-21 resulted in Hmga2 repression and increased expression of senescence-associated β-galactosidase (SA-β-gal) and cell cycle inhibitors p16Ink4a/p19Arf. Furthermore, simultaneous transduction of miR-10A* and miR-21 together resulted in more pronounced lin− BMC senescence (Online Figure III). Remarkably, overexpression of miR-10A* and miR-21 reduced the self-renewal potential of young lin− BMCs compared with miR-Ctr (Figure 3A–3D). In contrast, inhibition of miR-10A* or miR-21 by lentivirus-mediated overexpression of anti-miR-10A* and anti-miR-21 in aged lin− BMCs upregulated Hmga2 expression, decreased SA-β-gal and p16Ink4a/p19Arf expression, and increased self-renewal potential, compared with miR-Ctr (Figure 3A–3D and Online Figure IV). Moreover, anti-miR-10A* and anti-miR-21 resulted in increased proliferating cell nuclear antigen staining and increased cell numbers by 3-[4,5-dimethylthiazol-2 yl]-2,5-diphenyltetrazolium bromide assay relative to control infected cells (Figure 3E and 3F).

miR-10A* and miR-21 Induce EPC Functional Impairment

Cell migration and vascular tube formation are key functions of EPCs and play important roles in the angiogenic process. Lin− BMCs are enriched for EPCs and can differentiate into spindle-shaped cells, which express EC surface markers (Online Figure V). To investigate whether miR-10A* and miR-21 affected lin− BMC functions as a result of senescent changes, we first performed a wound closure assay to evaluate cell migration. Lentivirus-mediated overexpression of miR-10A* or miR-21 reduced the ability of young lin− BMCs to close the scratch to a level equivalent to that of aged cells, whereas control infection did not show any effects (Figure 4A, top panel). Furthermore, inhibition of miR-10A* or miR-21 by anti-miR-10A* and anti-miR-21 resulted in increased ability of aged lin− BMCs to close the scratch to a level comparable with that of young cells (Figure 4A, bottom panel). These effects were further confirmed by the relative migration distances of the infected cells (Figure 4B). Next, we tested whether miR-10A*
or miR-21 overexpression affected angiogenesis in vitro; we performed Matrigel tube formation assay. Young lin− BMCs infected with lentiviral vectors coding for miR-10A* or miR-21, or miR-Ctr were loaded onto Matrigel. miR-10A*-infected and miR-21-infected cells showed a significant impairment of capillary tube formation after10 hours of incubation (Figure 4C and 4D, top panel). Conversely, in aged lin− BMCs, inhibition of miR-10A* and miR-21 rescued tube formation in Matrigel assay (Figure 4C and 4D, bottom panel). Importantly, the miR-10A*-induced and miR-21-induced functional changes were associated with reciprocal changes in Hmga2 and p16Ink4a/p19Arf expression (Figure 5A and Online Figure VI). These data indicate that miR-10A* and miR-21 have opposite functional roles in senescence and the expression of Hmga2 and p16Ink4a/p19Arf, and these changes were associated with reciprocal effects on cell function.

**Hmga2 Mediates the Effects of miR-10A* and miR-21 in lin− BMCs**

Because miR-10A* and miR-21 negatively regulate Hmga2 expression and Hmga2 has been shown to regulate the senescence and functions of NSCs and umbilical cord blood-derived mesenchymal stem cells, we asked whether Hmga2 mediated the effects of miR-10A* and miR-21 on lin− BMC senescence, self-renewal potential, and angiogenic functions. Short hairpin RNA (shRNA)-mediated Hmga2 silencing in young lin− BMCs resulted in decreased Hmga2 expression, increased SA-β-gal and p16Ink4a/p19Arf expression, decreased self-renewal potential and cell proliferation (Figure 5A–5D), to a degree similar to that of miR-10A* and miR-21 overexpression. These cells also showed decreased tube formation (Figure 5E). We then infected aged lin− BMCs with lentiviral vectors coding for Hmga2 open reading frame (ORF) with 3′UTR deletion (Hmga2-3′del), a form of Hmga2 resistant to miRNA repression, or WT Hmga2. Hmga2-3′del transduction resulted in increased Hmga2 expression, decreased SA-β-gal and p16Ink4a/p19Arf expression, increased self-renewal potential and cell proliferation, and increased vascular tube formation (Figure 5F–5H). These effects were similar to that achieved with miR-10A* and miR-21 repression by their anti-miRs. Although WT Hmga2 overexpression transiently improved self-renewal potential...
and cell proliferation (Figure 5F–5G), it had no effects on lin− BMC senescence and vascular tube formation after 72 hours incubation, compared with miR-Ctr, suggesting that WT Hmga2 expression might be inhibited by endogenous miR-10A*, miR-21, and other miRNAs (Figure 5F and 5H). Remarkably, when young lin− BMCs were infected with lentiviral vectors encoding miR-10A* and miR-21, together with Hmga2-3′del, or WT Hmga2, overexpression of Hmga2-3′del, but not WT Hmga2, rescued the effects of miR-10A* and miR-21 in young (G, top) and aged (G, bottom) lin− BMCs to form capillary tubes in Matrigel assay were determined 10 hours after seeding. The images are representatives of at least 3 independent experiments. Relative tube lengths from 5 microscopic fields are shown in (D). **P<0.01 relative to miR-Ctr.

P16Ink4a/p19Arf Mediate the Effects of miR-10A*/miR-21 and Hmga2

We have demonstrated that miR-10A* and miR-21 promote lin− BMC senescence and functional impairment via regulating Hmga2 expression, and that these effects were associated with alterations in p16Ink4a/p19Arf expression. To test whether p16Ink4a and p19Arf mediated the effects of miR-10A*/miR-21 and Hmga2, we infected young lin− BMCs with lentiviral vectors encoding p16Ink4a and p19Arf cDNAs. These cells have low expression levels of endogenous miR-10A*/miR-21 and a considerable amount of Hmga2. The p16Ink4a and p19Arf overexpression increased SA-β-gal expression, decreased self-renewal potential, and decreased vascular tube formation, resembling Hmga2 shRNA-transfected young cells or cells overexpressing miR-10A* and miR-21 (data not shown). We also infected aged lin− BMCs with lentivirus coding for p16Ink4a or p19Arf shRNA. These cells displayed decreased SA-β-gal expression, increased self-renewal potential, and increased vascular tube formation, similar to Hmga2-overexpressing cells or cells in which miR-10A* and miR-21 were repressed (data not shown). Next, we transduced young lin− BMCs with lentiviral vectors coding for miR-10A*, miR-21, or Hmga2 shRNA in the presence and absence of shRNA for p16Ink4a and p19Arf. Remarkably, knockdown of p16Ink4a and p19Arf largely blocked the senescent effects of miR-10A* and miR-21 overexpression or Hmga2 repression (Online Figure VIIA). We then infected aged lin− BMCs with lentiviral vectors encoding anti-miR-10A* and anti-miR-21 and Hmga2-3′del in the presence and absence of p16Ink4a and p19Arf cDNAs. As expected, p16Ink4a and p19Arf overexpression mostly inhibited the effects of miR-10A* and miR-21 repression or Hmga2 overexpression-induced rejuvenation and functional improvement of aged lin− BMCs (Online Figure VIIIB). Taken together, these results indicate that p16Ink4a and p19Arf act in...
tandem with miR-10A*/miR-21 and Hmga2, mediating at least partially their effects on lin− BMC senescence and functions.

**miR-10A*/miR-21 and Hmga2 Regulate Angiogenesis In Vivo**

To determine the role of miR-10A*/miR-21 and Hmga2 in regulating the angiogenic capability of lin− BMCs in vivo, we first performed Matrigel plug assay. We determined the extent of and the time required for neovascularization of Matrigel plugs containing young vs aged lin− BMCs. As shown in Figure 7A, Matrigel containing aged lin− BMCs showed minimal vascularization, and there was no increase in vascularization in the plugs over a 7- to 14-day period. In contrast, plugs containing young lin− BMCs demonstrated a substantial
degree of neovascularization with a maximum increase in the number of vessels and tube length at day 11 after plug injection, as shown by the deep red color. Consistent with our in vitro angiogenesis study, Matrigel containing young lin− BMCs overexpressing miR-10A* and miR-21 or ShRNA-mediated Hmga2 silencing showed a marked reduction in neovascularization, compared with miR-Ctr (Figure 7B).

Quantification of angiogenesis revealed that the angiogenic activity of the miR-10A*, miR-21, and ShRNA-mediated Hmga2 silencing overexpressing young lin− BMCs resembled that of aged lin− BMCs. In contrast, plugs containing aged lin− BMCs overexpressing anti-miR-10A* and anti-miR-21 or Hmga2-3′del displayed significantly improved neovascularization, similar to young cells (Figure 7C–7E).

To validate the role of miR-10A*/miR-21 and Hmga2 in regulating lin− BMC-induced angiogenesis in vivo, we injected genetically modified young and aged lin− BMCs intramuscularly in the hindlimbs of mice whose femoral arteries were ligated on 1 side (hindlimb ischemic model) and measured blood flow by serial laser Doppler perfusion imaging analyses and angiogenesis by immunofluorescence staining. Similar to the in vivo Matrigel plug assay, young lin− BMCs overexpressing miR-10A*, miR-21, or ShRNA-mediated Hmga2 silencing showed a marked reduction in
the ratio of blood flow (ischemic/nonischemic leg) and in the degree of neovascularization compared with miR-Ctr-transduced cells (Figure 8A–8C and Online Figure VIIIB). In contrast, aged lin− BMCs overexpressing anti-miR10A*, anti-miR-21, or Hmga2-3′del significantly improved blood flow and neovessel formation, whereas lin− BMCs overexpressing WT Hmga2 were not effective (data not shown). Remarkably, the combined modulation of miR-10A* and miR-21 was more effective in improving blood flow at 7 days, but not at 21 days postoperatively. Furthermore, the rate of green fluorescent protein+ lin− BMC incorporation (endothelial differentiation) was increased in mice treated with aged lin− BMCs transduced with anti-miR-10A* and anti-miR-21, whereas the endothelial differentiation rate was decreased in mice receiving young cells overexpressing miR-10A* and miR-21, indicating that the effects of miR-21 and miR-10A* on angiogenesis correlate with their effects on endothelial differentiation. Collectively, these findings support the notion that miR-10A* and miR-21 inhibit the angiogenic capability of lin− BMCs via negatively regulating Hmga2 expression.

**Discussion**

As humans age, the functions of multiple systems deteriorate, resulting in the development of degenerative disease, such as atherosclerosis. Adult stem cells fuel the renewal of many tissues. Thus, aging problem may lie, at least in part, within the tissue-specific stem cells.25 Several human studies showed that the proliferation, differentiation, and migration capacity of circulating EPCs were reduced in the elderly.26,27 Aged mice have reduced number of EPCs, and these cells

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**Figure 7.** MicroRNA (miR)-10A* and miR-21 regulate angiogenesis in vivo. Matrigel (250 μL per plug) was mixed with 1.3×10^6 young or aged lineage negative bone marrow cells (lin− BMCs) transduced with lentivirus coding for miR-10A*, miR-21, and ShRNA-mediated Hmga2 silencing (shRNA-Hmga2), or their anti-miRs, and Hmga2 open reading frame (ORF) with 3′ untranslated region (UTR) deletion (Hmga2-3′del) miR-Ctr, respectively, and was injected subcutaneously into C57BL/6J mice (n=3). Noninfected or control infected lin− BMCs from young and aged mice were also included. After 11 days, Matrigel plugs were removed, and capillary tube formation was detected by confocal microscopy. Representative pictures of indicated groups are shown in (A) and (B) (×100 magnification). Quantitative analysis of tube formation in Matrigel plugs is shown in (C–E). Data represent means±SD. *P<0.05 and **P<0.01 vs control group. The capillaries in green and red are newly formed vessels. Red fluorescence indicates blood vessels stained by DiL, green fluorescence indicates green fluorescent protein (GFP)-positive infected cells, double-labeling indicates capillaries derived from the injection of GFP-positive lentiviral transduced lin− BMCs.
have decreased migratory, differentiating, and proliferative activities. Bone marrow-derived EPCs from young apoE−/− mice prevent atherosclerotic lesion formation, whereas progenitor cells from aged atherosclerotic apoE−/− mice do not. In the current study, we present evidence showing that lin− BMCs undergo senescent changes in association with aging. These aged cells display decreased self-renewal potential and reduced proliferative and migratory activity. We also have demonstrated that age-related lin− BMC senescence is associated with decreased angiogenic capability. Because EPCs play an important role in cardiac repair by promoting angiogenesis and improving blood supply, these findings indicate that lin− BMC senescence may not only promote atherogenesis but also diminish cardiac repair by impairing angiogenesis, thus serving as a double-edged sword affecting the development and prognosis of cardiovascular disease.

Increasing evidence supports the role of miRNAs as a common molecular mechanism that underlies cellular senescence in different cell types. MicroRNAs act via inhibiting silent information regulator 1-p53 pathway. Other miRNAs involved in the senescence of different cell types include miR-29, miR-30, and miR-128a. To thoroughly characterize the miRNA changes in the senescence of lin− BMCs, cells enriched for EPCs, we utilized a miRNA microarray platform to profile genomewide miRNA expression changes. To effectively identify miRNA candidates and their downstream pathways relevant to lin− BMC senescence, we also analyzed genomewide gene expression profiles. We performed in silico analysis to search for putative mRNA targets for the candidate miRNAs that showed differential expression changes in young vs aged lin− BMCs. We then overlaid the 3 datasets to identify the miRNA candidates whose expression levels correlated inversely with their predicted target genes. Through these robust and stepwise analyses and extensive experimental approaches, we provide evidence showing that miR-10A* and miR-21 serve as novel miRNAs regulating Hmga2 expression.

**Figure 8. MicroRNA (miR)-21 regulates neovascularization in hindlimb ischemia model.** Reperfusion in the ischemic hindlimb (A and B) and neovascularization (C) were improved when young endothelial progenitor cells (EPCs) infected with lentivirus coding for miR-Ctr or aged EPCs transduced with scrambled anti-miRNA control (anti-miR-Ctr), anti-miR-21 or Hmga2-3′ deletion (Hmga2 3′ del) were injected, as compared with young EPCs infected with lentivirus coding for miR-21 or shRNA for Hmga2 or aged cells transduced with anti-miR-Ctr. Laser Doppler perfusion imaging was obtained at 0, 7, and 21 days after operation, and recovery of perfusion was assessed. **P<0.01 vs miR-Ctr (n=4 for each group).**
in lin− BMCs, where established miRNAs for Hmga2, including the let-7 family, miR-23a, miR-26a, and miR-30a miRNAs, do not show expression changes in association with cell senescence.\textsuperscript{21,22}

Hmga2 is abundantly and ubiquitously expressed and plays a crucial role during embryonic development.\textsuperscript{22} Hmga2 also is highly expressed in fetal NSCs but its expression declines with age. Hmga2 deficiency reduced NSC frequency and self-renewal by increasing the expression of p16\textsuperscript{ink4a}/p19\textsuperscript{arf}, which are cell cycle inhibitors that regulate the pRB and p53 pathways, respectively. Activation of Ink4a/Arf promotes cell cycle arrest that contributes to both organismal aging and tumor suppression. Induction of p16\textsuperscript{ink4a} contributes to the decline of hematopoietic and NSC function in aged animals.\textsuperscript{18,22} In the present study, we have demonstrated that the effects of miR-10A* and miR-21 overexpression on lin− BMC senescence are rescued by the overexpression of mutant Hmga2 with 3′ UTR deletion, but not WT Hmga2. Remarkably, the overexpression of mutant Hmga2 with 3′ UTR deletion, but not WT Hmga2, in aged lin− BMCs rejuvenates the cells, indicating that WT Hmga2 might be subjected to repression by endogenous miR-10A*, miR-21, and perhaps other unidentified miRNAs. Furthermore, p16\textsuperscript{ink4a}/p19\textsuperscript{arf} overexpression rescues the effects of Hmga2-induced lin− BMC rejuvenation. These findings demonstrate that Hmga2 and p16\textsuperscript{ink4a}/p19\textsuperscript{arf} act downstream of miR-10A* and miR-21, regulating lin− BMC senescence. Importantly, using in vivo Matrigel plug assay and the more relevant hindlimb ischemic model, we show that modification of the miR-10A*/miR-21–Hmga2–p16\textsuperscript{ink4a}/p19\textsuperscript{arf} axis improves senescent lin− BMC/ECP-induced angiogenesis, indicating that modulation of this pathway rejuvenates lin− BMCs/ECPs. Remarkably, the combined treatment with miR-10A* and miR-21 antagonists promotes the initiation of angiogenesis but, given enough time, cells treated with each miRNA antagonist alone are able to catch up with the multiple treated cells. Considering that restoration of blood supply in the early stage after myocardial infarct is crucial, these findings may have clinical implications for the justification of multiple miRNA inhibition.

It is important to note that other miR-10A* and miR-21 targets also may have a role in regulating lin− BMC senescence and angiogenesis. For example, it has been recently shown that reactive oxygen species and angiogenic factor RhoB are targets of miR-21.\textsuperscript{15,23} Based on miRNA TargetScan software analysis, other target genes, including Smad7, VEGFC, SOX2, SOX5, KLF2, PTEN, BCL-2, may be involved in mediating the senescence and antiangiogenic effects of miR-21. Similarly, miR-10A* may act via Rgs13, Bmi-1, Myb, Wnt2, RhoB, Smad7, and CDK1 to exert its effects on cell senescence and angiogenesis. KLF2 was significantly upregulated in aged lin− BMCs and was found to be a key factor in regulating EC differentiation, which will be reported in our follow-up study. None of the other putative target genes showed meaningful changes in expression with aging, and thus were not selected for further study. Given that multiple factors are likely to work together to induce lin− BMC senescence and differentiation, it is conceivable that additional miRNAs and target genes are involved and are awaiting further investigation. The less prominent expression changes of miR-10A* and miR-21 relative to that of Hmga2 in young vs aged lin− BMCs underscores the potential involvement of other miRNAs and perhaps other epigenetic mechanisms in regulating their senescence and rejuvenation.

In summary, our data reveal the existence of a novel pathway that regulates lin− BMC senescence; miR-10A* and miR-21 expression increases with aging, resulting in downregulation of Hmga2, which, in turn, activates p16\textsuperscript{ink4a}/p19\textsuperscript{arf} expression, causing decreased self-renewal potential and impaired angiogenic capability. These findings not only may be helpful in developing approaches to rejuvenate lin− BMCs and to enhance angiogenesis for cardiovascular repair, but also may be beneficial in finding new ways to inhibit angiogenesis in the case of cancer treatment.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Endothelial progenitor cells (EPCs) have been suggested to be essential for the formation of new blood vessel formation and vascular repair. EPC number and angiogenic functions decline as a function of aging.
- Treatment with exogenous bone marrow stem/progenitor cells, such as EPCs, can repair injured vessels and induce angiogenesis.
- MicroRNAs (miRNAs) can regulate diverse cellular processes, including cellular senescence and angiogenesis.

**What New Information Does This Article Contribute?**

- Aging is associated with functional impairment and numerical exhaustion of EPCs.
- In comparison with EPCs from young mice, aged mouse EPCs have elevated levels of miRNAs, miR-10A*, and miR-21, leading to the downregulation of Hmga2 expression, a key regulator of stem and progenitor cell self-renewal and functionality, resulting in p16Ink4a-p19Arf-mediated cellular senescence.
- Application of modified EPCs, in which miR-10A* and miR-21 expression is inhibited or Hmga2 is overexpressed, improves new blood vessel formation and blood perfusion in a mouse model of hindlimb ischemia.

EPCs play important roles in maintaining cardiovascular health by promoting vascular regeneration and repair. EPC functionality and number, however, are reduced as a consequence of biological or pathological aging. Therefore, we sought to understand the molecular mechanisms underlying age-associated EPC senescence. Using high-content genomic approaches, we found that the regulatory pathways controlled by miR-10A* and miR-21 are necessary for the maintenance of EPC viability and functionality. We show that overexpression of miR-10A* and miR-21 in EPCs from young animals leads to Hmga2 silencing, which, in turn, promotes the expression of the pro-senescent genes p16Ink4a/p19Arf. The upregulation of p16Ink4a/p19Arf significantly impairs the angiogenic functions of mouse EPCs. In contrast, targeted silencing of miR-10A* and miR-21 in aged EPCs results in upregulation of Hmga2, rejuvenation of EPCs, and improved angiogenic activity. These findings suggest that the miR-10A*/miR-21–Hmga2–p16Ink4a/p19Arf pathway regulates EPC senescence and, therefore, genetic manipulation of this pathway could be a novel therapeutic intervention to improve EPC-mediated angiogenesis and vascular repair.
MicroRNA-10A* and MicroRNA-21 Modulate Endothelial Progenitor Cell Senescence Via Suppressing High-Mobility Group A2
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MicroRNA-10A* and MicroRNA-21 Modulate Endothelial Progenitor Cell Senescence via Suppressing Hmga2

*Shoukang Zhu, MD; Shanming Deng, MD, PhD; Qi Ma, PhD; Taifang Zhang, PhD; Chunling Jia, MD, PhD; Degen Zhuo, PhD; Falin Yang, MD, PhD; Jianqin Wei, MD; Liyong Wang, PhD; Joshua M Hare, MD; Pascal J Goldschmidt-Clermont, MD; *Chunming Dong, MD

METHODS

Animals

C57BL/6J apoE\(^{-}\) and wild type (wt) mice (6–8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, Maine). Pups were weaned at 3 weeks (wks) of age and fed either regular chow or a Western diet #88137 (Harlan-Teklad; 42% fat, 1.25% cholesterol, Teklad, Madison, WI). Animals were housed in sterile microisolator cages in which they received autoclaved food and autoclaved acidified drinking water in a specific pathogen-free facility throughout the study. Aged wt mice (>29 months) were purchased from National Institute on Aging (Bethesda, MD). Mice were euthanized at the age of 3 wks, 6 months, 1.5 years or >2.5 years, respectively, according to the experimental design. At the time of euthanization, bone marrow cells were collected. The University of Miami Institutional Animal Care and Use Committee approved all the procedures.

Isolation and Culture of Lin\(^{-}\) BMCs and CD34\(^{+}\) BMCs
Lin− BMCs were isolated from the wt or ApoE−/− C57BL/6J mice of different ages by magnetic separation using mouse Lineage Cell Depletion Kit (Miltenyi Biotec Inc.) according to the protocol provided by the manufacturer. In brief, whole bone marrow cells are magnetically labeled with a cocktail of biotinylated antibodies against a panel of lineage antigens (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119 antibodies) and anti-biotin microbeads. The magnetically labeled lineage+ cells are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled lineage negative cells (lin− BMCs) pass through the column. For lin− BMC culture, cells were plated onto mouse plasma fibronectin (Sigma-Aldrich) coated 24-well plates at the density of 2 x 10^5/well and maintained in endothelial basal medium-2 (Lonza) supplemented with EGM-2MV single aliquots (Lonza) containing vascular endothelial growth factor (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor, ascorbic acid, hydrocortisone, gentamycin, amphotericin-B, and 20% fetal bovine serum (FBS). Cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere. For CD34+ BMC isolation, lin− BMCs were further labeled with biotinylated CD34+ antibody and anti-biotin microbeads. Labeled cell suspension was subjected to immunomagnetic separation where magnetically labeled cells retain in the column while the unlabeled cells pass through the column. After washing, the column was removed from the magnet and the retained CD34+ cells were eluted. CD34+ cells were cultured in endothelial basal medium supplemented with EGM SingleQuots (Clontech) and 15% FBS.

**Isolation of LMW RNA and HMW RNA and Quantitative RT-PCR**

Both Low-molecular weight RNA (LMW RNA) including miRNA and High-molecular weight RNA (HMW RNA) were isolated from untransfected lin− BMCs and CD34+ BMCs or cells transfected with miR-10A*, miR-21, their anti-miRs or scrambled miRNA mimic or empty lentiviruses with miRNA isolation kit (Qiagen). Taqman microRNA reverse transcription and quantitative RT-PCR was then performed with specific primers and probes supplied in Taqman
MicroRNA Assay kits. Amplification and detection of specific products were performed according to the manufacturer's protocol with the ABI PRISM 7300 system (Applied Biosystems). The U6 small nucleolar RNA was used as the housekeeping small RNA reference gene. The relative microRNA expression was normalized to U6 small nucleolar RNA. Each reaction was performed in duplicate, and analysis was performed by the $2^{-\Delta\Delta C_T}$ method, as described previously. Primer identification numbers (Applied Biosystems) were as follows: has-miR-10A*, 002288; miR-21, 000014; U6, 001973. HMW RNA was also reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Samples were analyzed by TaqMan qRT-PCR for Hmga2, P16$^{\text{Ink4a}}$ and P19$^{\text{Arf}}$ transcripts and normalized to the 18S ribosome housekeeping gene (primer and probe sets from Applied Biosystems). Relative expression was calculated using the comparative cycle threshold method as described for miRNA expression.

**MicroRNA Microarray Hybridization and Data Analysis**

LMW RNA was sent to Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL) for analysis using customized multi-species microarrays containing 676 probes covering 686 mouse mature microRNAs present in miRBase version 13.0. Three hundred nanograms of (LMW) RNA samples were 3’-end labeled with Oyster-550 fluorescent dye using the Flash Taq RNA labeling Kit (Genisphere, Hatfield, PA). Labeled LMW RNA samples were hybridized to the MicroRNA microarrays according to conditions recommended in the Flash Taq RNA labeling Kit manual. The microarrays were scanned on an Axon Genepix 4000B scanner, and data was extracted from images using GenePix V4.1 software. Expression values were normalized by removing system-related variation (sample amount variations, different labeling dyes, and signal gain differences
of scanners) by a locally weighted regression method. Data adjustment included data filtering, log2 transformation, gene centering, and normalization. The data filtering removed miRNAs with normalized intensity values below a threshold value of 11 across all samples. For statistical analysis, samples were binned in four treatment groups: old apoE−/− (AO), young apoE−/− (AY), old wild type (WO), and young wild type (WY). The log2-transformed and normalized spot intensities for the 282 detectable mouse probes were examined for differences between the groups by 1-way ANOVA using National Institute of Ageing (NIA) Array Analysis software. The ANOVA was conducted using the Bayesian Error Model and 20 degrees of freedom. A total of 56 probes showed significant differences with P < 0.01 and FDR < 0.05. The statistical significance was determined using the False Discovery Rate (FDR) method which was proposed by Benjamini and Hochberg. It is the proportion of false positives among all probes with p values lower or equal to the p value of the probes that we consider significant. It can also be viewed as an equivalent of a p-value in experiments with multiple hypotheses testing. Principal Component Analysis was also performed on the 282 detectable probes using the module built in to the NIA software.

**Hierarchical Clustering of miRNA Array Data**

Data for the 282 mouse microRNA probes were clustered using Cluster 3.0 software. Three rounds of gene median centering and gene median normalization were used to pre-process the data. Hierarchical clustering was conducted using Centered Correlation as the similarity metric and Complete Linkage as the clustering method. Intensity scale shown is arbitrary.

**Gene Expression Microarray Hybridization and Data Analysis**
One microgram of the HMW RNA fraction from each sample was used as starting material for hybridization to the mouse exonic evidence-based oligonucleotide (MEEBO) microarrays containing 38,083 70-mer oligonucleotides probes complementary to constitutive exons of most mouse genes, as well as alternatively spliced exons, and control sequences. Spot intensities for each probe were calculated by subtracting median local background from median local foreground for each spot. Spot intensities were transformed by taking the base 2 logarithm of each value. The spot intensities were then normalized by subtracting the 70th percentile of the spot intensities of probes against mouse constitutive exons and adding back a scaling factor (grand mean of 70th percentile). After removing data for low quality spots, control sequences, and non-mouse probes, 35,000 mouse probe intensities remained. The mouse probes intensities were filtered to identify all probes with intensity above a normalized threshold $\log_2 (3 \text{ standard deviation of raw local background}) + \text{mean of log}_2\text{-transformed negative controls}$, to arrive at 17,805 probes complementary to coding mRNA above threshold in all samples from at least one treatment group.

For statistical analysis, samples were binned into four treatment groups (AO, AY, WO, WY). The log2-transformed and normalized spot intensities for the 17,805 detectable probes were examined for differences between the experimental groups by 1-way ANOVA using National Institute on Ageing (NIA) Array Analysis software as described in miRNA data analysis. A total of 1,135 probes showed significant differences with $p<0.05$ and FDR (false discovery rate) also less than 0.05. Principal Component Analysis was performed using the module built into the NIA software. Data for the detectable probes complementary to both coding and non-coding mRNA were clustered using Cluster 3.0 software.
**MiRNA and mRNA Inverse Correlation Analysis**

MicroRNA gene targets were determined using multiple online miRNA databases including the MicroCosm Targets Version 5 for *Mus musculus* (mouse) from European Bioinformatics Institute, (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/download.pl), TargetScan, PicTar, PITA and EMBL. Pearson correlations and associated $p$ values were calculated for all miRNA–mRNA pairs across all samples, and the Benjamini-Hochberg false discovery correction was used to adjust for multiple comparisons. Pairs of miRNA–mRNA with statistically significant inverse correlations were then cross-referenced with two target prediction databases PicTar or The MicroCosm Targets, version 5.

**Luciferase Assay**

Mammalian expression vector contains the *Renilla* luciferase reporter and the wt full-length mouse Hmga2 3′-UTR—“Addgene plasmid 14785” (Luc-wt)—was purchased from Addgene. The mutations in the miR-10A* and miR-21 binding sites within the Hmga2 3′UTR were introduced with the QuikChange II XL site-directed mutagenesis kit (Stratagene), according to the manufacturer’s protocol, and designated as M3’UTR1 and M3’UTR2 for miR-10A* and M3’UTR for miR-21, respectively. Plasmid Luc-wt and mutant Hmga2 3’UTR were further cloned into psiCHECK-2 vector which contains both firefly luciferase and Renilla luciferase reporter genes (Promega) by Choo-Choo Cloning kits (MCLAB). The mutagenesis primers and all other primers are listed in Table I. Sequences of the mutant Hmga2 3′UTR vectors were confirmed by direct sequencing.
The regulatory effects of miRNAs on their target mRNA expression were examined by luciferase reporter assay in HEK293T cells. Briefly, HEK293T cells were cultured in 6-well plates for 24 hours prior to transfection. Cells were co-transfected with 200 ng of Renilla luciferase reporter plasmid containing the wt Hmga2 3’UTR or mutant 3’UTRs and 20 ng of reference firefly reporter plasmids pGL3 (Promega), and synthetic oligonucleotide miR-10A* and miR-21 or their anti-miRs at the concentration of 50 nM with appropriate negative controls (n = 6 per group) using Lipofectamine 2000 reagents, per the recommended conditions (Invitrogen). After 48 hours of transfection, cells were lysed in 75 µl of lysis buffer (Promega) for 30 min at room temperature. Firefly and Renilla luciferase activity was measured using the Promega Dual-glo Luciferase Assay kit following the manufacturer’s instructions with microplate luminometer (FLUOstar Omega). All measurements were normalized with Renilla luciferase activity to correct for variations in transfection efficiencies and for the non-miR-10A*- or non-miR-21-related effects of miRNA transfection on enzymatic activity. Results were confirmed by co-transfection of miR-10A* or miR-21 and psiCHECK-2 containing wt Hmga2 3’UTR or mutant 3’UTRs with appropriate controls.

**Senescence-Associated β-galactosidase Staining**

Lin^-^ BMC senescence was determined by in situ staining for senescence-associated β-galactosidase (SA-β-gal) using senescence cell histochemical staining kit (Sigma). Briefly, Lin^-^ BMCs after transfection were first fixed for 10 min at room temperature in fixation buffer. After washing with PBS, cells were incubated with β-gal staining solution for 12 h at 37°C without CO₂. The reaction was stopped by the addition of PBS. Images of the staining were taken in 5-
10 random microscopic fields per sample at ×200 magnification. Percentages of positive staining and statistical analysis were determined by counting ≥600 cells for each sample.

Flow Cytometry for P16\textsuperscript{Ink4A}/P19\textsuperscript{Arf}

Cells were incubated in control mouse gammaglobulins (20 minutes at 4°C, 2% in PBS; Sigma Aldrich) to block nonspecific Fc receptors. Immunolabeling was performed with allophycocyanin (APC)–conjugated rat anti-mouse p16\textsuperscript{Ink4A} antibody and pacific blue-conjugated rat anti-mouse p19\textsuperscript{Arf} (Biolegend) antibody. Cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

Immunofluorescence Staining

Endothelial progenies were identified by the expression of endothelial specific-surface markers and Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) uptake. Briefly, after washing with PBS, cells were fixed with 4% paraformaldehyde for 10 minutes, blocked for 30 minutes with 2% BSA in PBS and then incubated for 1 hour with primary antibodies against CD31 (1:50), VEGFR2 (1:50), VWF (1:50) or Ulex lectin or VE-Cadherin (CD144) (1:50) (R&D Systems). Secondary antibodies and Hoechst 3342 for DNA staining (Molecular Probes) were used at concentrations of 1:500 to 1:1000 and incubated for 30 minutes. Between each step, cells were washed with PBS in 2% BSA. All procedures were performed at room temperature in dark. For Dil-Ac-LDL uptake, cells were incubated in EGM2 serum-free medium containing 10 µg/ml Dil-Ac-LDL (Molecular Probes) for 4 hours. Cells were then washed and observed by fluorescence microscopy.
**MiRNA Transfection**

To study the biological effects of miRNAs on target gene expression, we performed transfection of pre-miR mimic or anti-miR inhibitors using Lipofectamine 2000 (Invitrogen). Before transfection, lin− BMCs were cultured in EBM-based complete medium and were replated onto six-well plates at a density of $2.5 \times 10^5$ cells/well and incubated overnight. For transient overexpression of miR-10A* and miR-21, cells were transfected with 100 nmol/l of miR-10A*, miR-21 mimic, anti-miR-10A*, anti-miR-21 or scramble miRNA mimic (miR-Ctr), according to the manufacturer's protocol. After 72 h of transfection, cells were harvested for further analysis.

**Lentivirus Production and EPC Cell Lines**

Lentiviral supernatants were produced using standard procedures. Lentivirus transduced EPC cell lines were established by spin-infection followed by FACS sorting or puromycin selection. Lentivirus was generated by co-transfection of GFP-expressing lentiviral vectors coding for the miRNA or gene of interest and other 2 plasmids coding for lentiviral envelope (pCMV-VSV-G) and for packaging (pCMV delta R8.2), respectively, into 293T cells for viral packaging using Lipofectamine 2000 (Invitrogen). The lentivectors encoding miR-10A*, miR-21, miR-control, miR-199b (System Biosystem Inc.), their respective anti-miRs, Hmga2 open reading frame (ORF) with wt 3'UTR (Plasmid 25405: HMGA2+GFP), Hmga2 open reading frame (ORF) with mutant type 3'UTR (mHmga2), Hmga2 open reading frame (ORF) with 3'UTR deletion-a form of Hmga2 resistant to miRNA repression (Plasmid 25406: HMGA2 3'UTR DEL+GFP), shRNA for Hmga2 (plasmid 25408: HMGA2 shRNA) and shRNA for p19 (plasmid 14091: p19Arf.
shRNA -2) were purchased from Addgene. ShRNAs for p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} were purchased from Santa Cruz. Mouse p16\textsuperscript{Ink4a} cDNA clone (IMAGE: 5342027) in a GFP expression vector was purchased from Labome. To obtain viral particles, 293T cells (7 x 10\textsuperscript{6}) were plated in 10 cm dishes and cultured overnight in DMEM medium containing 10% serum. Then, cells were transfected with a mixture of 3 plasmids (for example, 6µg miR-10A*, 4µg pCMVdeta R8.2 and 2µg pCMV-VSV-G per dish). The following day, culture media were collected and replaced with fresh DMEM containing 20% serum. Lentivirus was filtered from the supernatant 48h after transfection, concentrated by Lenti-X Concentrator (Clontech) overnight at 4\textdegree C and centrifuged at 1500g for 30 min to pellet the precipitated viral particles. Viral particles were then concentrated by 10-fold in PBS and stored at -80\textdegree C.

To obtain stable EPC cell lines, lin\textsuperscript{-} BMCs were transduced with lentiviral supernatants containing the above vectors by the centrifugation method (1000g, 1.5h) with addition of 5 µg/ml potamine at room temperature. The supernatant was removed and the pellet was resuspended in culture media and incubated for 36 to 72 hours. The transduction efficiency was monitored by counting the number of GFP-positive cells or by qPCR for the transduced targets. Stably transduced cells were grown in culture under puromycin (5µg/ml) selection or sorted by FACS.

**Self-Renewal Assay and Cell Proliferation**

Self-renewal potential was examined by colony formation assay as described previously \textsuperscript{1,7} with minor modifications. Briefly, equal numbers of freshly isolated lin\textsuperscript{-} BMCs were plated in 24-well plates (Corning) that had been coated with 2 µg/ml fibronectin (Sigma) in M199 medium
containing 1% FBS, 100ng/ml SCF, 20ng/ml IL3, 50ng/ml IL6 and 10ng/ml IGF-1. The next day, cells were infected with lentivirus encoding miR-10A*, miR-21 or their anti-miRs (MOI=5) in the presence of transdux or protamine 500µg/ml and spin at 500g for 2 hours at room temperature. The supernatant was removed, and the cell pellet was resuspended into MethoCult GF M3434 (StemCell Technologies), and colonies were scored after 9–15 days incubation at 37°C and in 5% CO2. Secondary and tertiary colony-forming unit (CFU) assays were performed by isolating individual colonies, washing in PBS/3% FBS, and dissociation by vortexing thoroughly prior to replating in 96-well plates at a clonal density of 300 cells per well with 1% methycellulose containing medium supplemented with the following growth factors (GF) for 9-15 days: 50 ng/ml vascular endothelial growth factor (VEGF), 20 ng/ml stem cell factor (SCF), 50 ng/ml epidermal growth factor (EGF), 20 ng/ml interleukin-3, 50 ng/ml insulin-like growth factor-1 (IGF-1), 50 ng/ml basic fibroblast growth factor (bFGF), 2 U/mL heparin (R and D Systems, MN), and 15% FBS. Colonies were identified by visual inspection with an inverted microscope under ×200 magnification. The mean number or frequency of 20 CFU per well from lin` BMCs were counted.

For proliferation studies, cells were fixed for 10 min at room temperature in 4% paraformaldehyde, blocked, and then stained with anti-PCNA antibody (1:1000, Cell signaling). Cells were counterstained for 10 min at room temperature with 10µg/ml of Hoechst 33258 nuclear staining (Sigma). PCNA-positive and total cells were counted in 10 random microscopic fields per sample at ×100 magnification. Cell viability and proliferation were further analyzed using a methylthiazol tetrazolium (MTT) assay. The colorimetric assay is based on the ability of live cells to reduce the yellow MTT reagent (Promega) to a purple formazan product. Lentiviral
infected cells were seeded in 96 wells for 48 to 96 h. A total of 15 µl of a MTT dye solution was added to each well, and the cells were then incubated at 37°C and in 5% CO2 for 4 h. Then, 100 µl DMSO was added to each well. Cell viability was assessed by measuring the absorbance at 570 nm.

**Western Blotting**

Cells were suspended in ice-cold cell lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (sigma) and incubated for 15 to 30 min on ice. After centrifugation for 10 min at 12,000 g (4°C), the protein concentration of the samples was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (60 µg) were loaded. SDS-PAGE was done in 4%–20% Tris-Glycine Gels (Invitrogen) and transferred to Polyvinylidene Difluoride (PVDF) membranes (Millipore). The membranes were blocked in Tris-buffered saline with 0.05% Tween-20 and 5% milk, incubated with primary and secondary antibodies, and washed according to standard procedures. Primary antibodies were rabbit polyclonal anti-Hmga2, anti-p53 (Cell Signaling), rabbit polyclonal anti-p16\textsuperscript{Ink4a}, anti-p19\textsuperscript{Arf} (Santa Cruz Biotechnology), rabbit polyclonal anti-p21, and mouse monoclonal anti-β-actin. Rabbit polyclonal anti-GAPDH (Sigma) was used as a loading control. For detection, secondary antibodies were horseradish peroxidase-labeled anti-rabbit (1:4,000) or anti-mouse (1:5,000) antibodies. Bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and quantified with Quantity One System (Bio-Rad).

**Scratch Wound Migration Assay**
The scratch wound migration assay was used to assess the potential effects of miR-10A*, miR-21 and Hmga2 on endothelial cell migration and wound healing. Lin- BMCs transduced with miR-10A*, miR-21, their anti-miRs, or scrambled miRNA control were trypsinized 48 h post-transduction. 5 x 10^4 cells were seeded in a 48-well plate in 350 ml EGM-2 medium (Lonza) and incubated for 24 h to reach confluence. Using a 20 µl pipette tip, a wound was made in the monolayer (at time 0). The cells were then washed with PBS and incubated with EBM-2 medium containing 5% FBS, 10 ng/ml recombinant bFGF (Promega), and 50 ng/ml recombinant VEGF for 20 h. Photos of the wound closure were taken. The distance between the two sides of the wound was measured and calculated with ImageJ software. The distance between the two sides of the wound after 20 h of migration was subtracted from the distance at time 0.

**In Vitro Angiogenesis Assay**

The *in vitro* angiogenic activity of lin- BMCs was determined by Matrigel tube formation assay, as described previously\(^1\) with minor modifications. Briefly, lin- BMCs untransduced or transduced with lentivirus encoding miRNAs, their anti-miRs or miR-Ctr were plated at a density of 5 x 10^4 cells/well in 48-well plates precoated with 150µl/well growth factor-reduced Matrigel (BD Biosciences). After 15 h of incubation, tube formation was observed under light microscope (Nikon). Tube formation was defined as a tube-like structure exhibiting a length four times its width. Images of tube morphology were taken in 10 random microscopic fields per sample at ×100 magnification, and the cumulative mean tube lengths per field of view (n = 5 picture/condition) were quantified by ImageJ software 1.45 (NIH).

**In Vivo Mouse Angiogenesis Assay**
The mouse Matrigel plug model was performed to study the effects of miR-10A*, miR-21 and their anti-miRs on angiogenesis in vivo. Briefly, female C57BL/6J mice (6–8 weeks old) were injected subcutaneously into the franks and back with 0.5 ml ice-cold mixture of high protein concentrated growth-factor reduced Matrigel™ and 1.3 \( \times 10^6 \) lin- BMCs (per plug) transduced with lentivirus coding for miR-10A*, miR-21, anti-miR-10A*, anti-miR-21, Hmga2, truncated Hmga2 with 3’UTR deletion or shRNA against Hmga2 (3 plugs/group). The Matrigel™ rapidly formed a single, solid, gel plug. After 11 days, mice were perfused with 5 ml of PBS, followed with Dil solution (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate). Tissues were fixed by subsequent perfusion with 4% paraformaldehyde through the heart. The skin was then pulled back to expose the intact plug, which was dissected out, pressed into 2 glass slides and immersed in PBS. Blood vessels were directly labeled by Dil solution, a lipophilic carbocyanine dye, which incorporates into endothelial cell membranes upon contact by cardiac perfusion. Visualization of blood vessels with red fluorescence can be immediately examined by conventional and confocal fluorescence microscopy. Lentivirus transduced cells containing GFP protein also show green fluorescence, indicating the origin of the new vessel in the plug. Images of blood vessels were taken in 5-10 random microscopic fields per sample at \( \times 100 \) magnification. The number of tubes and the cumulative tube lengths were measured by ImageJ software 1.45 (NIH).

**Hindlimb Ischemia Model and Cell Therapy**

The mouse model of hindlimb ischemia was generated by ligating the proximal femoral arteries and veins of 4 to 5-week-old C57BL6/J or FVB/n mice on the right side. Young and aged lin-
BMCs, infected with lentivirus encoding miR-10A*, miR-21, anti-miR-10A*, anti-miR-21, shRNA against Hmga2, wt Hmga2, truncated Hmga2 with 3’UTR deletion, or miR-Ctr, were injected at 3 different sites (gastrocnemius, gracilis and quadriceps muscles, respectively, 50 µl per injection) of the ischemic leg. To evaluate limb perfusion ratio (ischemic limb (right)/normal limb (left)), laser Doppler perfusion imaging analysis (LDPI, Moor Instruments) was performed at days 0, 7, and 21 post ischemia. After 21 days, mice were perfused with 5 ml of PBS, followed by DiI solution through the heart. Ischemic and non-ischemic muscle tissues were collected, fixed with 4% paraformaldehyde and embedded with OCT medium. Frozen muscle sections of 60 µM were made. Images of blood vessels were taken in 10 random microscopic fields per sample at ×200 magnification. We counted the vessels staining positive for both GFP and for the lipophilic tracer Dil (vessels derived from the cells applied experimentally) and for Dil only (all vessels) in 10 random microscopic fields per group at ×200 magnification. The rate of GFP+ cell incorporation was calculated as the number of GFP and Dil double positive vessels/the number of Dil only positive vessels x 100%.
FIGURE LEGEND

Figure I: *In silico* analysis predicts Hmg2 as a target gene for miR-10A* and miR-21. Predicted pairing of 3’ UTR region of wild type (WT) or mutated (M) Hmg2 with miR-10A* and miR-21 mature sequences indicates that Hmg2 is a shared molecular target for miR-10A* and miR-21. One of the potential miR-21 binding sites is conserved in different species.

Figure II: Differential MiR-10A*, miR-21 and hmg2 expression in young and dge CD34+ BMCs. QRT-PCR validation of miR-10A*, miR-21 (A) and their target gene Hmg2 expression (B) reveals similar reciprocal expression changes in young and aged CD34+ BMCs. Aged wt CD34+ BMCs were infected with lentivirus encoding anti-miRs for miR-10A*, miR-21 or miR-Ctr. Hmg2 expression was detected by qRT-PCR after 48 h (C). Data are presented as fold changes (n = 6).

Figure III: Combined miR-10A* and miR-21 transduction synergistically regulates lin\(^{-}\) BMC senescence. Lin\(^{-}\) BMCs from 3-week-old WT mice were infected with lentivirus encoding mature miR-10A* and miR-21 individually or together as described in Materials and Methods. Simultaneous transduction of miR-10A* and miR-21 synergistically increased SA- β-gal staining as shown in A&B and up-regulated p16\(^{Ink4a}\)/P19\(^{Arf}\) expression as detected by FACS analysis (C&D). * p< 0.05, ** p<0.01 relative to miR-Ctr.
Figure IV: miR-10A* and miR-21 overexpression or inhibition regulate Hmga2 and p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} Expression. Lin\textsuperscript{−} BMCs from 3-week- or 18-month-old WT mice were infected with lentivirus encoding mature miR-10A* and miR-21 sense sequences or their anti-miRs as described in Materials and Methods. The effects of miR-10A* and miR-21 overexpression and inhibition on the protein expression of Hmga2 and p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} were detected by Western blotting as shown in Figure 4A and quantified by relative densitometry as shown here in A-C. Relative densitometry data represent mean ± SD from 3 independent experiments after normalization with GAPDH. *\(p<0.05\), **\(p<0.01\) versus corresponding controls (n ≥ 3).

Figure V: Phenotypic characterization of Lin\textsuperscript{−} BMC progeny cells. Lin\textsuperscript{−} BMCs from 3-week-old WT mice were cultured in endothelial basal medium-2 supplemented with EGM-2MV single aliquots (Lonza) containing vascular endothelial growth factor (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor, ascorbic acid, hydrocortisone, gentamycin, amphotericin-B, and 15% fetal bovine serum. The endothelial identity of the cells derived from lin\textsuperscript{−} BMCs is determined by the expression of endothelial surface markers VEGFR2 and VWF (Red and Green, Top Panel); CD31 and Ulex Lectin (Red and Green, Middle Panel); and Ac-LDL Uptake and CD144 (Green and Red, Bottom Panel). Nuclear staining (Blue) was used as counterstaining. These data confirm results from previous studies, providing evidence for lin\textsuperscript{−} BMCs to differentiate into ECs.

Figure VI: Hmga2 regulates p16\textsuperscript{Ink4a}/p19\textsuperscript{Arf} protein expression. Modification of Hmga2 expression by shRNA-induced knockdown and by WT Hmga2 cDNA or Hmga2-3’UTR deletion
(Hmga2-3’del) transduction regulates p16<sup>Ink4a</sup>/p19<sup>Arf</sup> protein expression. Suppression of Hmga2 resulted in increased p16<sup>Ink4a</sup>/p19<sup>Arf</sup> expression in young cells (A), whereas Hmga2 overexpression by Hmga2-3’del, and to a less degree, by WT Hmga2 cDNA transduction suppress p16<sup>Ink4a</sup>/p19<sup>Arf</sup> expression (B). The protein expression of Hmga2 and p16<sup>Ink4a</sup>/p19<sup>Arf</sup> was detected by Western blotting and presented in Figure 5 and quantified by relative densitometry and presented here. Relative densitometry data represent the mean ± SD from 3 independent experiments after normalization with GAPDH. *p<0.05, **p<0.01 versus corresponding Hmga2-3’del (n ≥ 3).

**Figure VII: Modification of P16<sup>Ink4a</sup> rescues the effects of Hmga2 on lin<sup>−</sup> BMC senescence and functions.** ShRNA-mediated knockdown of p16<sup>Ink4a</sup>/p19<sup>Arf</sup> in young lin<sup>−</sup> BMCs rescued the effects of Hmga2 repression on β-gal expression, self-renewal potential, and tube formation (A), whereas p16<sup>Ink4a</sup> overexpression inhibited Hmga2 overexpression-induced rejuvenation and functional improvement in aged lin<sup>−</sup> BMCs overexpressing miR-10A*- and miR-21-resistant Hmga2-3’del (B). *p< 0.05, **p< 0.01 represent the difference between p16<sup>Ink4a</sup>/p19<sup>Arf</sup> repression or overexpression and control infection. Experiments performed at least in triplicate.

**Figure VIII: MiR-10A* regulates neovascularization in hindlimb ischemia model.** Reperfusion in the ischemic hindlimb was improved, when young EPCs infected with lentivirus coding for miR-Ctr or aged EPCs infected with anti-miR-10A*, and combined anti-miR-10A* and anti-miR-21 were injected, as compared with young EPCs infected with lentivirus coding for miR-10A* or aged EPCs transduced with anti-miR-Ctr. Laser Doppler perfusion imaging was
obtained at 0, 7, and 21 days after operation, and recovery of perfusion was assessed. Representative blood flow images at day 0 and day 21 after operation are shown in A. Quantitative data of blood flow ratio of ischemic over non-ischemic limbs at day 0, 7 and 21 are shown in B; combined miR-10A* and miR-21 transduction was more effective than anti-miR-10A* or anti-miR-21 alone in improving blood flow in the ischemic limbs at day 7, but not day 21. Angiogenesis data obtained at day 21 are presented in C, which did not detect a significant difference between anti-miR-10A* alone and combined anti-miR-10A* and anti-miR-21 transduction. **P<0.01 vs. miR-Ctr (n=4 for each group).

**Figure IX:** The Effects of miR-10A* and miR-21 on Endothelial Differentiate Rate of Lin-BMCs. To determine the effects of miR-10A*/miR-21 and Hmga2 on endothelial differentiation rate of lin^- BMCs, the number of vessels staining positive for both GFP and for the lipophilic tracer Dil (vessels derived from the cells applied experimentally) and for Dil only (all vessels) in 10 random microscopic fields per group at ×200 magnification in the hindlimb ischemic mice was counted. The rate of GFP+ cell incorporation was calculated as the number of GFP and Dil double positive vessels/the number of Dil only positive vessels x 100%. The rate of GFP+ cell incorporation is increased in mice treated with aged lin^- BMCs transduced with anti-miR-10A* and/or anti-miR-21, whereas the GFP+ cell incorporation rate is decreased in mice receiving young cells overexpressing miR-10A* and/or miR-21. *p<0.05, **p<0.01. Data are presented as means ± SE.
**Table I: Primers and probes for PCR and Real time PCR**

**Mutant primers for miR-10A* binding sites:**

mhmg2-m1: 5’-gttttgagtcaagttgcgcgtacgttcttaaggacaagtttttttcagtgac-3’
mhmga2-m2: 5’-atgcttgtggcagcttgcgtgccacattgttacattcagactgaaacagttttgagt-3’
mhmga2-m3: 5’-ctagctgagtaaaatgatgcggttttggcagttaatgaaaggttaacagtaccc-3’
Mhmga2-M4: 5’-ctgggtcggcatctcaagcatacagactttctcgaccaattctctgtactc-3’
mhmga2-m5: 5’-gatttcaagttttggagcacaagaatagttgtcgggtctatgac-3’

**Mutant primers for miR-21 binding sites:**

miR-21-mp1: 5’-caatttctactccgcaacacttgatcttttgaag-3’
miR-21-mp2: 5’-tttttgatctcatacgcaagggtgttg-3’
MiR-21-mp3: 5’-cagcaggagtcatcagcccaacag-3’
miR-21-mp4: 5’-ctgtgtgctcagtagaaggtaatactgaagcc-3’
miR-21-mp5: 5’-gactcctacttctccgacgaagttattcc-3’

**Choo-choo cloning primers:**

hmga2-forward-psi: 5’-gcgatcgcgtcgcgcatctcagcatcagttgg-3’
hmga2-reverse-psi: 5’-cggccagcggccgctagggcacacaaaggaacg-3’

**Other primer and probes:**

mouse-hmg2-F: 5’-tctctcctctctctctctcc-3’
mouse-hmg2-R: 5’-taacacgagctctctcacttg-3’
mouse-hmg2-P: 5’-FAM-tccgtgaagaacagcatc-3’TAMRA
mouse-P19arf-F: 5’-gctctggctttcgtgaacat-3’
mouse-P19arf-R: 5’-gttgccatcatcatcactc-3’
mouse-P19arf-P: 5’-FAM-cttgagaagagggccgcacc-3’TAMRA
mouse-P16ink4a-F: 5’-cgctgggtgctcttgg-3’
mouse-P16ink4a-R: 5’-gctctgtctttgggattggg-3’
mouse-P16ink4a-P: 5’-FAM-cacaagaccacccgag-3’TAMRA
m18s rRNA-F: 5’-cggctaccacatccaaggaa-3’
m18s rRNA-R: 5’-gctggaattaccgcggc-3’
m18s probe: 5’-VIC-tgctggcaccagacttgccctc-TAMRA-3’
Ambion pre-miR miRNA precursor product: has-miR-21 product ID: PM10206
Ambion anti-miR miRNA inhibitor product: has-miR-21 product ID: AM10206
Ambion pre-miR miRNA precursor product: has-miR-10A* product ID: PM12998
Ambion anti-miR miRNA inhibitor product: has-miR-10A*, product ID: AM12998

Table II. Fold changes of normalized, log-transformed expression of top 20 differentially expressed miRNAs in young and aged ApoE<sup>−/−</sup> and WT mice.

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AY: Young ApoE<sup>-/-</sup>; AO: Aged ApoE<sup>-/-</sup>; WY: Young WT and WO: Aged WT.

Table III. Fold changes of normalized, log-transformed expression of the top 100 differentially expressed mRNAs in young and aged ApoE<sup>-/-</sup> and WT mice.
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AY: Young ApoE-/-; AO: Aged ApoE-/-; WY: Young WT and WO: Aged WT.
REFERENCES


Figure I

miR-10A* 3'AUAAGGGGAUCUAUGCUUAAAC 5'
mouse Hmga2 WT 3'UTR
860:5'AUCCUGUUUUGGAAUUUA 3'
1504: 5'UUUCCUGCUAGAUUGUUACAUUUAAUUUGA 3'
1548: 5'GCUCUAGGGGCUUAAAGGAAUUUU 3'
mouse Hmga2 M 3'UTR
860:5'AUGCAAGUUUGGUAUUAUUA 3'
1504: 5'UUUGCAAGCUAGAUUGUUACAUUUAAUUGA 3'
1548: 5'GCACAUACGUUCUUAAGGAGUAAUUU 3'

Seed sequence

miR-21
Human 3' GUUGUAGUCAGACUAUUCGAU 5'
Chimpanzee 5' AUAGUUUGUUGAUCAUUGAAGGAAGAGU 3'
mouse (WT 3'UTR)-1 5' AUAGUUUGUUGAUCAUUGAAGGAAGAGU 3'
mouse (M 3'UTR) 5' AUAGUUUGUUGAUCAUUGAAGGAAGAGU 3'

miR-21
mouse (WT 3'UTR)-2 3'GUUGUAGUCAGACUAUUCGAU 5'
mouse (M 3'UTR) 5'ACAGCAGCAGGAGUAAAGCC 3'
5'ACAGCAGCAGGAGUAAAGCC
Figure II

A

Relative Expression in CD34+ BM Cells

miR-10A*  miR-21

WO  WY

B

Relative Hmga2 Expression in CD34+ BM Cells

WO  WY

C

Relative Hmga2 Expression in CD34+ BM Cells

Ctrl  10A*  21

Anti-miRs
Figure III

A

SA-β-gal Staining

B

% SA-β-gal Staining

C

P19Arf

D

% of P16\textsuperscript{ink4A} Expression
Figure IV

A. Relative densitometry of Hmgb2
   - miRs in Young Cells
   - Anti-miRs in Aged Cells

B. Relative densitometry of P16
   - miRs in Young Cells
   - Anti-miRs in Aged Cells

C. Relative densitometry of P19
   - miRs in Young Cells
   - Anti-miRs in Aged Cells

Legend:
- Ctr
- 10A*
- 21

Statistical significance:
- * p < 0.05
- ** p < 0.01
Figure V

- VEGF2R
- VWF
- nuclear merge

- CD31
- Ulex lectin
- nuclear merge

- Ac-LDL uptake
- CD144
- nuclear merge
Figure VII

A

B

% of SA-β-gal staining and # of Colony and Tubes

β-gal
Colonies
# of Tubes

ShRNA-Hmgα2
Hmgα2-3′del
ShRNA-P16
P16

**

**

**

**
Figure VIII

A

Blood Flow

Day 0
Day 21

miR-Ctr
miR-10A#
Anti-miR-Ctr
Anti-miR-10A#

B

Blood Flow Ratio (ischemic/normal leg)

Days

0
7
21

NS

C

New Vessel Formation

Dil
GFP
Overlay

Young
miR-Ctr
anti-miR-Ctr

Aged
anti-miR-Ctr
Figure IX

The bar chart shows the percentage of GFP positive vessel formation across different treatments. The treatments include:

- miR-Ctr
- miR-10A*
- miR-21
- ShRNA-Hmga2
- anti-miR-Ctr
- anti-miR-10A*
- anti-miR-21
- anti-miR-10A*/21
- Hmga2-3'del

Significance levels are indicated with asterisks: * indicates p < 0.05, ** indicates p < 0.01.