Estrogen Receptor–Mediated Regulation of MicroRNA Inhibits Proliferation of Vascular Smooth Muscle Cells

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Objective—Estradiol (E2) regulates gene transcription by activating estrogen receptor-α and estrogen receptor-β. Many of the genes regulated by E2 via estrogen receptors are repressed, yet the molecular mechanisms that mediate E2-induced gene repression are currently unknown. We hypothesized that E2, acting through estrogen receptors, regulates expression of microRNAs (miRs) leading to repression of expression of specific target genes.

Methods and Results—Here, we report that E2 significantly upregulates the expression of 26 miRs and downregulates the expression of 6 miRs in mouse aorta. E2-mediated upregulation of one of these miRs, miR-203, was chosen for further study. In cultured vascular smooth muscle cells (VSMC), E2-mediated upregulation of miR-203 is mediated by estrogen receptor-α (but not estrogen receptor-β) via transcriptional upregulation of the primary miR. We demonstrate that the transcription factors Zeb-1 and AP-1 play critical roles in mediating E2-induced upregulation of miR-203 transcription. We show further that miR-203 mediates E2-induced repression of Abl1, and p63 protein abundance in VSMC. Finally, knocking-down miR-203 abolishes E2-mediated inhibition of VSMC proliferation, and overexpression of miR-203 inhibits cultured VSMC proliferation, but not vascular endothelial cell proliferation.

Conclusion—Our findings demonstrate that E2 regulates expression of miRs in the vasculature and support the estrogen receptors-dependent induction of miRs as a mechanism for E2-mediated gene repression. Furthermore, our findings demonstrate that miR-203 contributes to E2-induced inhibition of VSMC proliferation and highlight the potential of miR-203 as a therapeutic agent in the treatment of proliferative cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2013;33:257-265.)

Key Words: estrogen ■ gene regulation ■ microRNA ■ muscle, smooth ■ proliferation

Estrogen regulates diverse biological processes involving the reproductive, skeletal, neurologic, and cardiovascular systems via activation of the 2 known estrogen receptors, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ).1,2 Estrogen has important effects on vascular physiology and pathophysiology, with potential therapeutic implications. ERα and ERβ are both expressed in vascular smooth muscle cells (VSMC) and vascular endothelial cells (VEC), identifying these cells as direct targets of E2 action.3-9 Estrogen inhibits VSMC proliferation and migration, in vivo and in vitro.8,10-13 In contrast, estrogen increases VEC proliferation and migration and induces nitric oxide production.14,15 Estrogen receptors (ERs) are ligand-activated transcription factors, and as such, they regulate gene expression in response to estrogen. The molecular mechanisms by which ERs upregulate gene expression are now understood in some detail.5,16-17 However, we recently demonstrated that many ER-regulated genes in the mouse aorta are downregulated, rather than upregulated.18,19 Similar findings have been demonstrated in other, nonvascular cells as well.20 The molecular mechanisms by which ERs repress gene expression are currently unknown.

MicroRNAs (miRs) are small noncoding RNAs that repress gene expression at the posttranscriptional level.21,22 Recent studies have shown that miRs are involved in the regulation of a variety of biologic processes, including development, signal transduction, apoptosis, cell proliferation, and tumorigenesis. Although miRs are postulated to participate in estrogen regulation of gene expression in other systems, this has not been examined in vascular cells or tissues. Further, although estrogen has recently been shown to alter expression of specific miRs in nonvascular cells, the molecular mechanisms by which estrogen regulates these miRs have not been explored, nor have any physiologic consequences of these changes been shown.23-28

In this report, we demonstrate that ex vivo estrogen treatment of mouse aorta regulates miR expression. Among the estrogen upregulated miRs, miR-203 has been reported to inhibit cell proliferation in a variety of malignant tumor cells.29-32 We then focused our study on estrogen regulation of miR-203 in cultured VSMC and elucidate some of the molecular mechanisms by which E2 regulates miR-203. We demonstrate further that miR-203 contributes to estrogen-mediated inhibition of VSMC proliferation, and that miR-203 can, by itself, specifically inhibit VSMC proliferation.

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Materials and Methods

Mice and Tissue Collection

Aortas harvested from 10-week-old wild-type C57BL/6 mice, 7 days after ovariectomy, were treated ex vivo with 10 nmol/L 17β-estradiol (E2) or vehicle for 4 hours, as previously described. Three aortas were pooled for each assay. For each treatment group, miR arrays and real-time (RT)-PCR were performed in duplicate and quadruplicate, respectively, as described above. The Animal Care and Use Committee at Tufts Medical Center approved all animal procedures.

Plasmid Constructions

Plasmids for promoter activity assays were cloned into pGL3-basic vector (Promega). The region 1.1 kb upstream to the mmu-miR-203 gene was generated by PCR using primers 5'-GCTAGCCGTGGAACCTCTTCGAAG-3' (NheI) and 5'-AAGCCTGCTGCACACAGAAGTCTG-3' (HindIII), with genomic DNA template. The PCR fragments were ligated into Zero Blunt TOPO vector (Invitrogen), and subcloned into pGL3-miR-pro1, a 1.9-kb upstream region of mmu-miR-203 gene, amplified by PCR using primers 5'-GAGAATTTACACCCT-3' (NheI/HindIII sites), creating construct pGL3-miR-203-pro1. To generate pGL3-miR-pro1, a 1.9-kb upstream region of mmu-miR-203 gene was amplified by PCR using primers 5'-GCTAGCCGTGGAACCTCTTCGAAG-3' (NheI) and 5'-GAGAATTTACACCCT-3'. Constructs for deletion analysis were generated by PCR reaction with appropriate primers. Plasmids bearing specific mutations were generated from miR-203pro1 construct using Quick Change Site-Directed mutagenesis approach (Stratagene).

Cell Culture

Mouse Aortic Smooth Muscle Cell (MAoSMC) Harvest and Culture

MAoSMCs were obtained using the explant procedure, as previously published. Briefly, several mouse aortas or carotid arteries were obtained sterilely and placed into a 100-mm dish containing media. The adventitia was cleaned off, and the aorta was cut horizontally into 10 to 15 pieces. Each piece was placed into a 6- or 12-well collagen Bioclot plate (Fisher). Explants were cultured in Dulbecco’s modified Eagle’s medium containing antibiotics and 10% bovine serum for 3 to 7 days. When the well was 50% to 75% confluent, the explants were removed, and the smooth muscle cells were cultured in low-glucose, phenol red-free Dulbecco’s modified Eagle’s medium containing antibiotics and 10% fetal bovine serum. Before experimental use, smooth muscle cells were cultured in low-glucose Dulbecco’s modified Eagle’s medium supplemented with 5% dextan-coated, charcoal-treated fetal bovine serum.

Human Aortic Smooth Muscle Cell (HAoSMC) Harvest, Culture, and Immortalization

HAoSMC were derived by the explant method from discarded aortic tissue from Tufts Medical Center operating room, immortalized at passage 2 with retrovirus E6/E7 ELXN, and selected with G418 (300 μg/mL). The cells were cultured in low-glucose, phenol red-free Dulbecco’s modified Eagle’s medium containing antibiotics and 10% fetal bovine serum.

Cell Transfection

Plasmid transfection was performed with Lipofectamine 2000 (Life Technologies), according to the manufacturer’s protocol. Transfection with anti-miR (Life Technologies) and miR mimics (Life Technologies) was performed using RNAiMAX (Invitrogen), according to the supplier’s instructions.

Antibodies

The following antibodies were used: ERα (Santa Cruz Biotechnology, sc-7207), ERβ (Affinity BioReagents, PA1-310B), α-smooth muscle actin (Sigma-Aldrich, A2547), Flag M2 (Sigma-Aldrich, F1804), c-Abl (Calbiochem, OP20), p63 (Thermo Scientific, MS-1084), Zeb-1 (Santa Cruz Biotechnology, sc-10573), and β-actin (Santa Cruz Biotechnology, sc-6161) for Western blot; ERα (Santa Cruz Biotechnology, sc-542) for chromatin immunoprecipitation (ChIP); and Zeb-1 (Santa Cruz Biotechnology, sc-25388) for Western blot, immunoprecipitation, and ChIP.

Array-Based miR Profiling

Aortas were treated ex vivo with 10 nmol/L E2 or vehicle for 4 hours. Three aortas were pooled, and total RNA was isolated using the miR-Neasy kit (Qiagen). MiRs expression profiling was performed utilizing the TaqMan Rodent MicroRNA Array Set (Applied Biosystems). This array contains 517 mouse miRs, as well as endogenous controls. Normalization was performed with small nuclear RNA-202. For each treatment group, miR arrays were performed in duplicate.

Quantitative RT-PCR

Quantitative RT-PCR confirmation of miR array results was performed in quadruplicate by using RNA isolated from independently obtained aortas, treated as described above. MAoSMC were infected with adenovirus expressing human ERα (Ad-ERα), adenovirus expressing human ERβ (Ad-ERβ), or adenovirus expressing green fluorescent protein (Ad-GFP) and treated with 10 nmol/L E2 for 8 or 24 hours. Total RNA was extracted from aorta or MAoSMCs using the miRNeasy kit (Qiagen). Mature miR and primary miR-203 expression were measured using Taqman MicroRNA Assay and TaqMan Pri-miRNA Assay, respectively (Applied Biosystems). Quantitative RT-PCR analysis was performed using the Mastercycler ep realplex (Eppendorf).

Luciferase Assays

Firefly luciferase assays were carried out as follows: 20 μL cell lysate was added to 100 μL of firefly luciferase assay buffer (Promega). The samples were placed in a luminometer (Luminoscan Ascent, Labsystems) and light output was determined over a 10-second interval. Activity of β-galactosidase was measured with a Tropix Galacto-Light Plus kit (Applied biosystems), according to the manufacturer’s instructions. Firefly luciferase activity was normalized to β-galactosidase activity.

Cell Proliferation Assay

MAoSMC, HAoSMC, and human umbilical vein endothelial cells were transiently transfected with miR-203 precursor molecule (pre-miR-203, Ambion), or pre-miR precursor’s negative control no.1 (negative control, Ambion) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instructions. The final concentration of pre-miR was 50 nmol/L. Cells were seeded in 96-well plates. Cell proliferation was measured at 0, 2, 3, and 4 days postseeding using Cell TiterGlo Luminescence Cell Viability assay (Promega), according to the manufacturer’s protocol. Each assay was performed in triplicate and repeated at least 3 times independently. To study the effects of E2 on MAoSMC in which miR-203 is depleted, the Ad-ERα-infected MAoSMC were transfected with anti-miR-203 or anti-miR control (Ambion) and seeded into 96-well plates. E2 treatment was initiated 6 hours after transfection. Cell proliferation was measured at 0, 2, 3, and 4 days postseeding.

ChIP Assay

ChIP assays were performed using the protocol for ChIP Assay Kit (Millipore). Immunoprecipitates were dissolved in 50 μL of distilled water. Two microliter of the DNA was used for a 20 μL PCR reaction using the QuantiTect SYBR Green PCR Kits (Qiagen). Primers sequence for amplification of miR-203 promoter region are as follows: ERBS-Rev, 5'-TGATTCATCCCTTCTTCCTTCC-3'; ERBS-Rev, 5'-GCGCCCTGCCCTCTTAGTCACC-3'; E-Box1-For, 5'-TTCCAATCGCTCTCCTTTTCC-3'; E-Box1-Rev, 5'-GCGCCCTGCCCTCTTAGTCACC-3'; E-Box3-For, 5'-TGATTCATCCCTTCTTCCTTCC-3'; E-Box3-Rev, 5'-GCGCCCTGCCCTCTTAGTCACC-3'; E-Box5-For, 5'-TTCCAATCGCTCTCCTTTTCC-3'; E-Box5-Rev, 5'-GCGCCCTGCCCTCTTAGTCACC-3'; E-Box7-For, 5'-TTCCAATCGCTCTCCTTTTCC-3'; E-Box7-Rev, 5'-GCGCCCTGCCCTCTTAGTCACC-3'; E-Box9-For, 5'-TTCCAATCGCTCTCCTTTTCC-3'; E-Box9-Rev, 5'-GCGCCCTGCCCTCTTAGTCACC-3'.
Co-Immunoprecipitation

MAoSMCs were infected with Ad-ERα and treated with E2. At 24 hours posttreatment, cells were washed once with cold PBS and lysed with cold radioimmunoprecipitation assay buffer at 4°C for 30 minutes. The lysates were incubated with anti-zeb1 antibody (sc-25388, Santa Cruz) overnight at 4°C, followed by incubation with protein G-coated agarose beads (Millipore) for another 1 hour at 4°C. Afterwards, the samples were washed 5 times with ice-cold radioimmunoprecipitation assay buffer and supernatants were removed by centrifugation at 2000g for 1 minute. The proteins were then separated from beads using immunoblot loading buffer for 5 minutes at 95°C. The supernatants were collected for immunoblotting.

Apoptosis Assay

Apoptotic cells were measured by TUNEL assay (In Situ Cell Death Detection Kit, Roche), according to the manufacturer’s instructions.

Bioinformatic Analysis

ER-Binding Elements Analysis

ER-binding Elements Analysis was carried out using the CREAD suite of programs (STORM and MULTISTORM) that allows for the prediction of transcription factor binding sites (TFBS), and their conservation among various species. Mouse Genome Assembly mm9 (July 2007, NCBI Build 37) was used for all the calculations.

TFBS Analysis

TFBS analysis was carried out using the program STORM in the CREAD suite of programs, and TRANSFAC matrices for TFBS in the 91-bp sequence of interest. Only those matches that had a functional depth of at least 0.9 were considered as significant. The functional depth is defined as $F = (S - S_{\text{min}})/(S_{\text{max}} - S_{\text{min}})$, where $S$ is the score of occurrence for a motif in a sequence, and $S_{\text{min}}$ and $S_{\text{max}}$ are the minimum and maximum that a motif match can score. A functional depth of 1.0 signifies the best match of a motif to the sequence.

Statistical Analysis

Between-group differences were tested using the Student $t$ test. All data are shown as mean±standard deviations. A $P$ value of <0.05 was considered statistically significant. Significantly differentially expressed miR from TaqMan miR array results was defined as a mean fold change ≥1.4.

Results

E2 Regulates miRs Expression Profiles in Mouse Aorta

MiR expression profile analysis revealed that 318 of 517 miRs were detected at a significant level in vehicle-treated mouse aorta. Thirty-two miRs were significantly regulated by 4 hours of ex vivo E2 treatment, including 26 upregulated and 6 downregulated miRs (Figure 1A). Using independently obtained RNA samples, RT-PCR confirmed E2-mediated regulation of 6 of the 10 miRs chosen for testing, including E2-mediated upregulation of 5 miRs (miR-128a, miR-182, miR-200b, miR-203, and miR-205) and downregulation of 1 miR (miR-208), compared with vehicle-treated aorta (Figure 1B).

ERα Mediates E2 Regulation of miR-203 Expression in VSMC at the Transcriptional Level

Given prior reports demonstrating that miR-203 inhibits proliferation in a variety of malignant tumor cells, we hypothesized that E2-mediated upregulation of miR-203 contributes to the inhibitory effects of E2 on VSMC proliferation, and therefore...
focused the remainder of our studies on E2-mediated regulation of miR-203 in VSMC. To investigate whether E2 can regulate miR-203 expression in cultured vascular cells, we performed RT-PCR for miR-203 in MAoSMC. To allow us to determine which ER subtype mediates specific effects of E2, we used passaged MAoSMC that express low levels of ERs (Figure 2C) and infected them with Ad-ER\textsubscript{α} or Ad-ER\textsubscript{β}, as specified below. After 8 hours of treatment, E2 significantly upregulated miR-203 expression in MAoSMC infected with Ad-ER\textsubscript{α} (Figure 2A), but not Ad-ER\textsubscript{β} (Figure 2B), despite similar levels of overexpression of each receptor (Figure 2C), and ER\textsubscript{α}-mediated upregulation was blocked by the ER antagonist ICI 182780 (Figure 2D). Similar results were observed after 24 and 48 hours of E2 treatment (data not shown).

To begin to study the molecular mechanisms by which E2 regulates miR-203 biogenesis, we examined the effects of E2 on primary miR-203, the precursor to mature miR-203. E2 significantly increased the abundance of primary miR-203 in MAoSMC (Figure 3A, left panel) and in aorta (Figure 3A, right panel), suggesting that E2 regulation of miR-203 expression occurs at the transcriptional level.

**Identification of the Promoter Region for miR-203**

To confirm transcriptional regulation of miR-203 by E2, and to identify important transcriptional regulatory control elements for miR-203, we analyzed the 5-kb upstream region of mouse miR-203 gene using the CpG Island track, conservation track, and ORegAnno track (open, regulatory annotation database) in the UCSC genome browser and identified several potentially interesting regulatory elements, including the following: (1) a highly conserved CpG island (−474 to +154), with 52% identical sequence between human and mouse; (2)
Characterization of the miR-203 Gene Promoter

To characterize the miR-203 gene promoter, 2 DNA regions, which we named miR-203-pro-1 (a 2.6-kb upstream fragment) and miR-203-pro-2 (a 1.1-kb upstream fragment), were fused to a firefly luciferase reporter gene and transfected into E2- or vehicle-treated MAoSMC (Figure 3C). The reporter activity of both promoter constructs was increased similarly, ~3-fold by E2 treatment, in Ad-ERα–infected cells, supporting that the predicted ERE is not required for E2-mediated induction of miR-203.

To determine whether E2 regulation of miR-203 transcription is mediated by the known ERα-binding site in the promoter region, deletion analyses were performed (Figure 3D). Several truncated reporter constructs, named Truncated pro 1 to 4, were transfected into E2- or vehicle-treated mouse carotid artery smooth muscle cells. The ERα-binding site alone was insufficient to mediate the induction of E2 miR-203 expression (Truncated pro 1), and removal of the ERα-binding site resulted in loss of E2 induction (Truncated pro 2). These findings suggest that the known ERα-binding site has no promoter activity by itself, but is required for the effect of E2 on miR-203 expression. With Truncated pro 3 and 4, E2 still significantly increased reporter activity. Comparison of results with Truncated pro 2 and 4 localized the minimal region required for E2-mediated regulation to a 91-bp DNA sequence located at –752 to –662 in the miR-203 promoter.

AP-1 Is Required for E2 Regulation of miR-203 Expression

To identify other cofactors for E2 regulation of miR-203 transcription, we performed a TFBS analysis of the 91-bp DNA sequence required for E2 induction of miR-203 promoter. Computational analysis revealed potential binding sites for AP-1, a previously identified ERα-binding protein. We examined the effect of E2 on miR-203 promoter reporters bearing a mutated AP-1–binding site. Mutation of the AP-1–binding site suppressed E2 induction of reporter activity (Figure 4). These findings suggest that the AP-1–binding site is required for E2 regulation of miR-203 expression.

Zeb-1 Is Involved in E2 Regulation of miR-203 Expression

A previous study showed that the transcription factor Zeb-1 inhibits transcription of the human miR-203 gene by binding to conserved E-Boxes 1 and 3 in the promoter region of miR-203. To investigate whether Zeb-1 is involved in E2 regulation of miR-203 in VSMC, we mutated the 2 conserved E-Boxes 1 and 3 in the mouse miR-203 promoter in a luciferase-driven reporter plasmid, and transfected it into vehicle- or E2-treated mouse carotid artery smooth muscle cells. Mutations in either E-Box or in both E-Boxes significantly increased E2 activation of the miR-203 promoter reporter construct (Figure 5A). These results support that Zeb-1 represses E2 upregulation of miR-203 expression by binding to the 2 conserved E-Boxes.

To confirm that Zeb-1 inhibits E2-mediated induction of miR-203 expression, we knocked down Zeb-1 in Ad-ERα–infected MAoSMC and quantified the effect of E2 treatment on miR-203 expression. The RT-PCR results showed that knockdown of Zeb-1 enhances E2 induction of miR-203 expression (Figure 5B), supporting that Zeb-1 is a repressor of E2 regulation of miR-203 expression in VSMC.

To further study whether Zeb-1 exerts its function on E2 regulation of miR-203 by interacting with ERα, we performed co-immunoprecipitation experiments in MAoSMC. Zeb-1 co-immunoprecipitated with ERα, and E2 treatment enhanced the co-immunoprecipitation (Figure 5C). These data suggest that Zeb-1 forms a complex with ERα in VSMC, and that E2 promotes this interaction.

E2 Reduces ERα and Zeb-1 Binding to the miR-203 Promoter Region

Having demonstrated that ERα and Zeb-1 are involved in regulating miR-203 promoter activity, and that unliganded ERα at its binding site may inhibit miR-203 promoter activity, we performed ChIP assay with antibodies against ERα and Zeb-1 in vehicle- or E2-treated MAoSMC. The locations of primers used for ChIP assays are shown in Figure 5D. Enrichment of ERα-associated and Zeb-1–associated promoter fragments were confirmed by RT-PCR. Interestingly, we found less enrichment of both promoter fragments in E2-treated samples (Figure 5E and 5F). These results indicate that both ERα and Zeb-1 bind to the miR-203 promoter, and E2 treatment, although increasing ERα–Zeb-1 complex formation, reduces their binding to the miR-203 promoter.
E2 Downregulation of Abl1 and p63 in MAoSMC Is Mediated by miR-203

To explore whether E2 upregulation of miR-203 leads to repression of target gene expression, we chose 2 previously identified miR-203 targets, Abl1 and p63, and studied the relationship between their protein abundance and E2 treatment in VSMC. Overexpression of miR-203 by transfecting cells with a precursor miR-203–containing plasmid decreased Abl1 and p63 protein abundance in mouse aortic smooth muscle cells (MAoSMCs). E2 treatment decreased both Abl1 and p63 protein abundance in Ad-ERα–infected MAoSMC, which confirmed that both genes are miR-203 targets in VSMC. E2-mediated repression of Abl1 and p63 gene expression was abolished in miR-203–depleted MAoSMC, supporting that E2 downregulation of Abl1 and p63 in VSMC is mediated by miR-203. MiR-203 expression was significantly reduced as quantified by qRT-PCR in cells treated with anti–miR-203 (Figure 6B, right panel). The densitometry results of Abl1 and p63 blots are shown in Figure 6C.

E2 Inhibition of VSMC Proliferation Is Diminished by miR-203 Knockdown

MiR-203 has been shown to inhibit proliferation in a variety of malignant tumor cells. Given that E2 has previously
Overexpression of miR-203 Specifically Inhibits VSMC Proliferation

To examine whether miR-203 by itself has an antiproliferative effect on VSMC, we introduced precursor miR-203 into MAoSMC and did proliferation assays. Overexpression of miR-203 significantly reduced proliferation up to 4 days after transfection (Figure 7B). We performed TUNEL assays to determine whether inducing apoptosis might be a mechanism of the antiproliferative effect of miR-203. The mean percentage of TUNEL-positive cells in scrambled miR-treated and miR-203–treated cells was 0.54±0.62% and 0.24±0.40%, which suggested that overexpression of miR-203 did not result in an induction of apoptosis. To determine whether the antiproliferative effect of miR-203 is specific to VSMC, we studied the effect of overexpressing miR-203 in HAOaSMC and human umbilical vein endothelial cell. MiR-203 significantly inhibited HAOaSMC proliferation, but caused no change in human umbilical vein endothelial cell growth (Figure 7C), despite similar expression levels of miR-203 (data not shown). These results indicate that the antiproliferative effect of miR-203 is specific for VSMC.

Discussion

We have previously shown that many vascular genes are downregulated by E2,19,20 but the molecular mechanisms by which E2 elicits target gene repression are poorly understood. So far, several mechanisms have been proposed for E2-mediated gene repression, including physiological interference of cofactors, direct action of corepressors, and participation of elements of the basal transcripational machinery.36 Given the known role of miRs in other cells and tissues, we hypothesized that E2-mediated regulation of miR expression might be a mechanism for gene repression in the vasculature, and thereby be involved in suppressing VSMC proliferation. Therefore, we studied the role of E2 in regulating miR expression in the vasculature. Our screen of E2-treated mouse aortas revealed that 26 of the 32 E2-responsive miRs were upregulated, whereas only 6 were downregulated. Several previous studies of E2 regulation of miRs expression in breast cancer cells26 and mouse uterus28 also showed that the majority of the E2-regulated miRs are upregulated. Although several of the E2-regulated miRs were of potential interest, we chose to focus on miR-203 as it had previously been identified through a tethering mechanism in which AP-1 directly binds to DNA. AP-1–binding sites have been shown to inhibit VSMC proliferation, both in vitro and in vivo,8,10–13 we hypothesized that miR-203 contributes to the inhibitory effect of E2 on VSMC proliferation. To test this hypothesis, we studied the effects of E2 on proliferation in ERα-overexpressing MAoSMC in which miR-203 was knocked down. In control cells, E2 significantly inhibited MAoSMC proliferation on day 3, and this effect was abolished by depletion of miR-203 (Figure 7A). These results indicate that E2 inhibition of VSMC proliferation is partially mediated by miR-203.
VSMC proliferation contributes significantly to the pathobiology of vascular proliferative disorders, such as atherosclerosis, postangioplasty restenosis, and transplant vasculopathy. Estrogen has previously been shown to inhibit VSMC proliferation, both in vitro and in vivo. In the current study, we demonstrate that knockdown of miR-203 abolished E2-mediated inhibition of VSMC proliferation in vitro (without increasing apoptosis), identifying miR-203 as contributing to this antiproliferative effect of E2.

Drug-eluting stents have been increasingly used to reduce VSMC proliferation after percutaneous coronary intervention. Recent studies have demonstrated that these drug-eluting stents may increase the risk of late thrombosis, which partially results from inhibition of VEC proliferation, thereby impairing reendothelialization. Identification of antiproliferative agents selective for VSMC will represent a significant advance in the treatment of restenosis and other VSMC-proliferative diseases. In the present study, we found miR-203 specifically inhibits proliferation in VSMC, but not VEC, in vitro. A limitation of the current study includes that we have only partially characterized the role of miR-203 on VEC proliferation. Further studies are required to determine whether miR-203 may serve as a valuable therapeutic agent in the treatment of proliferative cardiovascular disease.

In conclusion, our study provides the first evidence that E2 selectively regulates miRs that, in turn, contribute to repression of target gene expression in vascular cells and tissues, and identify an important role for miR-203 in E2-mediated inhibition of VSMC proliferation. The specific inhibitory effect of miR-203 on VSMC proliferation supports its potential as a therapeutic agent in proliferative cardiovascular diseases.

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None.

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