

MicroRNA-210 Plays a Critical Role in the Angiogenic Effect of Isoprenaline on Human Umbilical Vein Endothelial Cells via Regulation of Noncoding RNAs

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Abstract

Background: β -adrenoceptors play a crucial regulatory role in blood vessel endothelial cells. Isoprenaline (ISO, a β -adrenergic agonist) has been reported to promote angiogenesis through upregulation of vascular endothelial growth factor (VEGF) expression; however, the underlying mechanism remains to be investigated. It is widely accepted that certain noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), can regulate endothelial cell behavior, including their involvement in angiogenesis. Therefore, we aimed to investigate whether noncoding RNAs participate in ISO-mediated angiogenesis using human umbilical vein endothelial cells (HUVECs).

Methods: We evaluated VEGF-A messenger RNA (mRNA) and protein levels in ISO-treated HUVECs by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. To establish whether noncoding RNAs are associated with ISO-mediated angiogenesis, we measured expression of the miRNAs miR-210, miR-21, and miR-1, as well as that of the lncRNAs growth arrest-specific transcript 5 (GAS5), maternally expressed 3 (MEG3), and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in HUVECs exposed to ISO. Furthermore, to ascertain its importance in ISO-mediated angiogenesis, we constructed the HUVECs with overexpressing miR-210 and detected the subsequent expression of VEGF-A and noncoding RNAs. All statistical analyses were performed using SPSS 16.0 software. Intergroup comparisons were carried out by one-way analysis of variance.

Results: VEGF-A mRNA levels were elevated in the ISO group (1.57 ± 0.09) compared to those in the control group ($P < 0.01$). Moreover, concentrations of VEGF-A in culture supernatants significantly differed between the control (113.00 ± 19.21 pg/ml) and ISO groups (287.00 ± 20.27 pg/ml; $P < 0.01$). Expression of miR-1, miR-21, and miR-210 was higher (3.89 ± 0.44 , 2.87 ± 0.87 , and 3.33 ± 1.31 , respectively) in ISO-treated cells than that in controls ($P < 0.01$), whereas that of GAS5 and MEG3 (0.22 ± 0.10 and 0.58 ± 0.16 , respectively) was lower as a result of ISO administration ($P < 0.05$). There was no significant difference in the expression of MALAT1 between the groups. Interestingly, miR-210 overexpression heightened the levels of VEGF-A and miR-21 (5.87 ± 1.24 and 2.74 ± 1.15 , respectively; $P < 0.01$) and reduced those of GAS5 and MEG3 (0.19 ± 0.01 and 0.09 ± 0.05 , respectively; $P < 0.01$).

Conclusions: ISO-mediated angiogenesis was associated with altered expression of miR-210, miR-21, and the lncRNAs GAS5 and MEG3. The effects of miR-210 on the expression of VEGF-A and noncoding RNAs were similar to those of ISO, indicating that it might play an important role in ISO-mediated angiogenesis.

Key words: Angiogenesis; Isoprenaline; Long Noncoding RNAs; MicroRNAs

INTRODUCTION

β -adrenoceptors (β -ARs), comprising β_1 , β_2 , and β_3 subtypes, have been reported to play a critical regulatory role in blood vessel endothelial cells.^[1] β -AR stimulation can promote angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF), a crucial angiogenic

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protein.^[2] Isoprenaline (ISO; a synthetic catecholamine and β -adrenergic agonist) has been shown to increase VEGF-A expression and VEGF receptor-2 (VEGFR-2) activity in hemangioma-derived endothelial cells.^[3] Similarly, ISO promotes angiogenesis in Matrigel cultures of human umbilical vein endothelial cells (HUVECs),^[4] however, the underlying mechanism remains to be clarified.

Certain noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), are known to regulate angiogenesis and other aspects of endothelial cell behavior.^[5] miRNAs have been extensively investigated in this respect. For example, miR-210 has been found to promote angiogenesis in HUVEC Matrigel cultures by enhancing the expression of VEGF and VEGFR-2.^[6] Furthermore, miR-21 promotes the tube-forming capacity of primary bovine retinal microvascular endothelial cells,^[7] and its inhibition restricts the antiangiogenic effect of cardamomin on HUVECs.^[8] In addition, upregulation of miR-1 expression enhances the formation of vascular tubes on Matrigel.^[9] Besides miRNAs, recent studies have shown that some lncRNAs also regulate angiogenesis.^[5,10-12]

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been reported to enhance VEGF expression and promote pathological angiogenesis,^[13] whereas maternally expressed 3 (MEG3) inhibits angiogenesis by reducing the levels of this protein.^[14] The lncRNA growth arrest-specific transcript 5 (GAS5) also participates in angiogenesis.^[15]

In this study, we evaluated the effect of ISO on VEGF-A expression in HUVECs. To elucidate the mechanism underlying this effect, we also examined the impact of ISO treatment on the expression of the miRNAs miR-1, miR-21, and miR-210 and the lncRNAs MALAT1, MEG3, and GAS5.

METHODS

Cell culture

HUVE-12 cells were obtained from the China Center for Type Culture Collection (Wuhan, China) and maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO₂. The culture medium was replaced for every 2 days.

Cell viability assay

Cell viability was evaluated by methyl thiazolyl tetrazolium (MTT) assay. In brief, 2 × 10⁴ HUVECs were seeded on 96-well plates, to which phosphate-buffered saline (PBS) or ISO (100 μ mol/L) was then added. After 18 h, cells were incubated with MTT (500 μ g/ml) for 4 h, before addition of dimethyl sulfoxide. Absorbance at 570 nm was subsequently measured using a microplate reader (Thermo Scientific, Wilmington, DE, USA).

Cell transduction

HUVE-12 cells were transduced with lentiviral particles containing a miR-210-green fluorescent

protein (LV-mir-210-GFP) or control GFP (LV-GFP) construct. In brief, HUVE-12 cells (40–60% confluent) were seeded on 6-well plates and cultured for 20 h before being exposed to the LV-mir-210-GFP or LV-GFP vector coated with Lipofectamine 2000 (Invitrogen, Guangzhou, China). miR-210 expression was confirmed using quantitative real-time polymerase chain reaction (qRT-PCR), and GFP expression was observed under a fluorescence microscope 72 h after transfection.

Detection of vascular endothelial growth factor-A in cell-free culture supernatants

HUVE-12 cells were placed on 6-well plates and treated with PBS or ISO (100 μ mol/L) for 18 h before harvesting the cell-free supernatants to measure VEGF-A concentration using human VEGF-specific enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

RNA isolation and real-time quantitative reverse transcription polymerase chain reaction

For expression measurements, miRNA was extracted from plasma samples using a miRcute miRNA isolation kit (TIANGEN, Beijing, China). Poly(A) tailing and reverse transcription using a miScript reverse transcription kit (TIANGEN, Beijing, China) were then performed. Expression of miR-210, miR-21, and miR-1 was quantified using a TaqMan miRNA qRT-PCR assay as per the manufacturer's protocol. U6 RNA was used as an internal control. To measure lncRNA levels, 1 μ g total RNA was reverse transcribed using MuLV reverse transcriptase (TransGen, Beijing, China) and random hexamer primers in a 20 μ l reaction. A SYBR Green PCR Master Mix kit (TransGen, Beijing, China) was used for relative quantification of miRNAs and lncRNAs by qRT-PCR, employing U6 and 18S rRNA as internal controls, respectively. qRT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Naerum, Denmark). The primers (synthesized by Sangon, Shanghai, China) used were as follows: (1) miR-1 forward, 5'-GGGGTGAATGTAAAGAA-3' and reverse, 5'-TGCGTGTCTGGAGTC-3'; (2) miR-21 forward, 5'-CAGATCAGCCGCTGTCACA-3' and reverse, 5'-TGCCACCGCACAC-3'; (3) miR-210 forward, 5'-ACACTCCAGCTGGGTAGCTTATCAGACTGA-3' and reverse, 5'-GTGTCGTGGAGTCGGCAATTC-3'; (4) U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTTCAT-3'; (5) MALAT1 forward, 5'-CTTAAGCGCAGCGCCATTTT-3' and reverse, 5'-CCTCCAAACCCCAAGACCAA-3'; (6) MEG3 forward, 5'-CTGCCATCTACACCTCACG-3' and reverse, 5'-CTCTCCGCCGCTGCGCTAGGGGCT-3'; (7) GAS5 forward, 5'-GTTACCAGGAGCAGAACCATTA-3' and reverse, 5'-CATTGGCACACAGGCATTAG-3'; (8) 18S rRNA forward, 5'-CAGCCACCCGAGATTGAGCA-3' and reverse, 5'-TAGTAGCGACGGGCGGTGTG-3'.

Analysis of relative gene expression levels was performed using the 2^{- Δ CT} method, where Δ CT (cycle threshold) = CT (target gene) – CT (control).

Statistical analysis

Data were reported as mean \pm standard deviations (SD) or medians for general subject characteristics. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Intergroup comparisons were carried out by one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered highly statistically significant.

RESULTS

Effect of isoprenaline on the expression of vascular endothelial growth factor and cell viability in human umbilical vein endothelial cells

ISO has been reported to promote angiogenesis and hemangioma-derived endothelial cell proliferation by increasing VEGF-A expression and VEGFR-2 activity.^[3] In the current investigation, we also studied the effect of ISO on VEGF-A expression in HUVECs and their viability. VEGF-A messenger RNA (mRNA) levels were increased (1.57 ± 0.09) in the ISO group compared to those in the control group ($P < 0.01$) [Figure 1a]. The average concentration of VEGF-A in supernatants of control and ISO-treated cultures was 113.13 ± 19.21 and 287.42 ± 20.27 pg/ml, respectively ($P < 0.01$) [Figure 1b]. However, ISO exerted only a minor stimulatory effect on HUVEC proliferation, which did not significantly differ between the two treatment groups [Figure 1c].

Effect of isoprenaline on microRNA and long noncoding RNA expression in human umbilical vein endothelial cells

It is widely accepted that some noncoding RNAs, including miRNAs and lncRNAs, regulate endothelial cell functions, including those involved in angiogenesis.^[5] To investigate whether noncoding RNAs participate in ISO-mediated angiogenesis, we tested the effect of ISO on the expression of miRNAs and lncRNAs in HUVECs. Levels of miR-1, miR-21, and miR-210 were found to be increased (3.89 ± 0.44 , 2.87 ± 0.87 , and 3.33 ± 1.31 , respectively) in the ISO group compared to those in the control group ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively). In contrast, GAS5 and MEG3 expression decreased (0.22 ± 0.10 and 0.58 ± 0.16 , respectively) as a result of ISO treatment ($P < 0.01$ and $P < 0.05$, respectively). However, no significant difference in MALAT1 expression was identified between the ISO (0.96 ± 0.15) and control groups [Figure 2].

Effect of miR-210 on the expression of vascular endothelial growth factor-A and noncoding RNAs in human umbilical vein endothelial cells

Our data showed that ISO increased miR-210 expression in HUVECs. Moreover, this miRNA has been reported to upregulate VEGF-A expression and promote angiogenesis in HUVEC cultures.^[6] To determine whether miR-210 is associated with ISO-mediated angiogenesis, we tested

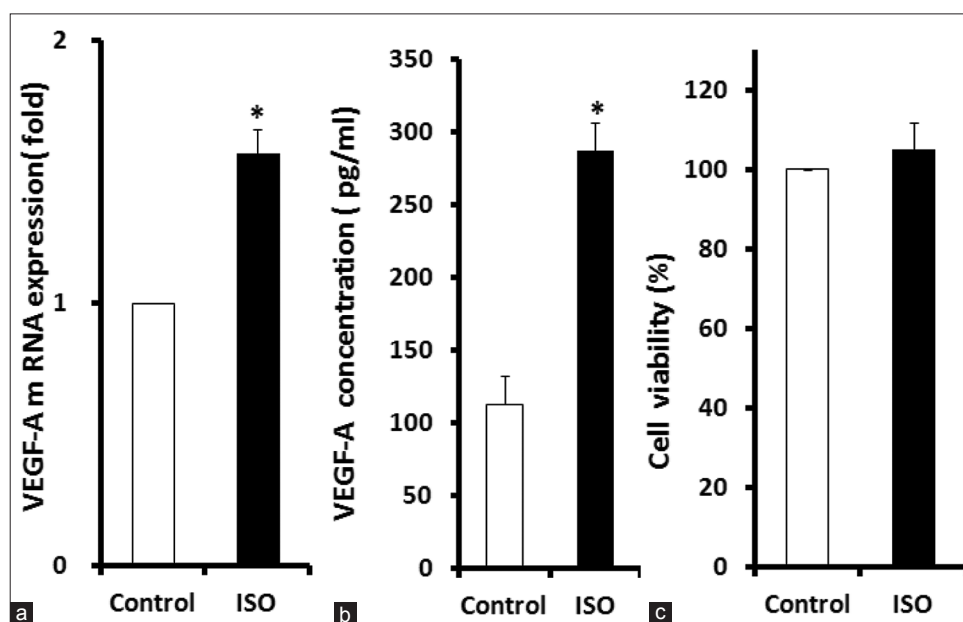


Figure 1: Effect of ISO on HUVEC viability and VEGF-A expression. Cells were exposed to PBS (control) or 100 $\mu\text{mol/L}$ ISO for 18 h. VEGF-A mRNA and protein levels were quantified by qRT-PCR and ELISA, respectively. Cell viability was measured by MTT assay. (a) VEGF-A mRNA expression in HUVECs treated with PBS or ISO. VEGF-A mRNA levels were higher (1.57 ± 0.09) in the ISO group than the control group. (b) VEGF-A concentration in the supernatants of HUVEC cultures treated with ISO or PBS. Higher VEGF-A concentrations were observed in the ISO group ($287.42.13 \pm 20.27$ pg/ml) than the control group (113.13 ± 19.21 pg/ml). (c) Effect of ISO on HUVEC viability. ISO was found to increase HUVEC viability ($105.02\% \pm 6.74\%$) compared with the control treatment (100%). Data are expressed as mean \pm standard deviations of three independent experiments. * $P < 0.01$ compared to the control. ISO: Isoprenaline; VEGF-A: Vascular endothelial growth factor-A; mRNA: Messenger RNA; HUVECs: Human umbilical vein endothelial cells; PBS: Phosphate-buffered saline; qRT-PCR: Quantitative real-time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; MTT: Methyl thiazolyl tetrazolium.

its effects on VEGF-A mRNA level and noncoding RNA expression. Overexpression of miR-210 in HUVECs was achieved by transduction with LV-miR-210-GFP; cells

transduced with LV-GFP were used as a control. Successful transduction of cells was confirmed by observing GFP expression under a fluorescence microscope [Figure 3a].

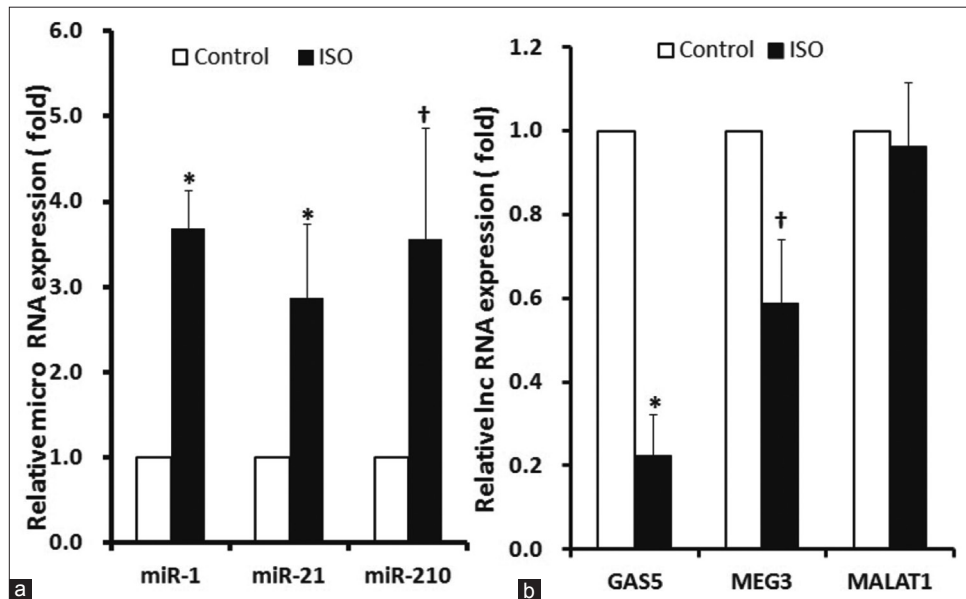


Figure 2: Effect of ISO on miRNA and lncRNA expression in HUVECs. Cells were exposed to PBS (control) or 100 μmol/L ISO for 18 h, and expression of the miRNAs miR-1, miR-21, and miR-210 and the lncRNAs MEG3, GAS5, and MALAT1 was measured by qRT-PCR. (a) Effect of ISO on miRNA expression in HUVECs. miR-1, miR-21, and miR-210 levels were increased (3.89 ± 0.44 , 2.87 ± 0.87 , and 3.33 ± 1.31 , respectively) in the ISO group compared with the control group. (b) Effect of ISO on lncRNA expression in HUVECs. GAS5 and MEG3 levels were decreased (0.22 ± 0.10 and 0.58 ± 0.16 , respectively) as a result of ISO treatment. There was no significant difference in MALAT1 expression (0.96 ± 0.15) between ISO-treated and control cells. Data are expressed as mean ± standard deviations of three independent experiments. * $P < 0.01$ and † $P < 0.05$ compared to the control. ISO: Isoprenaline; miRNA: MicroRNA; lncRNA: Long noncoding RNA; GAS5: Growth arrest-specific transcript 5; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; MEG3: Maternally expressed 3; PBS: Phosphate-buffered saline; HUVECs: Human umbilical vein endothelial cells; qRT-PCR: Quantitative real-time polymerase chain reaction.

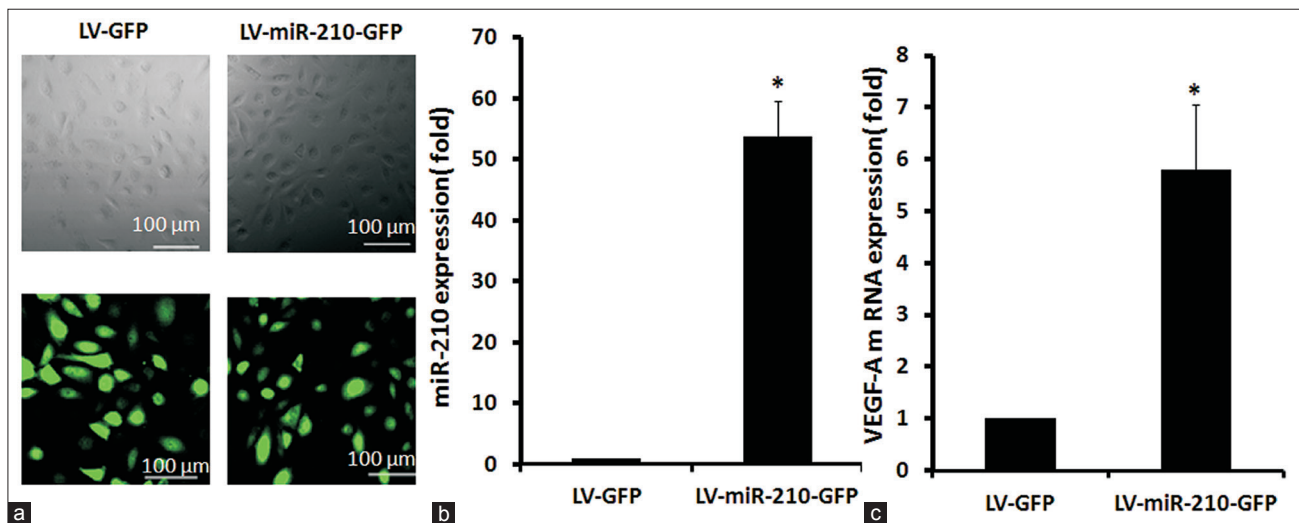


Figure 3: Effect of miR-210 on VEGF mRNA expression in HUVECs. Cells were transduced with LV-GFP or LV-miR-210-GFP constructs. Successfully transduced cells were identified by observing GFP expression with a fluorescence microscope or by detecting miR-210 expression using qRT-PCR. VEGF-A mRNA levels in transduced cells were also tested by qRT-PCR. (a) Detection of GFP expression by fluorescence microscopy after transduction. (b) Verification of miR-210 overexpression in HUVECs after transduction. miR-210 levels were increased (53.71 ± 5.90) in cells expressing LV-miR-210-GFP, as determined by qRT-PCR. (c) Effect of miR-210 on VEGF-A mRNA expression in HUVECs. VEGF-A transcript level was increased (5.87 ± 1.24) in cells transduced with LV-miR-210-GFP, as measured by qRT-PCR. Data are expressed as mean ± standard deviations of three independent experiments. * $P < 0.01$ compared to the control. mRNA: Messenger RNA; VEGF: Vascular endothelial growth factor; LV: Lentiviral vector; GFP: Green fluorescent protein; HUVECs: Human umbilical vein endothelial cells; qRT-PCR: Quantitative real-time polymerase chain reaction.

Levels of miR-210 increased up to approximately 60 (53.71 ± 5.90) in cells transfected with LV-miR-210-GFP, as determined by qRT-PCR [Figure 3b]. Furthermore, VEGF mRNA expression increased more than 5 (5.87 ± 1.24) in miR-210-overexpressing cells [Figure 3c].

We also evaluated the effect of miR-210 on the expression of other noncoding RNAs in HUVECs. As shown in Figure 4a, levels of miR-1 and miR-21 significantly decreased and increased, respectively, in HUVECs overexpressing miR-210, compared to control cells ($P < 0.01$ and $P < 0.05$, respectively). Moreover, the expression of GAS5 and MEG3 significantly declined (0.19 ± 0.01 and 0.09 ± 0.05 , respectively) as a result of miR-210 overexpression ($P < 0.01$ and $P < 0.01$, respectively); however, there was no significant difference in MALAT1 level (1.19 ± 0.19) between miR-210-overexpressing and control cells [Figure 4b].

DISCUSSION

Previous studies have shown that ISO promotes angiogenic activity in tumor cells and HUVECs through induction of VEGF.^[3,4,16] In keeping with previous findings, we also established that ISO enhanced VEGF-A expression in HUVECs. Furthermore, we found that ISO upregulated the levels of miR-210, miR-21, and miR-1 and downregulated those of the lncRNAs MEG3 and GAS5, indicating that ISO-mediated angiogenesis might be associated with the regulation of noncoding RNAs. Certain miRNAs,

including those examined here, have been implicated in the regulation of VEGF expression.^[9,17,18] This observation is also supported by other data. For instance, overexpressing miR-210 in HUVECs can promote angiogenesis through upregulation of VEGF and VEGFR-2.^[6] In addition, miR-21 has been found to induce tumor angiogenesis by raising VEGF expression.^[17]

The role of miR-1 in angiogenesis is contentious. Upregulation of miR-1 enhances the formation of vascular tubes on Matrigel;^[9] however, inhibition of this miRNA has also been shown to promote angiogenesis.^[19] In addition, miR-1 negatively regulates angiogenesis in zebrafish by inhibiting VEGF-A.^[20] Thus, the role of miR-1 in the angiogenic effect of ISO needs to be investigated. Besides miRNAs, lncRNAs also participate in the regulation of angiogenesis. MEG3 is thought to inhibit this process by reducing VEGF levels,^[14] and GAS5 knockdown increases retinal neovascularization and capillary leakage.^[15] In contrast, the lncRNA MALAT1 can enhance VEGF expression and promote pathological angiogenesis. *In vivo* genetic ablation of MALAT1 suppresses endothelial cell proliferation, and thus reduces neonatal retinal vascularization.^[13] However, ISO stimulation of HUVECs did not significantly alter MALAT1 expression in the present work.

Of the noncoding RNAs affected by ISO, miR-210 appeared to play an important role in ISO-mediated angiogenesis,

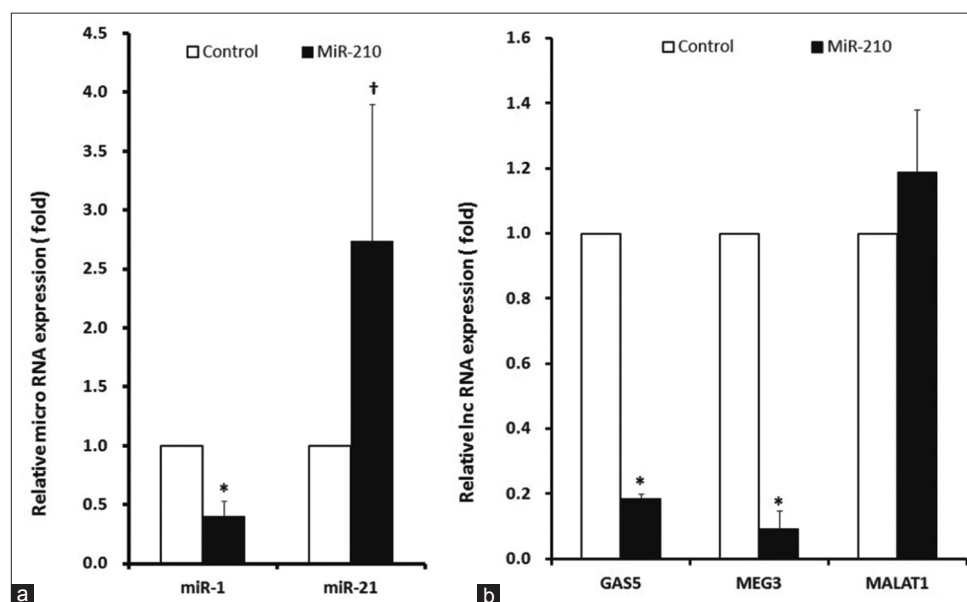


Figure 4: Effect of miR-210 on miRNA and lncRNA expression in HUVECs. Expression of the miRNAs miR-1, miR-21 and the lncRNAs MEG3, GAS5, and MALAT1 was tested in cells transduced with LV-GFP (control) or LV-miR-210-GFP constructs. (a) Effect of miR-210 on miRNA expression in HUVECs. Expression of miR-1 was significantly decreased (0.44 ± 0.12) and that of miR-21 significantly increased (2.74 ± 1.15) in miR-210-overexpressing HUVECs, compared with the control group. (b) Effect of miR-210 on lncRNA expression in HUVECs. GAS5 and MEG3 levels were significantly decreased (0.19 ± 0.01 and 0.09 ± 0.05 , respectively) in miR-210 overexpressing HUVECs, compared with the control group. No significant difference in MALAT1 expression (1.19 ± 0.19) was identified between miR-210-overexpressing and control cells. Data are expressed as mean \pm standard deviations of three independent experiments. * $P < 0.01$ and † $P < 0.05$ compared to the control. miRNA: MicroRNA; lncRNA: Long noncoding RNA; MEG3: Maternally expressed 3; GAS5: Growth arrest-specific transcript 5; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; HUVECs: Human umbilical vein endothelial cells; LV: Lentiviral vector; GFP: Green fluorescent protein.

as it exerted an effect similar to that of ISO on VEGF and noncoding RNAs. The underlying mechanism might involve upregulation of hypoxia-inducible factor (HIF)-1 α . It has been reported that ISO induces expression of HIF-1 α protein in a dose-dependent manner in tumor cells. HIF-1 α can increase miR-210 and miR-21 levels, which in turn, upregulates HIF-1 α ,^[18,21,22] indicating that these miRNAs might enhance each other's expression. HIF-1 α can inhibit miR-29 expression,^[23] while overexpression of the latter increases transcription of MEG3.^[24] HIF-1 α also augments the expression of miR-21,^[17] which inhibits GAS5.^[25] In addition, MALAT1 level positively correlates with the expression of HIF-2 α , but not with that of HIF-1 α in tumor cells.^[26]

In the present study, ISO exerted a minor stimulatory effect on HUVEC proliferation, which might be associated with the inhibition of GAS5 and MEG3 expression observed. The lncRNA GAS5 is believed to promote apoptosis and inhibit the growth of tumor cells through G0/G1-phase cell cycle arrest,^[27] and several studies have demonstrated that MEG3 can inhibit tumor cell proliferation.^[5,28-30] In the current investigation, we also found that ISO and miR-210 inhibited MEG3 expression, implying that ISO promoted cell proliferation through downregulation of MEG3.

It has been well established that miRNAs regulate gene expression in a posttranscriptional manner. A recent study showed that mouse miR-709 directly regulated miR-15a/16-1 biogenesis in the nucleus at the posttranscriptional level,^[31] indicating that one miRNA could affect another by direct targeting. Here, we also observed that one miRNA influenced the biogenesis of other miRNAs and lncRNAs.

This study had several limitations. We found that ISO upregulated VEGF-A expression, and that this was associated with altered noncoding RNA levels. Noncoding RNAs might regulate each other in this process; however, the underlying mechanism needs to be investigated further. In addition, the expression of many other miRNAs, including miR-329 and miR-126, has been shown to correlate with angiogenesis.^[32,33] Our study focused only on those implicated in angiogenesis whose expression is affected by ISO.^[34,35]

In conclusion, ISO-mediated angiogenesis was found to be associated with the regulation of noncoding RNAs, including the miRNAs miR-210 and miR-21 and the lncRNAs GAS5 and MEG3. Of these, miR-210 appeared to play an important role in ISO-mediated angiogenesis, exerting an effect similar to that of ISO on VEGF and noncoding RNAs.

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Conflicts of interest

There are no conflicts of interest.

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