Micro RNA-210 targets TET1 and suppresses pregnancy-mediated adaptation of BK_{Ca} channel expression and function in ovine uterine arteries

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Abstract

Gestational hypoxia inhibits large conductance Ca²⁺-activated K⁺ (BK_{Ca}) channel expression and function in uterine arterial adaptation to pregnancy. Given the findings that microRNA 210 (miR-210) is increased in hypoxia during gestation and preeclampsia, the present study sought to investigate the role of miR-210 in the regulation of BK_{Ca} channel adaptation in the uterine artery. Gestational hypoxia significantly increased uterine vascular resistance and blood pressure in pregnant sheep, and upregulated miR-210 in uterine arteries. MiR-210 bound to ovine ten-eleven translocation methylcytosine dioxygenase 1 (TET1) mRNA 3'UTR and decreased TET1 mRNA and protein abundance in uterine arteries of pregnant sheep, as well as abrogated steroid hormone-induced upregulation of TET1 expression in uterine arteries of non-pregnant animals. In accordance, miR-210 blocked pregnancy- and steroid hormone-induced upregulation of BK_{Ca} channel β 1 subunit expression in uterine arteries. Functionally, miR-210 suppressed BK_{Ca} channel current density in uterine arterial myocytes of pregnant sheep and inhibited steroid hormone-induced increases in BK_{Ca} channel currents in uterine arteries of non-pregnant animals. Blockade of endogenous miR-210 inhibited hypoxia-induced suppression of BK_{Ca} channel activity. In addition, miR-210 decreased BK_{Ca} channel-mediated relaxations and increased pressure-dependent myogenic tone of uterine arteries. Together, the results demonstrate that miR-210 plays an important role in the downregulation of TET1 and repression of BK_{Ca} channel function in uterine arteries, revealing a novel mechanism of epigenetic regulation in the maladaptation of uterine hemodynamics in gestational hypoxia and preeclampsia.

Keywords

hypoxia; preeclampsia; DNA methylation; blood pressure; uterine vascular resistance

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Introduction

The maternal cardiovascular system undergoes extensive adaptations during pregnancy.¹ The most striking change occurs in the uterine circulation. Uterine blood flow increases substantially to meet the demand of placental and fetal growth and to ensure well-being of the mother.^{2–4} Reduction of uterine vascular resistance plays a key role in the increased uterine blood flow during gestation.^{4–6} The large conductance Ca^{2+} -activated K^{+} (BK_{Ca}) channel is of critical importance in the regulation of vascular tone.^{7–9} The BK_{Ca} channel in vascular smooth muscle cells is a heteromer of channel-forming α subunits and regulatory $\beta 1$ subunits, encoded by *KCNMA1* and *KCNMB1*, respectively.¹⁰ The $\beta 1$ subunit increases the apparent Ca^{2+} sensitivity of the α subunit.¹¹ The upregulation of BK_{Ca} channel $\beta 1$ subunit and the subsequent increase in BK_{Ca} channel activity are essential in reducing uterine vascular tone and increasing uterine blood flow during pregnancy.^{6, 12–14}

Emerging evidence supports a critical role of epigenetic mechanisms in the regulation of cardiovascular function and pathogenesis of cardiovascular diseases.^{15, 16} We have recently demonstrated in an animal model of sheep that the expression of BK_{Ca} channel $\beta 1$ subunit in the uterine artery is governed by the methylation status of *KCNMB1* promoter.^{17, 18} DNA methylation/demethylation dynamics is achieved by the actions of DNA methyltransferases (DNMTs), ten-eleven translocation methylcytosine dioxygenases (TETs), and other enzymes.¹⁹ In general, DNA methylation accomplished by DNMTs represses gene expression, whereas active DNA demethylation initialized by TETs reactivates gene expression. The expression of BK_{Ca} channel $\beta 1$ subunit in uterine arteries of non-pregnant sheep was restrained due to heavily methylated Sp1 binding site (Sp1₋₃₈₀) at the *KCNMB1* promoter, which hampered the binding of transcription factors ER α and Sp1. In pregnancy, estrogen upregulated the expression of TET1 that in turn promoted demethylation of Sp1₋₃₈₀ binding site and facilitated ER α and Sp1 binding to the *KCNMB1* promoter, leading to enhanced BK_{Ca} channel $\beta 1$ subunit expression and BK_{Ca} channel activity in the uterine artery.²⁰

Chronic hypoxia during gestation has a major impact in the uteroplacental circulation and pregnancy complications. It has been shown that pregnancy at high altitude with gestational hypoxia increases uterine vascular resistance, which is associated with an increased incidence of preeclampsia and fetal growth restriction.^{21, 22} We have demonstrated in sheep that chronic hypoxia during gestation inhibits steroid hormone-mediated upregulation of BK_{Ca} channel activity^{17, 23, 24} and increases myogenic tone in uterine arteries.^{23–26} Of importance, our studies revealed that the *KCNMB1* promoter remained hypermethylated, resulting in the repression of BK_{Ca} channel $\beta 1$ subunit in uterine arteries of pregnant sheep acclimatized to high altitude hypoxia during gestation.²⁰ This suggests a possible mechanism of hypoxia-induced inhibition of TET1-mediated demethylation of the *KCNMB1* promoter in uterine arteries that normally occurs during gestation.

The enzymatic machineries of DNA methylation/demethylation are targets of microRNAs (miRs) and the expression of DNMTs and TETs are regulated by a subset of miRs.^{27–30} Given that miR-210 is *The Master Hypoxamir* of a specific group of miRs termed “*Hypoxamirs*” that are regulated by hypoxia,^{31–36} we sought to investigate whether hypoxia

during gestation increases miR-210 in the uterine artery, and whether miR-210 downregulates TET1 and suppresses BK_{Ca} channel upregulation in the uterine artery during gestation. MiRs are important players in the epigenetic control of gene expression patterns by targeting the mRNA 3'UTR and resulting in degradation of mRNAs or translational suppression of the target transcripts. Of interest, miR-210 appears to be a common denominator in preeclampsia and gestational hypoxia. Elevated placental expression of miR-210 has been demonstrated in both preeclampsia and pregnancy at high altitude.^{37–42} Circulating miR-210 levels also increased in both conditions.^{43–45}

Herein, we present a novel finding that gestational hypoxia increases miR-210 in the uterine artery and that miR-210 targets the 3'UTR of TET1 mRNA transcript and downregulates TET1 protein and mRNA abundance in the uterine artery. We further demonstrated that miR-210 inhibited steroid hormone-mediated upregulation of TET1 expression in the uterine artery. As a result, BK_{Ca} channel β 1 subunit was repressed in the uterine artery in pregnancy. Of importance, miR-210 suppressed BK_{Ca} channel current density, inhibited BK_{Ca} channel-mediated relaxations and increased pressure-dependent myogenic tone in uterine arteries of pregnant sheep. Moreover, blockade of endogenous miR-210 in uterine arteries inhibited hypoxia-induced suppression of BK_{Ca} channel activity. Thus, the results reveal a novel epigenetic mechanism of miR-210 in suppressing TET-mediated DNA demethylation and its functional role in modulating BK_{Ca} channel activity in uterine arterial adaptation to pregnancy, which is likely to be important in the maladaptation of uterine hemodynamics in gestational hypoxia and preeclampsia.

Methods

Tissue preparation and treatment

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After tissue collection, animals were killed via intravenous injection of 15 mL T-61 solution (Hoechst-Rousel, Somerville, NJ, USA), according to American Veterinary Medical Association guidelines.

Uterine arteries were harvested from non-pregnant or near-term (~142–145 days of gestation) pregnant sheep (*Ovis aries*) maintained at low altitude (~300 m above sea level, PaO₂: ~102 mm Hg) or exposed to high altitude hypoxia (3,801 m, PaO₂: ~60 mm Hg) for 110 days, as described in previous studies.^{25, 26} Animals were anesthetized with intravenous injection of propofol (2 mg/kg) followed by intubation, and anesthesia was maintained on 1.5% to 3.0% isoflurane balanced in O₂ throughout the surgery. An incision was made in the abdomen and the uterus was exposed. The fourth-generation branches of main uterine artery were isolated and removed without stretching and placed into a Krebs solution containing (in mmol/L) 130.0 NaCl, 10.0 HEPES, 6.0 glucose, 4.0 KCl, 4.0 NaHCO₃, 1.8 CaCl₂, 1.2 MgSO₄, 1.18 KH₂PO₄, and 0.025 EDTA (pH 7.4). Uterine arteries were placed in a culture dish containing 5 mL of phenol red-free DMEM supplemented with 1% charcoal-stripped fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin and incubated at 37 °C in humidified incubators for 48 hours, as described previously.^{23, 26, 46} For the hormonal treatment, uterine arteries from non-pregnant sheep were incubated in the absence

or presence of 17 β -estradiol (E2 β , Sigma, St. Louis, MO) (0.3 nmol/L) and progesterone (P4, Sigma) (100.0 nmol/L), as reported previously.^{6, 26, 46}

Hemodynamic measurements

Non-invasive measurements of blood pressure and uterine blood flow were obtained under anesthesia in the supine position. Blood pressure was measured with a SurgiVet Advisor Vital Signs Monitor (Smiths Medical, Waukesha, Wisconsin) with the pressure cuff placed on the upper left thoracic limb of sheep. Uterine blood flow was measured with transabdominal ultrasonography using a GE LOGIQ e Vet (Sound, Carlsbad, California) with a 10 MHz Probe 8C-RS (GE Healthcare, Wauwatosa, WI). Internal diameter of uterine artery lumen was determined from B-mode images. Uterine artery volume flow (Q_{UtA}) was quantified using the formula: Q_{UtA} (ml/min) = time-averaged mean velocity (cm/s) $\times$ cross-sectional area of the vessel (πr^2 , cm²) $\times$ 60 s/min. Uterine vascular resistance (UVR) was calculated as: UVR (mmHg-min/ml) = MAP (mmHg) $\div$ Q_{UtA} (ml/min).

Measurement of BK_{Ca} channel current

Arterial smooth muscle cells were enzymatically dissociated from resistance-sized uterine arteries, and whole-cell K⁺ currents were recorded using an EPC 10 patch-clamp amplifier with Patchmaster software (HEKA, Lambrecht/Pfalz, Germany) at room temperature as previously described.^{6, 23} Briefly, cell suspension drops were placed in a recording chamber, and adherent cells were continuously superfused with HEPES-buffered physiological salt solution containing (in mmol/L) 140.0 NaCl, 5.0 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10.0 HEPES, and 10.0 glucose (pH 7.4). Only relaxed and spindle-shaped myocytes were used for recording. Micropipettes were pulled from borosilicate glass and had resistances of 2 to 5 megaohm (m Ω) when filled with the pipette solution containing (in mmol/L) 140.0 KCl, 1.0 MgCl₂, 5.0 Na₂ATP, 5.0 EGTA, 10.0 HEPES (pH 7.2). CaCl₂ was added to bring free Ca²⁺ concentrations to 100 nmol/L as determined using WinMAXC software (Chris Patton, Stanford University). Cells were held at -50 mV, and whole-cell K⁺ currents were evoked by voltage steps from -60 mV to +80 mV by stepwise 10-mV depolarizing pulses (350-ms duration, 10-second intervals) in the absence and presence of 1 mmol/L BK_{Ca} channel blocker tetraethylammonium.^{6, 23} BK_{Ca} currents, determined as the difference between whole-cell K⁺ currents in the absence of tetraethylammonium and that in the presence of tetraethylammonium, were normalized to cell capacitance and expressed as picoampere per picofarad (pA/pF).

Relaxation studies

Uterine arteries were separated from surrounding tissues and cut into 2-mm ring segments. Isometric tension was measured in the Krebs solution in a tissue bath system (Radnoti, Monrovia, CA) at 37 °C, as described previously.^{26, 47} Briefly, each ring segment was equilibrated for 60 minutes and then gradually stretched to the optimal resting tension determined by responses to 3 $\times$ 120 mmol/L KCl challenges. Tissues were then pre-contracted with submaximal concentrations of serotonin that produced ~70% to 80% of the maximal contraction, followed by additions of the BK_{Ca} channel opener NS1619 in a cumulative manner.

Measurement of pressure-dependent myogenic tone

Pressure-dependent myogenic tone of resistance-sized uterine arteries was measured as described previously.^{6, 26} Briefly, the arterial segments (dia. ~150 μ m) were mounted and pressurized in an organ chamber (Living Systems Instruments, Burlington, VT). The intraluminal pressure was controlled by a servo-system to set transmural pressures, and arterial diameter was recorded using the SoftEdge Acquisition Subsystem (IonOptix LLC, Milton, MA). After the equilibration period, the intraluminal pressure was increased in a stepwise manner from 10 to 100 mmHg in 10-mmHg increments, and each pressure was maintained for 5 minutes to allow vessel diameter to stabilize before the measurement. The passive pressure-diameter relationship was conducted in Ca^{2+} -free PSS containing 3.0 mmol/L of EGTA to determine the maximum passive diameter. The following formula was used to calculate the percentage of pressure-dependent tone at each pressure step: % tone = $(D1 - D2)/D1 \times 100$, where D1 is the passive diameter in Ca^{2+} -free PSS (0 Ca^{2+} with 3.0 mmol/L of EGTA), and D2 is the active diameter with normal PSS in the presence of extracellular Ca^{2+} .

MiR transfection

For tissue transfection, the mixture of miScript miR-210 mimic (Qiagen, Germantown, MD) or AllStars Negative Controls (Qiagen), HiPerfect transfection reagent (Qiagen) and Opti-MEM 1 (ThermoFisher, Waltham, MA) was prepared and incubated for 10 minutes at room temperature. The mixture was subsequently added to Dulbecco's Modified Eagle's Medium (DMEM) containing 1% charcoal-stripped fetal bovine serum (FBS) in a 6-well plate maintained in an incubator at 37 °C for 48 hours. For cell transfection, uterine arterial VSMCs were prepared as described above and re-suspended in DMEM containing 10% fetal bovine serum (FBS). The mixture of FAM-labeled miR-210 (Integrated DNA Technologies, Coralville, IA) or FAM3™ Dye-Labeled Pre-miR Negative Control #1 (ThermoFisher), HiPerfect transfection reagent (Qiagen) and Opti-MEM I (ThermoFisher) was prepared and incubate for 10 minutes at room temperature. Subsequently, freshly isolated VSMCs were added on top of the mixture in a 24-well plate and maintained in an incubator at 37 °C for 48 hours. FAM-labeled miR-210/Negative control were used to permit optic selection in electrophysiological studies. The working concentrations of miRs were 100 nmol/L for miR-210 and the negative control, respectively. To block endogenous miR-210, cells were transfected with 100 nmol/L miR-210-antimiR LNA (miR210-LNA)^{48–50} or its negative control (Exiqon, Woburn, MA). DMEM contained no antibiotics.

Real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, CA) and subjected to reverse transcription with iScript cDNA Synthesis system (Bio-Rad, Hercules, CA). The mRNA abundance of TET1, BK_{Ca} channel β 1 subunit and Ras association domain family member 7 (RASSF7) was measured with real-time polymerase chain reaction (PCR) using iQ SYBR Green Supermix (Bio-Rad), as described previously.¹⁷ Primers used were 5'-acactagaacaagtagtggaata-3' (forward) and 5'-tttaagtttggtcttgagggtct-3' (reverse) for TET1, 5'-ctgtaccacacggaggacact-3' (forward) and 5'-gtagaggcgctggaataggac-3' (reverse) for BK_{Ca} channel β 1 subunit, and 5'-gtggtttgtgggtttcaga-3' (forward) and 5'-

tcaggacaaactggacatcg-3' (reverse) for RASSF7. GAPDH was used as an internal reference, and serial dilutions of the positive control were performed on each plate to create a standard curve for the quantification. PCR was performed in triplicate, and threshold cycle numbers were averaged for each sample. Quantification of mature miR-210 was performed using the miScript II RT kit and the miScript SYBR Green PCR kit with miScript Primer Assay kit (Qiagen) according to the manufacturer's instructions, as described previously.⁵¹ Primers included miScript Universal Primer, miR-210 miScript Primer Assay and SNORD61 miScript Primer Assay (Qiagen). Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification. PCR was done in triplicate and threshold cycle numbers were averaged for each sample. SNORD61 miScript Primer (Qiagen) was used as the internal control. The relative expression levels of mature miR-210 were computed and expressed as fold of SNORD61.

Western immunoblotting

Protein abundance of TET1 and BK_{Ca} channel β 1 subunit in uterine arteries was measured, as described previously.^{6, 20, 23} Briefly, tissues were homogenized in a lysis buffer followed by centrifugation at 4 °C for 10 minutes at 10,000g, and the supernatants were collected. Samples with equal proteins were loaded onto 7.5% polyacrylamide gel with 0.1% SDS and were separated by electrophoresis at 100 V for 2 hours. Proteins were then transferred onto nitrocellulose membranes. After blocking nonspecific binding sites by dry milk, membranes were incubated with primary antibodies against TET1 (Millipore, Billerica, MA) and BK_{Ca} channel β 1 subunit (Santa Cruz Biotechnology, Santa Cruz CA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software (Kodak, Rochester, NY). The target protein abundance was normalized to the abundance of β -actin as a protein loading control.

Luciferase reporter assay

Luciferase reporter assay was performed as previously described.^{17, 46, 51} A 244-bp 3'-UTR of ovine TET1 mRNA harboring the mature miR-210 target motif was cloned into the pmirGLO vector (Promega, Madison, WI) between NheI (5') and SalI (3'). The final construct was designated pmirGLO-TET244. For luciferase assays, uterine arterial smooth muscle cells were transfected with pmirGLO-TET244 or pmirGLO (for the vector control) together with miR-210-miScript miRNA mimic or the negative control at 7 nmol/L (Qiagen) using X-tremeGENE HP DNA transfection reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. The cells were processed using the Dual-Luciferase Reporter Assay System (Promega) 48 hours after transfection.

Statistical analysis

Data were expressed as means $\pm$ S.E.M. obtained from the number of experimental animals given. Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA). Differences were evaluated for statistical significance ($P < 0.05$) by ANOVA or *t* test where

appropriate; and the concentration-response relationship was analyzed with repeated measure ANOVA.

Results

High altitude acclimatization elevates miR-210 in uterine arteries

Considering that miR-210 is strongly induced by hypoxia in various types of tissues and cells,³² we determined and compared the expression of miR-210 in uterine arteries from non-pregnant and pregnant sheep residing at low altitude and high altitude. The expression of miR-210 was low in uterine arteries of non-pregnant and pregnant animals at low altitude. However, high altitude acclimatization significantly increased miR-210 levels in uterine arteries regardless of the status of pregnancy (Figure 1). In sheep genome miR-210 and RASSF7 genes are located on chromosome 21 and are separated by only 3.31 kb. Thus, it is possible that they are coordinated in the expression in response to hypoxia due to genomic proximity. Indeed, RASSF7 mRNA abundance coordinated with miR-210 in response to hypoxia and was significantly increased in uterine arteries of animals exposed to high altitude hypoxia (Supplemental Figure S1).

MiR-210 directly targets 3'UTR of TET1 mRNA transcript

Given the concurrence of robust increase of miR-210 and decrease of TETs in preeclamptic/hypoxic placenta,^{37–42, 52} we reasoned that TET1 in uterine arteries might be the target of miR-210. An application of the sequence alignment program MultAlin (<http://multalin.toulouse.inra.fr/multalin/multalin.html>) revealed a potential miR-210 complimentary binding site in TET1 mRNA 3'-untranslated region (3'UTR) (Figure 2). To examine whether TET1 mRNA is indeed a direct target of miR-210, a 244-bp 3'UTR of ovine TET1 mRNA harboring the predicted miR-210 target motif was cloned into the pmirGLO vector. Uterine arterial smooth muscle cells were transfected with pmirGLO-TET244 or pmirGLO (for the vector control) together with the miR-210 mimic or the negative control. As shown in Figure 3, a significant decrease in luciferase activity was observed following co-transfection of pmirGLO-TET244 and miR-210 mimic ($P < 0.05$), demonstrating a direct interaction of miR-210 with the 3'UTR of TET1 mRNA transcript.

MiR-210 represses TET1 expression

We then determined the role of miR-210 in the regulation of TET1 protein and mRNA abundance. Uterine arteries were transfected with miR-210 mimic to emulate preeclamptic/hypoxic induction of miR-210. Not surprisingly, transfection of miR-210 mimic, but not the negative control, significantly reduced the expression of TET1 at the levels of mRNA and protein in uterine arteries of pregnant sheep ($P < 0.05$, Figure 3A). Estrogen has been shown to function as an initiator to upregulates TET1 in uterine arteries in pregnancy.²⁰ We then examined whether the regulation of TET1 by estrogen is altered by miR-210. Consistent with the previous findings,²⁸ the *ex vivo* treatment of uterine arteries of non-pregnant sheep with estrogen in conjunction with progesterone for 48 hours significantly increased the expression of TET1 at both protein and mRNA levels when the tissues were concurrently transfected with the miR-210 negative control (Figure 3B). However, the upregulation of TET1 by the hormonal treatment was ablated in the presence of miR-210 (Figure 3C).

MiR-210 suppresses BK_{Ca} channel β 1 subunit expression and channel activity

Given that TET1-induced active demethylation of KCNMB1 promoter plays an important role in pregnancy- and steroid hormone-induced upregulation of BK_{Ca} channel β 1 subunit in uterine arteries,²⁰ we examined the expression of BK_{Ca} channel β 1 subunit in uterine arteries following miR-210 transfection. As shown in Figure 4A, transfection of uterine arteries of pregnant sheep with miR-210 mimic significantly reduced the expression of BK_{Ca} channel β 1 subunit mRNA and protein by ~60%, respectively ($P < 0.05$). In uterine arteries of non-pregnant animals transfected with the negative control, estrogen and progesterone treatment enhanced the protein and mRNA expression of BK_{Ca} channel β 1 subunit (Figure 4B). However, miR-210 blocked the steroid hormone-induced upregulation of BK_{Ca} channel β 1 subunit in the uterine artery (Figure 4C).

Functionally, miR-210 treatment significantly decreased BK_{Ca} channel current density (e.g., 10.7 ± 1.3 pA/pF versus 26.9 ± 2.1 pA/pF at +80 mV; $P < 0.05$) in uterine arteries of pregnant animals, whereas the negative control had no effect (e.g., 25.9 ± 2.5 pA/pF versus 26.9 ± 2.1 pA/pF at +80 mV; $P > 0.05$, Figure 5A). As shown in Figure 5B, the *ex vivo* steroid hormone treatment significantly increased BK_{Ca} channel current density in negative control-transfected uterine arteries from non-pregnant animals (e.g., 36.4 ± 2.0 pA/pF versus 13.8 ± 1.3 pA/pF at +80 mV; $P < 0.05$). Although the steroid hormone treatment slightly but significantly increased the channel current density in uterine arteries of non-pregnant animals in the presence of miR-210 (e.g., 14.5 ± 1.4 pA/pF versus 10.8 ± 0.8 pA/pF at +80 mV; $P < 0.05$; Figure 5C), the hormonal effect was dramatically diminished compared to the negative control.

Blockade of endogenous miR-210 inhibits hypoxia-induced suppression of BK_{Ca} channel activity

We further determined a cause-and-effect role of endogenous miR-210 in uterine arteries in the regulation of BK_{Ca} channel activity under hypoxia. Similar to the findings *in vivo* in animals exposed to high altitude hypoxia, *ex vivo* hypoxic treatment (10.5% O₂ for 48 hours) of uterine arteries significantly increased miR-210 expression (Supplemental Figure S2A). Consistent with the previous finding²³, *ex vivo* hypoxia significantly decreased BK_{Ca} channel current density (e.g., 11.8 ± 1.0 pA/pF versus 31.2 ± 2.9 pA/pF at +80 mV, $P < 0.05$, Figure S2B). Of importance, blockade of endogenous miR-210 in uterine arteries with the miR-210-antimiR LNA (miR210-LNA)^{48–50} inhibited hypoxia-induced suppression of BK_{Ca} channel current density (e.g., 33.9 ± 3.1 pA/pF versus 15.8 ± 1.3 pA/pF at +80 mV, $P < 0.05$, Figure S2C).

MiR-210 inhibits BK_{Ca} channel-mediated relaxation

Impaired BK_{Ca} channel function contributes to increased uterine vascular tone.²³ To seek a link between elevated expression of miR-210 and uterine vascular dysfunction, we tested functional effects of miR-210 on BK_{Ca} channel-mediated vasorelaxation and myogenic response of uterine arteries. Consistent with our previous studies,^{24, 53} the BK_{Ca} channel opener NS1619 induced concentration-dependent relaxations of uterine arteries from pregnant sheep, and miR-210 treatment significantly inhibited NS1619-induced relaxations (Figure 6A). In uterine arteries of non-pregnant animals, NS1619-induced relaxations were

reduced as compared with those in pregnant animals, which were significantly increased by the steroid hormone treatment (Figure 6B). Of importance, the hormonal treatment failed to augment NS1619-induced relaxations following miR-210 transfection (Figure 6C). In addition, pressure-dependent myogenic tone in uterine arteries of pregnant sheep was significantly increased following the miR-210 treatment (Figure 6D). In uterine arteries of non-pregnant animals transfected with the negative control, the *ex vivo* steroid hormone treatment significantly decreased pressure-dependent myogenic tone (Figure 6E). However, the miR-210 treatment rescinded the steroid hormone-induced attenuation of uterine vascular tone (Figure 6F).

Gestational hypoxia increases uterine vascular resistance and blood pressure

As shown in Supplemental Figure S3A, pregnancy was associated with a decrease in systolic, diastolic and mean arterial blood pressure in low altitude animals. High altitude hypoxia had no significant effect on blood pressure in non-pregnant animals, but increased systolic and mean arterial blood pressure in pregnant sheep and abolished the pregnancy-induced decrease in blood pressure. Pregnancy resulted in a dramatic increase in uterine artery blood flow from 14.2 ± 2.0 ml/min in non-pregnant sheep to 598.1 ± 35.2 ml/min in near-term pregnant animals at low altitude, which were not significantly different in animals exposed to high altitude hypoxia (12.6 ± 1.0 ml/min versus 530.9 ± 43.4 ml/min). However, gestational hypoxia resulted in about 45% increase (0.16 ± 0.01 mmHg*min/ml versus 0.11 ± 0.01 mmHg*min/ml, $P < 0.05$) in uterine vascular resistance in pregnant animals (Figure S3B).

Discussion

Aberrant expression of miRs and DNA hypermethylation occurred in both preeclampsia and gestational hypoxia. However, the roles of these two important epigenetic modifications in the pathogenesis of pregnancy complications are not fully understood. The present study revealed that gestational hypoxia elevated miR-210 levels in the uterine artery. TET1 was identified as a target of miR-210 and its expression in the uterine artery was downregulated by miR-210. We also demonstrated that miR-210 impaired BK_{Ca} channel function due to the repression of the BK_{Ca} channel $\beta 1$ subunit, a downstream target of TET1. Of importance, blockade of endogenous miR-210 in uterine arteries with miR210-LNA inhibited hypoxia-induced suppression of BK_{Ca} channel current density, demonstrating a cause-and-effect role of endogenous miR-210 in uterine arteries in the regulation of BK_{Ca} channel activity under hypoxia. Thus, our findings provide a novel link between the two important epigenetic mechanisms of miRs and TET-induced demethylation, and implicate miR-210-mediated TET1 silencing as a key mechanism promoting the downregulation of BK_{Ca} channel $\beta 1$ subunit expression and the channel function, leading to uterine vascular dysfunction in gestational hypoxia and preeclampsia.

Hypoxia is the driving force behind the increased expression of miR-210. The induction of miR-210 is a typical hypoxic response in a broad variety of cells/tissues, including vascular smooth muscle cells and endothelial cells.^{32, 54–57} In the present study, we observed 3 to 5-fold increases in miR-210 in uterine arteries from animals acclimatized to high altitude

hypoxia regardless of the status of pregnancy, demonstrating that prolonged high altitude hypoxic environment promoted expression of miR-210. Consistently, high altitude also increased circulating and placenta levels of miR-210 in both non-pregnant and pregnant human subjects.^{41, 43, 58} Of importance, increased expression of miR-210 was also observed in preeclamptic placenta.^{37–40, 42} HIF-1 α is a major regulator of the cellular response to hypoxia.⁵⁹ MiR-210 transcription is mainly regulated by HIF-1 α , and the hypoxia response elements on the proximal miR-210 promoter are highly conserved across species between humans and animals, suggesting that HIF-1 α is phylogenetically conserved in the regulation of miR-210 *de novo* synthesis.^{32, 60, 61} Consistent with the present finding of increased miR-210, our previous studies demonstrated that high altitude acclimation elevated expression of HIF-1 α in uterine arteries of non-pregnant and pregnant sheep.⁴⁷ The miR-210 gene resides close to *HRAS* and *RASSF7*, the expression of which are also induced by hypoxia due to genomic proximity.³¹ Indeed, the expression of RASSF7 coordinated with miR-210 in response to hypoxia and its mRNA abundance was significantly increased in uterine arteries by chronic hypoxia, albeit its function remains to be explored.

MiRs are important players in the epigenetic control of gene expression patterns by targeting the mRNA 3'UTR and resulting in degradation of mRNAs or translational suppression of the target transcripts.³⁰ Several targets of miR-210 in placenta and their contribution to the pathogenesis of preeclampsia have been identified, including potassium channel modulatory factor 1 and iron-sulfur cluster scaffold protein (ISCU).^{39–41, 62–65} Similarly, miR-210 also contributes to the development of pulmonary hypertension by repressing ISCU⁵⁶ as seen in preeclamptic placenta. Of interest, repression of TETs were also observed in placenta and umbilical veins in preeclampsia.^{52, 66} The reciprocal changes in the expression between miR-210 and TET in preeclampsia imply that TET could be a target of miR-210. Indeed, this notion was validated with the bioinformatic analysis and, more importantly, was demonstrated with the luciferase reporter assay in the present study, in which TET1 was identified as a direct target of miR-210. Moreover, enforced expression of miR-210 in uterine arteries to simulate gestational hypoxia-induced elevation of miR-210 was able to suppress the expression of endogenous TET1 in the uterine artery, providing functionally supporting evidence. Likewise, various other miRs have also been shown to trigger TET repression *via* targeting 3'UTR of the genes in cell differentiation and cancer cell proliferation.^{28, 29, 67, 68} The functional significance of increased miR-210 in the maternal circulation remains to be determined. The elevated circulating miR-210 in gestational hypoxia and preeclampsia is likely to participate in the downregulation of TET1, as a recent study showed that extracellular miR-210 could be taken up by recipient cells to repress ISCU1/2.⁴³ Together, these findings suggest that the expression of TETs could be tuned by miRs in both physiological and pathophysiological conditions.

TET family-mediated demethylation regulates dynamic gene expression and plays a crucial role in epigenetic reprogramming.^{69, 70} We have recently demonstrated that reprogramming of KCNMB1 expression by estrogen is essential for the adaptive changes in uterine circulation during pregnancy.²⁰ Estrogen was found to induce TET1 expression, which in turn promoted active demethylation of KCNMB1 and increased expression of BK_{Ca} channel β 1 subunit in the uterine artery. Reduced uterine vascular tone was subsequently achieved as

the result of this TET1-BK_{Ca} channel regulatory axis upregulation. Thus, the repression of TET1 by miR-210 would be expected to impair BK_{Ca} channel β 1 subunit expression and channel function. Indeed, we demonstrated that ectopic expression of miR-210 decreased KCNB1 expression in parallel to TET1 repression and inhibited BK_{Ca} channel-mediated function in uterine arteries of pregnant animals. In addition, we demonstrated that *ex vivo* hypoxia treatment, in a way similar to gestational hypoxia at high altitude, increased miR-210 expression and suppressed BK_{Ca} channel activity in uterine arteries of pregnant animals. Of importance, blockade of endogenous miR-210 inhibited hypoxia-induced suppression of BK_{Ca} channel current density in uterine arteries. Moreover, miR-210 also diminished the ability of steroid hormones to upregulate TET1 and its downstream target KCNB1 in uterine arteries of non-pregnant animals. Ultimately, these alterations conferred by miR-210 lead to increased uterine vascular tone in pregnancy and/or impeded steroid hormone-induced attenuation of uterine vascular tone. These observations simulated the changes induced by high altitude and by the *ex vivo* treatment of uterine arteries with fumarate, a competitive TET inhibitor.^{17, 20, 23} Thus, our data define a novel role for aberrant miR-210 expression in the dysfunction of uterine vasculature conferred by high altitude hypoxia through targeting the TET1-KB_{Ca} channel axis. We have recently demonstrated that high altitude hypoxia upregulated DNMT3b expression in uterine arteries of pregnant sheep, which subsequently triggered downregulation of BK_{Ca} channel β 1 subunit and elevated uterine vascular tone.⁷¹ Thus, gestational hypoxia promoted hypermethylation of KCNB1 gene by enhancing methylation and diminishing demethylation machineries concurrently, leading to dysfunction of the uterine circulation. MiR-210 increased in uterine arteries of high-altitude non-pregnant sheep and might cause dysfunction of TET1. However, it had limited impact on KCNB1 expression since the promoter of this gene was already hypermethylated in uterine arteries of non-pregnant animals.¹⁷

Functionally, decreased BK_{Ca} channel activity and elevated myogenic tone lead to an increase in vascular resistance. Pregnancy is associated with a drastically decrease in uterine vascular resistance.^{5, 72} The present study demonstrated that gestational hypoxia at high altitude significantly increased uterine vascular resistance in pregnant animals. Increased uterine vascular resistance is often associated with increased risk for development of preeclampsia.^{73, 74} Similarly, high altitude acclimation increases the incidence of preeclampsia largely due to the increased uterine vascular resistance.^{21, 22, 75, 76} Indeed, we found that high altitude hypoxia increased systolic and mean arterial blood pressure in pregnant sheep. Similar findings were obtained in our previous study, showing that gestational hypoxia increased blood pressure in pregnant rats.⁷⁷ In pregnant women, the decline of mean arterial pressure peaks around 17–24 weeks of gestation, followed by a slow return to pre-pregnancy level at about 40-week of gestation.^{78, 79} Increased sympathetic activity during the third trimester of gestation is believed to bring mean arterial pressure to the non-pregnant level.⁸⁰ In the present study, blood pressure was measured noninvasively in animals under anesthesia, which is likely to suppress sympathetic nerve activity.^{81–83} Thus, the present study reveals the effect of gestational hypoxia primarily on the myogenic activity of peripheral vascular resistance independent of sympathetic nerve activity. Both human and ovine uterine arteries express the BK_{Ca} channel.^{6, 12–14, 84} Although dysfunction of the

BK_{Ca} channel in preeclampsia has yet to be determined, the present study and several lines of evidence support a potential mechanistic link between the miR-210-TET1-BK_{Ca} channel axis and the pathogenesis of preeclampsia. Thus, the previous studies showed that the induction of miR-210^{37–42} and downregulation of TETs^{52, 66} in uteroplacental circulation are common features in preeclampsia and gestational hypoxia.

Considering their roles in the pathogenesis of a variety of diseases, miRs have emerged as promising targets for therapeutic intervention.⁸⁵ The down- or up-regulated miRs in pathological conditions could be corrected by using synthetic miR mimics or chemically modified anti-miR oligonucleotides. For example, intraperitoneal delivery of a locked nucleic acid antagonist LNA-92a has been shown to inhibit endometrial cancer growth in a rodent model.⁸⁶ In addition, catheter-based delivery of LNA-92a protects against ischemia/reperfusion injury in a large-animal model.⁸⁷ Thus, local application of anti-miR-210 could potentially be used to counteract the uterine circulation dysfunction caused by the imbalance of miR-210 in preeclampsia and other pregnancy complications associated with hypoxia.

Perspective

Gestational hypoxia-induced increase in uterine vascular resistance is linked to increased incidence of preeclampsia and fetal growth restriction. Dysfunction of the BK_{Ca} channel has been shown to play an important role in the occurrence of aberrant uterine circulation. The present study provides novel evidence that hypoxia-induced miR-210 triggers KCNMB 1 gene repression *via* directly targeting TET1 in the uterine artery. The suppression of TET1-BK_{Ca} axis contributes to the maladaptation of uterine circulation during pregnancy by increasing uterine vascular resistance, which may lead to pregnancy complications including preeclampsia. These findings have important implications for our understanding of the etiology of pregnancy complications including preeclampsia and for the development of novel strategies to prevent and cure these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

What Is New?

- Gestational hypoxia increases miR-210 expression in uterine arteries.
- MiR-210 targets and represses TET1 expression, resulting in downregulation of BK_{Ca} channel β 1 subunit and a reduction in channel activity.
- MiR-210 rescinds pregnancy- and steroid hormone-induced augmentation of BK_{Ca} channel-mediated relaxations and attenuation of uterine vascular tone.

What Is Relevant?

- The study reveals a novel mechanism of increased miR-210 in uterine vascular dysfunction by targeting the TET1-BK_{Ca} channel axis, providing new insights in our understanding of the pathogenesis of preeclampsia and in the development of potential therapeutic strategies for pregnancy complications associated with gestational hypoxia.

Summary

In the present study, we demonstrated that gestational hypoxia increases miR-210 expression in uterine arteries. Of importance, miR-210 represses TET1 and its downstream target KCNMB1. The suppression of the TET1-BK_{Ca} channel axis by miR-210 impairs pregnancy- and steroid hormone-induced upregulation of BK_{Ca} channel function, resulting in elevated uterine vascular resistance and blood pressure. Together, our findings implicate aberrant expression of miR-210 as a key contributor to uterine vascular dysfunction during gestation and point to a critical role of epigenetic modifications in the development of preeclampsia.

Figure 1. Gestational hypoxia increases miR-210 expression in uterine arteries

MiR-210 was determined with real-time qPCR in uterine arteries isolated from low altitude (normoxic control) and high altitude (hypoxia) non-pregnant and pregnant sheep. Data are means $\pm$ S.E.M. from 5 animals of each group. *P < 0.05, hypoxia versus normoxic control.

Ovine TET1 mRNA

Figure 2. MiR-210 targets TET1 mRNA 3'UTR

The diagram shows ovine TET1 mRNA 3'UTR with the binding sites of miR-210. The pmiRGLO plasmid inserted with TET1 3'UTR sequences containing putative miR-210 binding sites (pmiRGLO-TET244 harboring a 244-bp 3'UTR of ovine TET1 mRNA) and pmiRGLO vector control were transfected into uterine arterial vascular smooth cells and were treated with miR-210 mimic or miR-210 scramble. Firefly and Renilla reniformis luciferase activities were measured in a luminometer using a dual-luciferase reporter assay system. Data are means $\pm$ S.E.M., n = 8. *P < 0.05, pmiRGLO-TET244+miR-210 versus pmiRGLO-TET244 alone.

Figure 3. MiR-210 represses TET1 in uterine arteries

TET1 mRNA and protein abundance was determined in uterine arteries. **A.** Uterine arteries from pregnant animals were treated with miR-210 mimic (miR-210) or negative control for 48 hours. Data are means $\pm$ S.E.M. from 4–5 animals of each group. * $P < 0.05$, miR-210 versus negative control. **B.** Uterine arteries from non-pregnant animals were treated with 17 β -estradiol (E₂ β ; 0.3 nmol/L)/progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 negative control. Data are means $\pm$ S.E.M. of 4–5 animals of each group. * $P < 0.05$; +E₂ β /P4 versus vehicle control. **C.** Uterine arteries from non-pregnant animals were treated with 17 β -estradiol (E₂ β ; 0.3 nmol/L)/progesterone (P4; 100.0

nmol/L) or vehicle control for 48 hours in the presence of miR-210 mimic. Data are means $\pm$ S.E.M. of 4–5 animals of each group.

Figure 4. MiR-210 decreases BK_{Ca} channel β1 subunit in uterine arteries

BK_{Ca} channel β1 subunit (BKβ1) mRNA and protein abundance was determined in uterine arteries. **A.** Uterine arteries from pregnant animals were treated with miR-210 mimic (miR-210) or negative control for 48 hours. Data are means ± S.E.M from 4 animals of each group. *P < 0.05, miR-210 versus negative control. **B.** Uterine arteries from non-pregnant animals were treated with 17β-estradiol (E₂β; 0.3 nmol/L)/progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 negative control. Data are means ± S.E.M. of 4–5 animals of each group. *P < 0.05; +E₂β/P4 versus vehicle control. **C.** Uterine arteries from non-pregnant animals were treated with 17β-estradiol (E₂β; 0.3

nmol/L)/progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 mimic. Data are means $\pm$ S.E.M. of 4–5 animals of each group.

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Figure 5. MiR-210 inhibits BK_{Ca} channel activity in uterine arteries

BK_{Ca} channel current density was determined in uterine artery smooth muscle cells. BK_{Ca} currents were normalized to cell capacitance and expressed as picoampere per picofarad (pA/pF; y axis), as a function of stepwise 10-mV depolarizing pulses (x axis). **A.** Uterine arteries from pregnant animals were treated in the absence (untransfected) or presence of miR-210 mimic (miR-210) or negative control for 48 hours. Inset image shows smooth muscle cells transfected with miR-210 mimic. Data are means $\pm$ S.E.M. of 7 cells from 5 animals of each group. *P < 0.05, miR-210 versus negative control. **B.** Uterine arteries from non-pregnant animals were treated with 17 β -estradiol (E₂β; 0.3 nmol/L)/progesterone (P₄;

100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 negative control. Data are means $\pm$ S.E.M. of 7 cells from 5 animals of each group. * $P < 0.05$; +E₂ β /P4 versus vehicle control. C. Uterine arteries from non-pregnant animals were treated with 17 β -estradiol (E₂ β ; 0.3 nmol/L)/progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 mimic. Data are means $\pm$ S.E.M. of 7 cells from 5 animals of each group. * $P < 0.05$; +E₂ β /P4 versus vehicle control.

Figure 6. MiR-210 impairs BK_{Ca} channel-mediated relaxations and increases pressure-dependent myogenic tone in uterine arteries

NS1619-induced relaxations were determined in: **A.** uterine arteries from pregnant animals treated with miR-210 mimic (miR-210) or negative control for 48 hours; **B.** uterine arteries from non-pregnant animals treated with 17β-estradiol (E₂β; 0.3 nmol/L)/progesterone (P₄; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 negative control; **C.** uterine arteries from non-pregnant animals treated with 17β-estradiol (E₂β; 0.3 nmol/L)/progesterone (P₄; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 mimic. Pressure-dependent myogenic tone was measured in: **D.** uterine arteries from

pregnant animals treated with miR-210 mimic (miR-210) or negative control for 48 hours; **E.** uterine arteries from non-pregnant animals treated with 17 β -estradiol (E₂ β ; 0.3 nmol/L)/progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 negative control; **F.** uterine arteries from non-pregnant animals treated with 17 β -estradiol (E₂ β ; 0.3 nmol/L)/progesterone (P4; 100.0 nmol/L) or vehicle control for 48 hours in the presence of miR-210 mimic. Data are means $\pm$ SEM of tissues from 5 animals of each group. *P < 0.05, miR-210 versus negative control or +E₂ β /P4 versus vehicle control.