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Original Article

Upregulated miR-222 targets BCL2L11 and promotes apoptosis of mesenchymal stem cells in preeclampsia patients in response to severe hypoxia

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Abstract: Abnormal maternal trophoblast invasion is a common finding in preeclampsia pregnancy. A hypoxic environment develops in the placenta after the 10th week of pregnancy and exerts a major influence over trophoblast activity. In the present study, we investigated expression of miR-222 and apoptosis-related BCL2L11 in preeclampsia placenta and in primary placenta mesenchymal stem cells (MSCs) under hypoxia. The results demonstrate that miR-222 is upregulated in the placenta of preeclampsia patients, along with the downregulation of BCL2L11 in both mRNA and protein levels. *In vitro* results demonstrate that miR-222 is upregulated either in preeclampsia placenta tissues or in the MSCs under hypoxia. Western blotting showed downregulation of BCL2L11 in the trophoblasts under hypoxia, along with an increased MSC apoptosis. miR-222 was also confirmed to downregulate BCL2L11 expression via targeting the 3' untranslated region (UTR) of the *BCL2L11* gene. miR-222 inhibitor transfection markedly ameliorated expression and transcriptional activity of *BCL2L11*. Altogether, the present study found that upregulation of miR-222 promotes apoptosis of mesenchymal stem cells in preeclampsia patients in response to hypoxia, via targeting BCL2L11. This suggests that a key regulatory role of miR-222 is in preeclampsia progression.

Keywords: Apoptosis, BCL2L11, hypoxia, miR-222, preeclampsia

Introduction

Preeclampsia is an abnormal pregnancy-related syndrome with proteinuria and hypertension following 20-weeks of gestation [1]. Preeclampsia affects 3-5% of pregnancies worldwide and 9.4-10.4% of Chinese pregnancies every year, and is a leading risk factor for maternal, fetal, and neonatal mortality [2-4]. Preeclampsia is currently recognized to associate with inadequate trophoblast invasion and placentation [3], with decreased circulation in the early placenta, and with continuous placental ischemia after 20 weeks of gestation [5, 6]. The sustained ischemia/hypoxia promotes oxidative stress, and thus induces production of excessive carbon monoxide, nitric oxide, and androgen in the local microcirculation [7]. Thus, increased apoptosis of trophoblast cells in the placenta, which are driven by the oxidative stress, can decrease trophoblast invasion and may be the major pathological cause of preeclampsia [8, 9].

Mesenchymal stem cells (MSCs) represent a group of cells with multi-differentiation potential [10]. MSCs are mainly from bone marrow and human umbilical cord and have high regenerative capacities [11, 12]. MSCs are capable of differentiating into a wide variety of cell types [13] and display key roles in tissue regeneration and repair [14]. Interestingly, human umbilical cord MSCs from preeclampsia patients show differentiation, indicating clinical potential for the preeclampsia treatment [15]. Recent findings in mouse models indicated that MSCs have a significant influence on the course of preeclampsia [16].

microRNAs (miRNAs) are a group of short non-coding single-stranded RNA molecules, which inhibit mRNA translation or promote mRNA degradation through specific binding mainly to the 3'-untranslated region (UTR) of targeted genes [17]. miRNAs have been recognized to be involved in the pathogenesis of preeclampsia. Abnormal miRNA profiles have been identified

in placental tissues [18], placental trophoblasts [19], and placental MSCs [20], plasma/whole blood samples [21]. Accumulating endothelial-related miRNAs have been reported to be potentially important in hypoxia-promoted endothelial damage in preeclampsia [20, 21]. In particular, miRNAs, such as miR-16, miR-181a, and miR-494 are abnormally expressed in placental MSCs and inhibit the angiogenesis potential at the maternal-fetal interface in preeclampsia [16, 22-24]. Therefore, miRNAs may act as an important regulators involved in the pathogenesis of preeclampsia.

In this study, we analyzed expression of miR-222 and BCL2L11 (B-cell lymphoma 2) in placental tissues, and in MSCs from patients with preeclampsia. We explored miR-222 or BCL2-L11 expression in MSCs under normoxia or hypoxia. Finally we investigated the role of miR-222 in hypoxia-induced apoptosis and its effect on expression of BCL2L11 in hypoxia-treated MSCs. Our findings contribute to a greater understanding of miRNA function in preeclampsia MSCs.

Materials and methods

Placenta specimens

17 pregnant women with preeclampsia and 17 normal pregnant subjects were included in this study. All included subjects were registered at the Department of Obstetrics and Gynecology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University and formally signed consent at the time of study enrollment. The present study was approved by the Ethics Committee of our hospital. Detailed clinic-pathological information was reported previously (https://doi.org/10.5114/aoms.2016.63261).

Isolation and culture of MSCs

Placentas from two groups of donor mothers were obtained immediately post-delivery. For BCL2L11 immuno-staining, placenta sections were fixed with 10% formalin and were embedded with paraffin. Decidua basalis was dissected from the maternal-facing surface of placenta, and were immediately set in ice-cold phosphate buffered saline (PBS) for the MSC isolation. The MSC isolation was performed according to a reported protocol [25]. In brief, decidua basalis was mechanically minced, and enzymatically digested with 0.1% collagenase IV (Thermo Fisher Scientific, Inc., Waltman, MA, USA) to produce the cell suspension. Following a series of centrifugation steps (with or without Ficoll) and washes with PBS, the cell pellet was suspended in the complete low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Ameresco, Framingham, MA, USA).

MSCs were seeded and cultured in 24-well plates with complete medium. 6-10 days post culture, the fibroblast-like cells adhered in the well during primary passage and were harvested using 0.25% trypsinase (Gibco-BRL, Hangzhou, China), and then seeded in 24-well plates at the density of 5×10^4 cells/cm^2 for secondary passage. The non-adherent cells were harvested and transferred into another 24-well plate by updating 1/2 medium. Cells were cultured at 37°C under 5% CO_2 and 20% O_2 routinely. Hypoxia treatment *in vitro* was simulated by the chemical hypoxia agent $CoCl_2$ (100 mmol/L).

Quantitative real-time polymerase chain reaction for BCL2L11 mRNA and miR-222

PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) were utilized for the mRNA and miRNA isolation respectively. Each sample of mRNA or miRNA was supplemented with ribonuclease inhibitor (Promega, Madison, WI, USA) and was was quantified for BCL2L11 mRNA level or for miR-222 level by the quantitative real-time PCR (qRT-PCR) method with Takara One Step RT-PCT kit (Takara, Tokyo, Japan) (for mRNA) or with mirVana™ qRT-PCR miRNA Detection Kit (Thermo Scientific, Rockford, IL, USA) (for miRNA). Relative levels of BCL2L11 mRNA or miR-222 were calculated using the ΔΔCt method using β-actin or U6 as internal control gene [26].

Immuno-staining for BCL2L11 in placenta tissues

Paraffinized placenta tissue slices were firstly deparaffinized, and subjected to antigen resurrection according to a general standard protocol. Polyclonal rabbit anti-BCL2L11 (Cell Signaling Technology Inc., Danvers, MA, USA) and HRP-coupled goat anti-rabbit IgG antibodies (Abcam, Cambridge, UK) were utilized for the BCL2L11 immuno-staining in the placental tissue. 3-amino-9-ethylcarbazole (AEC) (Sigma-Aldrich, St. Louis, MO, USA) was utilized as an HRP substrate for staining and the immune-

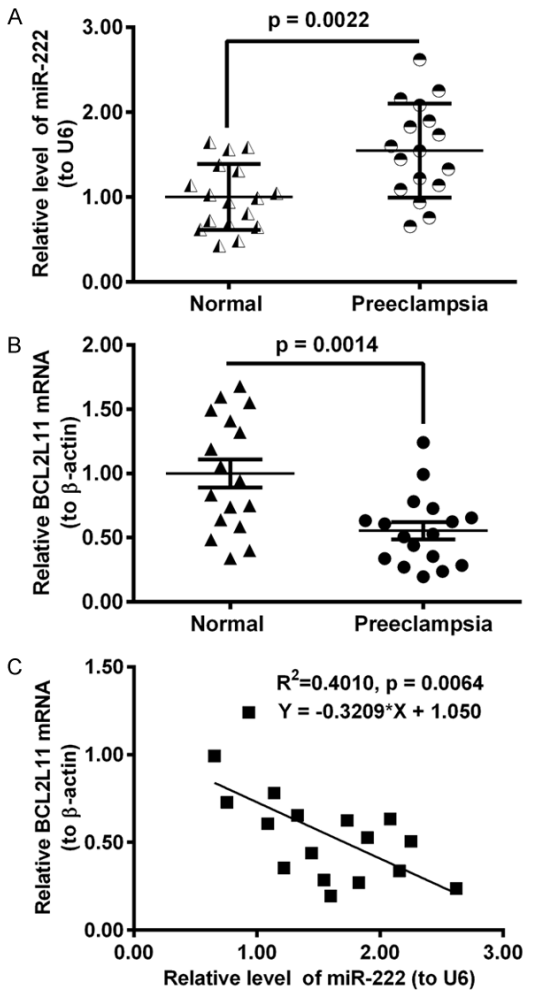

Figure 1. Quantification of miR-222 and BCL2L11 mRNA in preeclampsia placental specimens. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure the relative miR-222 (A) (to U6) and the relative BCL2L11 mRNA (B) (to β-actin) in the placental specimens from preeclampsia (N = 17) or normal (N = 17) subjects. (C) Correlation of miR-222 level with BCL2L11 mRNA in the placental specimens from preeclampsia subjects. Statistical significance with a *P* value <0.05 or less.

staining was semi-quantified by counting AEC-positive cells.

Western blot analysis

Protein samples from placental tissue or MSCs were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL, USA) under the guidance of kit's manual. For the Western blot of BCL2L11, each sample was separated by electrophoresis (12% SDS-PAGE gel), transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA), blocked with 2% BSA (Sigma-Aldrich, St. Louis, MO, USA) and then the membrane was incubated successively with rabbit polyclonal antibody against human BCL2L11 or β-actin respectively at room temperature for 2 hours. HRP-conjugated secondary antibody against rabbit IgG at 4°C was added for another one hour, and finally electrochemiluminescence (ECL) (Amersham, Uppsala, Sweden) was performed at room temperature for 25 minutes. The levels of BCL2L11 are presented as a percent gray value normalized to β-actin.

Luciferase reporting assay

The 3'UTR of *BCL2L11* and the CMV (Cytomegalovirus) promoter were cloned into the pGL3-luciferase basic vector (Promega, Madison, WI, USA). Sequences of primers and cloning strategy are available on request. For the luciferase assays, 50 nM hsa-miR-222 miRNA precursor (Thermo Scientific, Rockford, IL, USA) or scrambled Pre-miR™ miRNA Precursor Negative Control (Thermo Scientific, Rockford, IL, USA) were transfected with siPORT™ NeoFX™ Transfection Agent (Thermo Scientific, Rockford, IL, USA) into the cultured 80%-confluent MSCs, 2 hours later, MSCs were then transfected with the pGL3-luciferase reporter vector and the Renilla control vector (Promega, Madison, WI, USA) by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 24 h post transfection, the Dual Luciferase reporter assay kit (Promega, Madison, WI, USA) was utilized to examine luciferase activity.

Apoptosis assay

Annexin V/Propidium Iodide double staining was performed to determine the placental MSC apoptosis using an Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, UK). In brief, 1×10^4 MSCs per ml aliquot were suspended in PBS, and 5 μl annexin V-FITC and 10 μl PI were added, followed by incubation for 10 min at 4°C in the dark. Finally, the stained cells were visualized with a live cell imaging system (Olympus LCS SYSTEM, Tokyo, Japan). Early apoptotic MSCs (Annexin V+/PI-) and late apoptotic MSCs (Annexin V+/PI+) were expressed as apoptotic MSCs to total MSCs.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT cell viability assay kit (Biotium Inc, Beijing, China) was utilized to examine the MSC viabili-

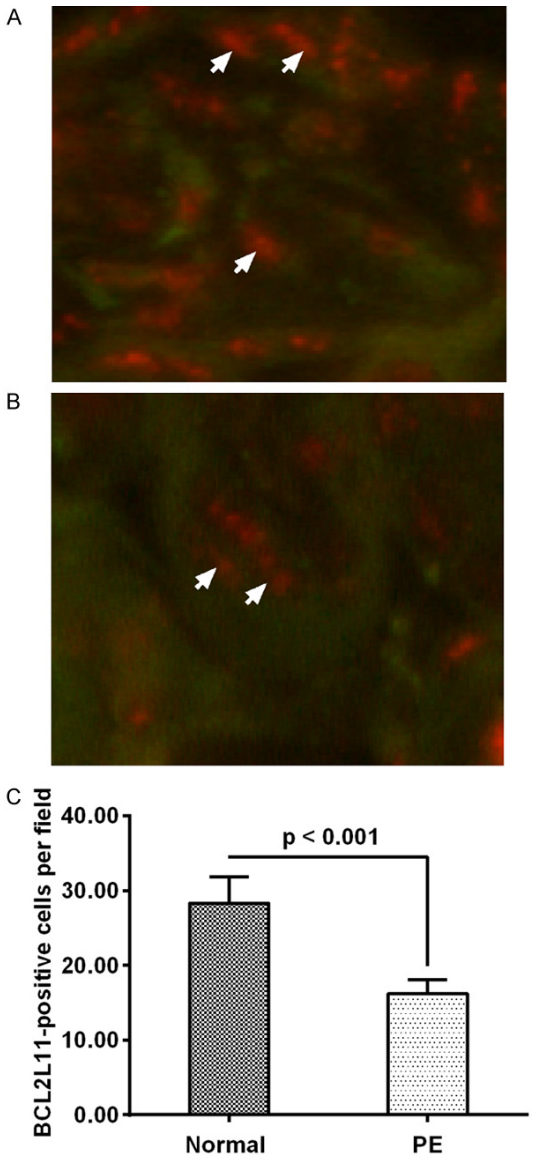

Figure 2. Immunohistochemical staining for BCL2L11 in preeclampsia placental specimens. BCL2L11 staining by immunofluorescence on the placenta tissues from normal (n = 17) (A) or from preeclampsia (n = 17) (B) subjects. The BCL2L11 positive staining is indicated with white arrows. (C) BCL2L11-positive cell number in preeclampsia and normal groups. Statistical significance with a *P* value <0.05 or less.

ty. MSCs were seeded in 96-well plates to approximately 85% confluence and then cells were incubated at 37°C under hypoxia or normoxia for 6 or 12 hours. 10 μL MTT was added to each well and was incubated at 37°C for another 2 hours. Finally, 100 μl DMSO was added into each well to dissolve the formazan, and the absorbance was measured with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Statistical analysis

Quantitative data were presented as means ± SEM. A nonparametric Mann-Whitney test or ANOVA StatView was used for data analysis on GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered when $P<0.05$ or less.

Results

Upregulation of miR-222 in association with BCL2L11 downregulation in preeclampsia placentas specimens

Significant increase of miR-222 has been found in maternal plasma at the time of severe preeclampsia [27]. However, it is not clear whether its expression in preeclamptic placental tissues is of functional significance. To investigate the miR-222 level in preeclampsia placentas, we quantified miR-222 (relative to U6) in 17 preeclampsia subjects. As indicated in **Figure 1A**, the relative miR-222 level was upregulated in the preeclampsia group ($P = 0.0022$). Various genes, including *BCL2L11,* are targeted by miR-222 [28, 29]. Therefore, BCL2L11 expression in the two groups of samples was examined and it is shown in **Figure 1B** that BCL2L11 mRNA level was significantly downregulated in the preeclampsia group ($P = 0.0014$). Moreover, there was a negative association of BCL2L11 downregulation with the miR-222 upregulation ($P = 0.0064$, $R^2 = 0.4010$, **Figure 1C**).

Immunohistochemical analysis was also performed to verify the BCL2L11 downregulation in the preeclampsia group. In contrast to staining for BCL2L11 in the placentas tissues of the control group (**Figure 2A**), there were fewer stained cells in the preeclampsia placentas tissues (**Figure 2B**), which was confirmed by quantitative analysis ($P<0.001$, **Figure 2C**). Thus, we found that upregulation of miR-222 in association with BCL2L11 downregulation was prominent in preeclampsia placentas specimens.

Hypoxia upregulates miR-222 and downregulates BCL2L11 in placental mesenchymal stem cells (MSCs)

The role of hypoxia in promoting preeclampsia has been identified previously [30, 31]. We were thus interested in whether hypoxia regulates both miR-222 and BCL2L11 in human placental cells. Human placenta mesenchymal

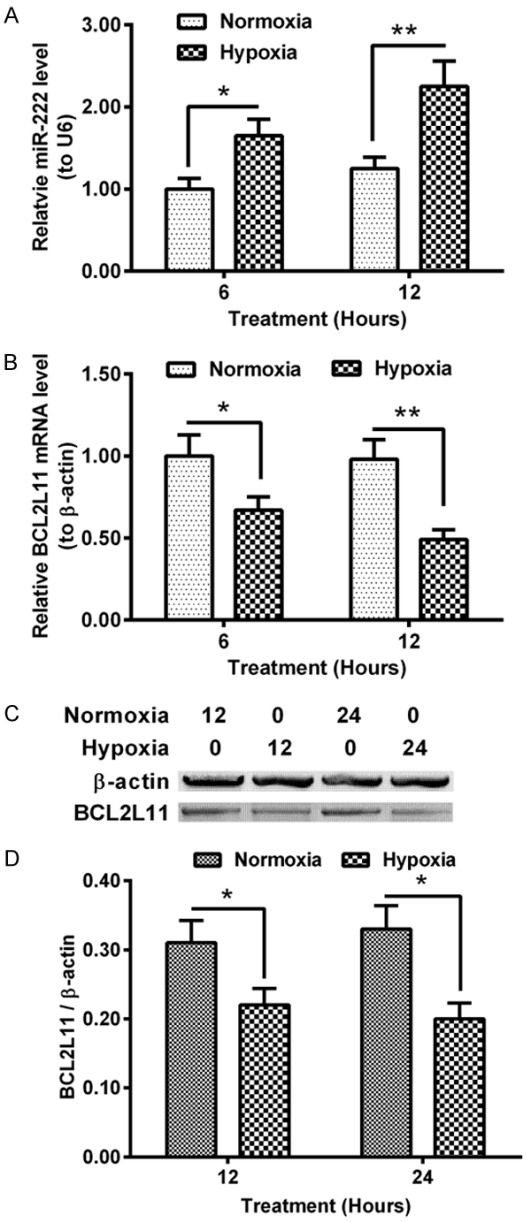

Figure 3. Expression of miR-222 and BCL2L11 in placental MSCs under hypoxia. Approximately 85%-confluent MSCs were cultured under normoxia or hypoxia for another 6, 12, or 24 hours, then qRT-PCR was performed to measure the miR-222 (A) and BCL2L11 mRNA (B) levels in MSCs under normoxia or hypoxia for 6 or 12 hours. (C and D) Western blotting (C) and quantification (D) of BCL2L11 in the normoxia- or hypoxia-treated MSCs under normoxia or hypoxia for 12 or 24 hours. Triple independent experiments were performed. **P*<0.05 or ***P*<0.01.

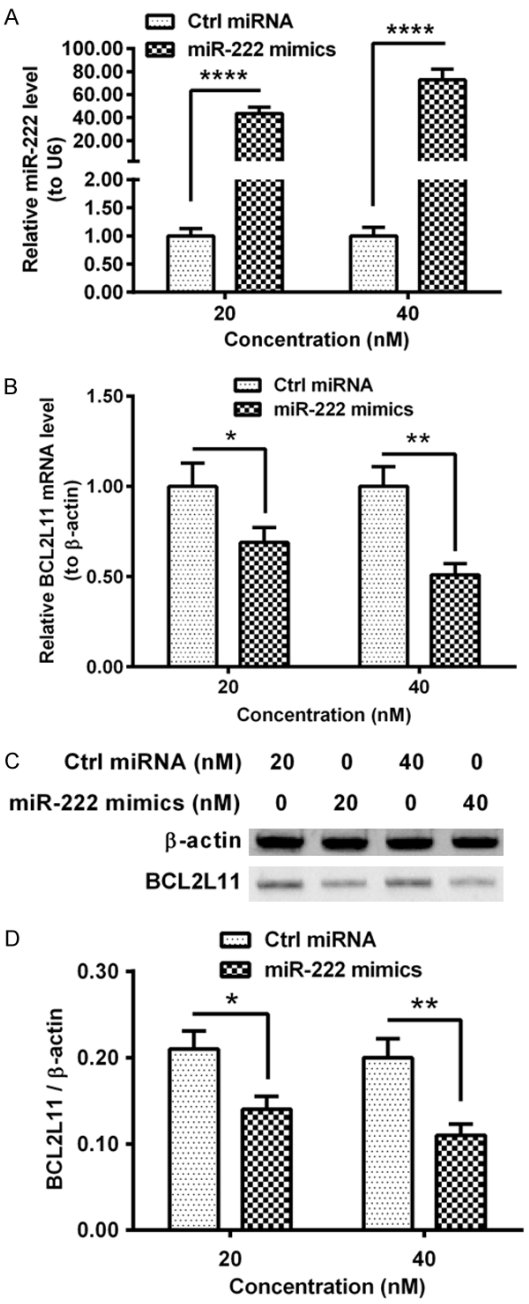

Figure 4. Downregulation by miR-222 on BCL2L11 in MSCs *in vitro*. Approximately 85%-confluent MSCs were transfected with 20 or 40 nM miR-222 mimics (to U6) or scrambled RNA (Ctrl miRNA) for 12 or 24 hours, and the relative miR-222 level (to U6) (A) and the relative mRNA level of BCL2L11 (to β-actin) (B) were quantified. (C and D) Western blot analysis (C) of and the quantification (D) of BCL2L11 protein in the miR-222 mimics- or the scrambled RNA-transfected (for 24 hours) MSCs. Triple independent experiments were performed. **P*<0.05, ***P*<0.01 or *****P*<0.0001.

stem cells (MSCs) were cultured under hypoxia and normoxia and then expression of both molecules was examined. **Figure 3A** demonstrates a higher level of miR-222 in hypoxia-treated MSCs, either at 6 or 12 hours post treatment (*P*<0.05 or *P*<0.01). On the other hand, BCL2L11 mRNA level (relative to β-actin) was

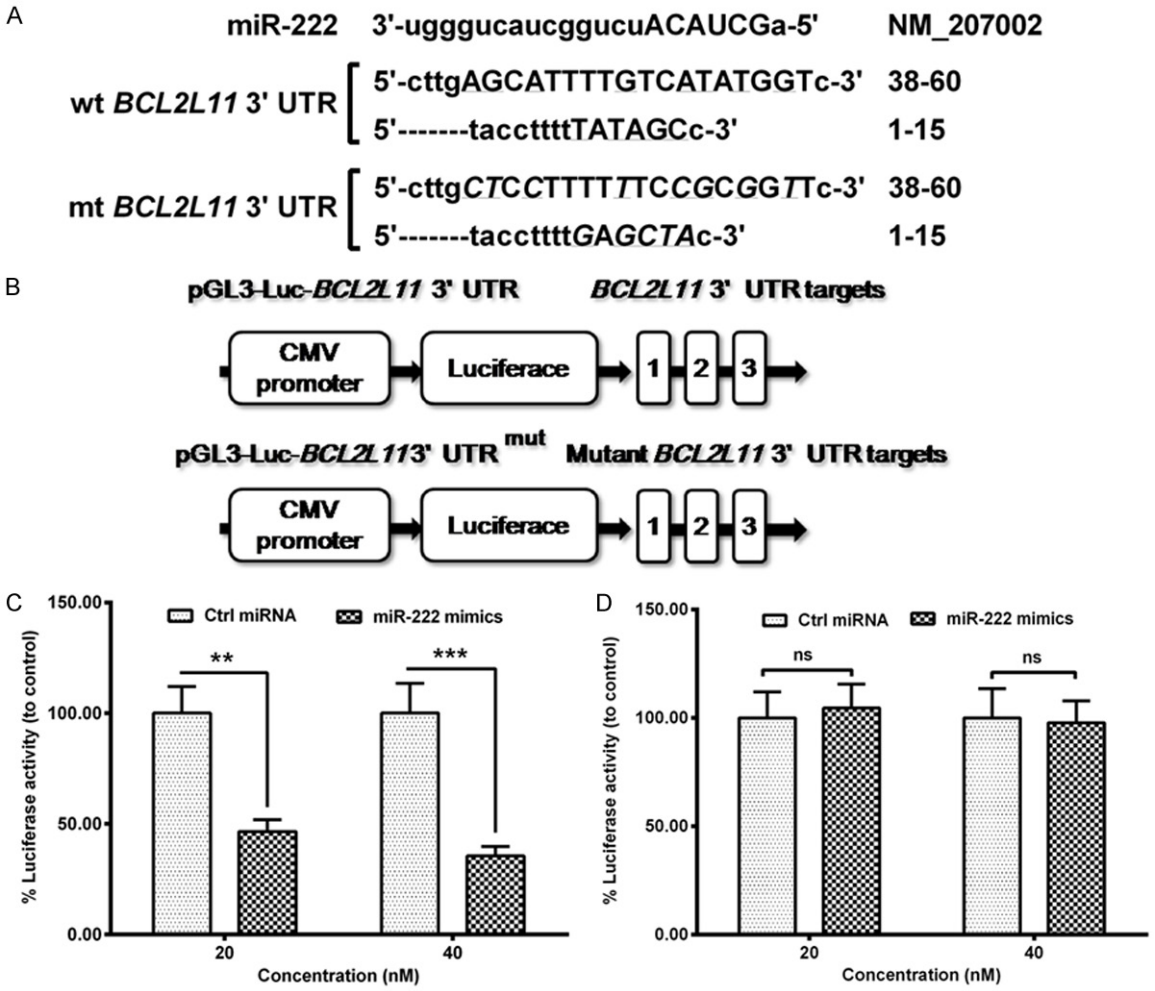

Figure 5. Regulation by miR-222 on BCL2L11 expression via targeting 3'UTR of *BCL2L11* in MSCs with a luciferase reporter. (A and B) Sequence analysis of *Homo sapiens* miR-222 and the 3'UTR of *Homo sapiens BCL2L11* (A) and a sketch (B) of the luciferase reporter with wild-type and mutant 3'UTR of *BCL2L11* inserted behind the cytomegalovirus promoter. (B and C) Relative luciferase in MSCs, which were transfected with the reporter with wild-type (C) or mutant (D) 3'UTR of *BCL2L11*. Triple independent experiments were performed. $^{**}P<0.01$, $^{****}P<0.0001$, ns: no significance.

downregulated in the hypoxia group ($P<0.05$ or $P<0.01$ for 6 or 12 hours, **Figure 3B**). Such downregulation was also confirmed at the protein level by Western blotting at 12 or 24 hour post hypoxia ($P<0.05$ or $P<0.01$, **Figure 2C** and **2D**).

miR-222 downregulates BCL2L11 via targeting the 3'UTR of BCL2L11 gene in MSCs

BCL2L11 is identified as one target for miR-222 [28, 29]. We then investigated such targeting regulation by miR-222 on the *BCL2L11* gene in placental MSCs. Manipulated upregulation of miR-222 with miR-222 mimics ($P<0.0001$, **Figure 4A**) markedly downregulated BCL2L11 mRNA level in MSCs with a transfection concentration of 20 or 40 nM, with scrambled RNA as a control (Ctrl miRNA) ($P<0.05$ or $P<0.01$, **Figure 4B**). The BCL2L11 protein level was also significantly downregulated by transfection of miR-222 mimics at a concentration of 20 or 40 nM ($P<0.05$ or $P<0.01$, **Figure 4C**).

In addition, we also constructed a luciferase-based reporter for the paired sequence (**Figure 5A**) to miR-222 in the 3'UTR of the *BCL2L11* gene (**Figure 5B**). A control reporter was also constructed with a mutant 3'UTR sequence (unpaired to miR-222) of the *BCL2L11* gene (**Figure 5B**). The luciferase reporter assay confirmed that transfection with 20 or 40 nM miR-

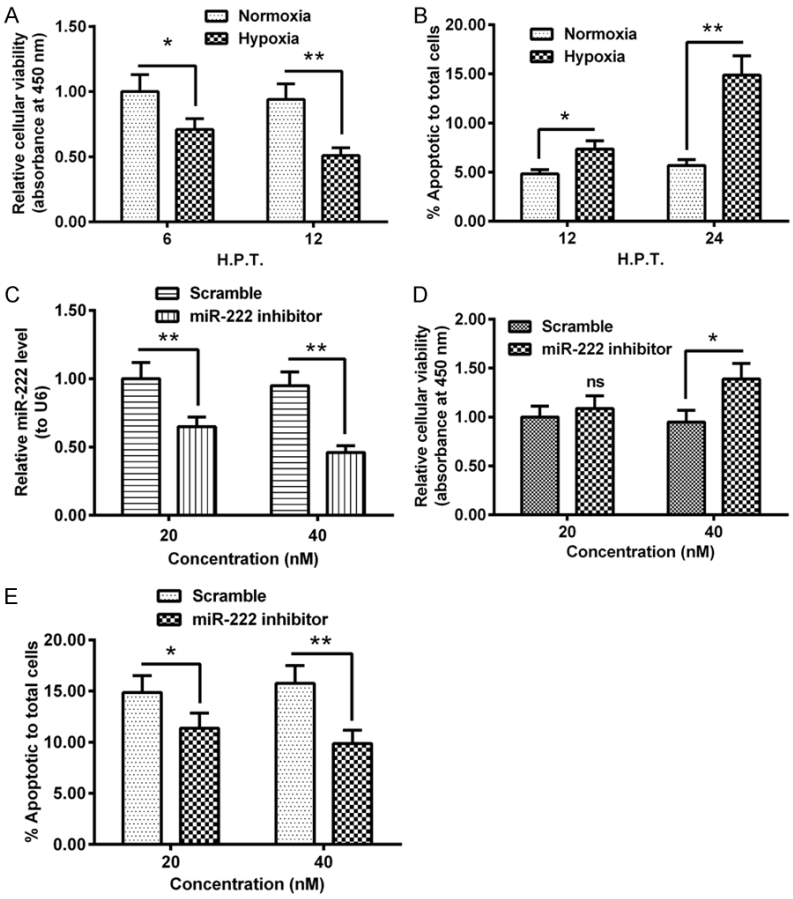

Figure 6. miR-222 regulates hypoxia-induced cellular viability reduction and apoptosis in MSCs. (A and B) Relative cellular viability (A) and apoptosis (B) of the MSCs, which were cultured under normoxia or hypoxia for 6, 12 or 24 hours. Cellular viability was examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and cell apoptosis was determined by flow cytometry analysis. (C) Relative miR-222 level (to U6) in the MSCs, which were transfected with 20 or 40 nM miR-222 inhibitor (with Scramble RNA as control) for 24 hours. (D and E) Relative cellular viability (D) and apoptosis (E) of the MSCs, post the miR-222 inhibitor- or scrambled RNA-transfection (20 or 40 nM) and under hypoxia. Triple independent experiments were performed. **P*<0.05, ***P*<0.01, ns: no significance.

222 mimics markedly reduced luciferase activity in MSCs, compared to control miRNA (*P*<0.01 or *P*<0.001, **Figure 5C**). However, such differential regulation was not found between miR-222 and control miRNA with the mutant reporter (**Figure 5D**).

miR-222 regulates sensitivity of MSCs to hypoxia

Cellular viability and apoptosis were examined respectively with MTT assay and flow cytometry analysis. Hypoxia treatment significantly reduced cellular viability of MSCs for either 6 or 12 hours (*P*<0.05 or *P*<0.01, **Figure 6A**). Moreover, a significantly higher apoptosis was also induced in MSCs by hypoxia for 12 or 24 hours (*P*<0.05 or *P*< 0.01, **Figure 6B**). To evaluate the role of hypoxia-promoted miR-222 in the hypoxia-mediated cellular viability reduction and apoptosis induction, we knocked down miR-222 in the hypoxia-treated MSCs, and then re-examined cell viability and apoptosis. **Figure 6C** demonstrates that transfection with 20 or 40 nM miR-222 inhibitor significantly knocked down the miR-222 level (*P*< 0.01 respectively). Moreover, the miR-222 inhibitor-mediated miR-222 knockdown with 40 nM markedly ameliorated the hypoxia-induced loss of MSC viability (*P*<0.01, **Figure 6D**). Hypoxia-induced apoptosis was also reduced (*P*<0.05 for 20 nM or *P*<0.01 for 40 nM, **Figure 6E**).

Discussion

Increasing evidence indicates that there are impairments in preeclampsia [32] affecting trophoblast-mediated implantation and placentation, which are essential for the future development of embryo organisms [33, 34]. In particular, apoptosis of trophoblasts is increased in preeclampsia [35-38]. Therefore, placental hypoxia developed during preeclampsia represents one of the most common causes of placental trophoblast damage via apoptosis [37, 39, 40]. Our previous study also showed a promoting role for hypoxia in preeclampsia [41].

Significant increases of miR-222 have been found in maternal plasma at the time of severe preeclampsia [27]. We found here that miR-222 is upregulated in preeclampsia placentas specimens. Therefore, we speculate that miR-222 might play a role in preeclampsia. We also

found that BCL2L11 was significantly downregulated in both mRNA and protein levels in preeclampsia placentas specimens. *BCL2L11* is targeted by miR-222 [28, 29] and there is a negative association between the two deregulated molecules. This data implies that miR-222-mediated regulation might depend on targeting *BCL2L11*. Our *in vitro* results confirmed negative regulation by miR-222 on BCL2L11 via targeting the 3'UTR of *BCL2L11* gene in human placental MSCs. Manipulated upregulation of miR-222 with miR-222 mimics also markedly downregulated BCL2L11 in both mRNA and protein levels in MSCs with a concentration of 20 and 40 nM. Moreover, a luciferase-based approach using the paired sequence of *BCL2L11* gene indicated that miR-222 transfection markedly reduces luciferase activity in MSCs. Thus, we confirmed negative regulation by miR-222 on BCL2L11 in human placental MSCs.

Hypoxia has been identified to promote the development of preeclampsia [30, 31]. Our results further demonstrate that hypoxia upregulates miR-222 and downregulates BCL2L11 in placental MSCs. A higher level of miR-222 and reduced mRNA and protein levels of BCL2L11 were found in hypoxia-treated MSCs. The mechanism of the promotion by hypoxia to preeclampsia has not been clear. Our study indicates that hypoxia markedly downregulates cellular viability and promotes apoptosis of MSCs *in vitro*. Furthermore, hypoxia-upregulated miR-222 regulates the sensitivity of MSCs to hypoxia. The knockdown of miR-222 with a miR-222 inhibitor in the hypoxia-treated MSCs also significantly ameliorated the hypoxia-caused cellular viability reduction and the hypoxia-induced apoptosis in MSCs.

Taken together, we included that miR-222 is upregulated in preeclampsia placenta, associating with a reduced BCL2L11. miR-222 also modulates hypoxia-induced damage to human placental MSCs by downregulating BCL2L11 and inducing apoptosis. Our study implies that miR-222/BCL2L11 might be an important signal in preeclampsia.

Acknowledgements

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Disclosure of conflict of interest

None.

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