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microRNA-29b contributes to pre-eclampsia through its effects on apoptosis, invasion and angiogenesis of trophoblast cells

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Abstract

PE (pre-eclampsia), a pregnancy-specific disorder, is characterized by increased trophoblast cell death and deficient trophoblast invasion and reduced trophoblast-mediated remodelling of spiral arteries. The present study was performed to determine the function of *miR-29b* (microRNA-29b) in trophoblast cells and its underlying role in the pathogenesis of PE. The prediction of *miR-29b* target genes was performed using computer-based programs, including Targetscan, Pictar and miRBase. The function of these target genes was analysed further by gene ontology (GO). The effects of *miR-29b* on apoptosis, and invasion and angiogenesis of trophoblast cell lines (HTR-8/SVneo, BeWo and JAR) were examined by flow cytometry and Matrigel assay respectively. We found that *miR-29b* induced apoptosis and inhibited invasion and angiogenesis of trophoblast cells. Further studies confirmed that *miR-29b* regulated the expression of *MCL1* (myeloid cell leukaemia sequence 1), *MMP2* (encoding matrix metallproteinase 2), *VEGFA* (vascular endothelial growth factor A) and *ITGB1* (integrin β1) genes by directly binding to their 3′-UTRs (untranslated regions). Moreover, we identified that there was an inverse correlation between *miR-29b* and its target genes in subjects with PE. Taken together, these findings support a novel role for *miR-29b* in invasion, apoptosis and angiogenesis of trophoblast cells, and *miR-29b* may become a new potential therapeutic target for PE.

Key words: microRNA, *miR-29b*, myeloid cell leukaemia sequence 1 (MCL1), matrix metalloproteinase 2 (MMP2), pre-eclampsia, pregnancy, trophoblast, vascular endothelial growth factor A (VEGFA)

INTRODUCTION

PE (pre-eclampsia), a pregnancy-specific disorder characterized by hypertension and proteinuria, is a major cause of maternal and fetal morbidity and mortality [1]. Although the aetiology of PE is uncertain, the core hypothesis is that poor trophoblast migration/invasiveness associated with a poor remodelling of the spiral arteries are key pathological features of the disease [2–4]. In addition, several reports have indicated that a typical hallmark of PE includes increased trophoblast cell apoptosis in the placenta [5–7]. However, an understanding of the underlying molecular mechanisms that are involved in the invasion and survival of trophoblast cells is still not clear [8].

miRNAs (microRNAs) are non-coding RNAs of approximately 22 nt that act as post-transcriptional regulators of gene expression. They function in diverse biological processes, including development, differentiation, apoptosis and oncogenesis [9]. Previous findings have shown that many miRNAs are abundantly expressed in the human placenta [10]. We and others [11–14] have reported that several miRNAs are differentially expressed in placenta tissue from subjects with PE. However, the effects of miRNAs in mediating trophoblast cell function have been addressed sparsely [15,16]. As our group has found the aberrant overexpression of seven miRNAs in subjects with PE (*miR-29b*, *miR-16*, *miR-195*, *miR-26b*, *miR-181a*, *miR-335* and *miR-222*) [14], it was necessary to investigate further their potential roles in the pathogenesis of the disease.

Several studies have emphasized the importance of *miR-29b* in the regulation of cell proliferation, differentiation and apoptosis [17–21]. Moreover, levels of *miR-29b* have been found to be higher in the plasma of smokers [22]. However, there has been no study to date addressing the impact of *miR-29b* on trophoblast

Abbreviations: *ACTB*, β-actin; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; ID, integrated density; *ITGB1*, integrin β1; MCL1, myeloid cell leukaemia sequence 1; miRNA (miR), microRNA; MMP2, matrix metalloproteinase 2; PE, pre-eclampsia; PI, propidium iodide; RT-PCR, real-time PCR; UTR, untranslated region; VEGFA, vascular endothelial growth factor A

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Table 1 Clinical characteristics of the study population
Values are means ± S.E.M. NS, not significant.

Parameter	PE ($n = 24$)	Control ($n = 26$)	P value
Age (years)	28.1 ± 1.3	28.7 ± 1.1	NS
Gestational age at delivery (week)	37.0 ± 0.2	38.8 ± 0.4	NS
Primiparae (*n*)	10 (41.6%)	15 (57.6%)	NS
Body mass index (kg/m^2)	29.0 ± 1.0	27.2 ± 1.2	NS
Systolic blood pressure (mmHg)	161.5 ± 4.1	119.6 ± 3.9	<0.05
Diastolic blood pressure (mmHg)	113.4 ± 2.8	81.7 ± 3.3	<0.05
Proteinuria (mg/24h)	2209.4 ± 23.6	0	<0.05
Alanine aminotransferase (units/l)	33.9 ± 8.6	30.5 ± 6.5	NS
Blood urea nitrogen (mmol/l)	4.1 ± 0.3	3.8 ± 0.2	NS
Platelets (*n*)	(157.9 ± 22.1)×10^9	(192.0 ± 31.4)×10^9	NS
Birth weight (g)	2835.6 ± 173.0	3415.3 ± 158.3	NS
Placenta weight (g)	485.8 ± 25.6	526.3 ± 28.4	NS

cells, and it has not been determined whether *miR-29b* may have diagnostic or prognostic values in PE.

The aim of the present study was to elucidate the functional role of *miR-29b* in trophoblast cells. We therefore examined the apoptosis, proliferation, invasion and angiogenesis of trophoblast cells after overexpression or down-regulation of *miR-29b*. We also studied its regulatory mechanism during this process and found that several key genes involved in PE were the direct targets of *miR-29b*. These findings highlight the important role of *miR-29b* in the pathogenesis of PE and provide new insight into the development of the disease.

MATERIALS AND METHODS

Patients and tissue samples

PE was defined as gestational hypertension (systolic pressure >140 mmHg or diastolic blood pressure >90 mmHg on two or more occasions after gestational week 20) with proteinuria (>0.3g/day). Severe PE was defined by the presence of more than one of the following: (i) severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >110 mmHg on two or more occasions after gestational week 20), (ii) severe proteinuria (⩾5 g of protein in a 24 h urine specimen), (iii) oliguria <500 ml in 24 h, (iv) cerebral or visual disturbances, (v) pulmonary oedema or cyanosis, (vi) epigastric or right upper-quadrant pain, (vii) impaired liver function, (viii) thrombocytopenia or (ix) fetal growth restriction [ACOG (American Congress of Obstetricians and Gynecologists) Practice Bulletin] [23]. We collected data from 24 pregnancies complicated by severe late-onset PE and delivered after 34 weeks, and 26 pregnant women during normal term pregnancy, who were recruited as healthy controls. The detailed clinical characteristics of the subjects is summarized in Table 1. For the control group, women with chronic hypertension, cardiovascular disease, renal disease, hepatitis, diabetes, any evidence of intrapartum infection or other pregnancy complications, such as fetal anomalies or chromosomal abnormalities, were excluded from the study.

Placental tissues were obtained from women who were hospitalized in the Department of Gynecology and Obstetrics of Nanjing Drum Tower Hospital and the Affiliated Hospital of Nanjing University Medical School. Written consent was received from women after a full explanation of the purpose, nature and risk of all procedures used before surgery. The hospital ethics committee approved the consent forms and the protocols to utilize the tissue.

For the placentas, only chorionic tissue blocks (∼1 cm^3) from the central part of the placenta were collected, and contamination with maternal decidua and amniotic membranes was excluded by morphological observation. All placental tissues were obtained at the time of Caesarean section, were stabilized in RNAlater (Qiagen) and then stored at −80 °C until used.

Cell culture

HTR-8/SVneo cells, an immortalized human trophoblast cell line established from first-trimester human cytotrophoblast cells, were kindly provided by Dr Charles H. Graham (Faculty of Health Sciences, Queen's University, Kingston, Ontario, Canada). Human placental cell line derived from a choriocarcinoma (BeWo and JAR cells) were obtained from the A.T.C.C. (Rockville, MD, USA). BeWo cells were cultured in F-12 medium (Gibco) supplemented with 10% FBS (fetal bovine serum) (Gibco), 100 units/ml penicillin, and 100 g/ml streptomycin. HTR-8/SVneo and JAR cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 100 units/ml penicillin, and 100 g/ml streptomycin. All cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Reverse transcription and RT-PCR (real-time PCR)

Total RNA was extracted from the cultured cells and placenta tissues using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. For quantitative RT-PCR analysis of the genes *MMP2* (matrix metalloproteinase 2), *MCL1* (myeloid cell leukaemia sequence 1), *ITGB1* (integrin β1), *VEGFA* (vascular endothelial growth factor A) and *ACTB* (β-actin), 1 μg of total RNA was reverse-transcribed to cDNA with oligdT and

Table 2 Primers used for quantitative RT-PCR amplification

Gene	Forward (5′→3′)	Reverse (5′→3′)
ACTB	CCACGAAACTACCTTCAACTCC	TCATACTCCTGCTGCTTGCTGATCC
ITGB1	CAAAGGAACAGCAGAGAAGC	ATTGAGTAAGACAGGTCCATAAGG
MCL11	GAAAGCTGCATCGAACCATT	ACATTCCTGATGCCACCTTC
MMP2	ACCCTCAGAGCCACCCCTAA	AGCCAGCAGTGAAAAGCCAG
VEGFA	CACACAGGATGGCTTGAAGA	AGGGCAGAATCATCACGAAG
miR-29b	ACACTCCAGCTGGGTAGCACCATTTGAAA	TGGTGTCGTGGAGTCG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Thermoscript (TaKaRa). RT-PCR for these genes was performed on an Applied Biosystems 7300 Sequence Detection System using SYBR green dye (Invitrogen). A 20 μl PCR mixture was used and included 1 μl of reverse-transcribed product, 1× Quanti-Tect SYBR green PCR Master Mix and 0.5 μM forward and reverse primers. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The housekeeping gene *ACTB* was used as an endogenous control for RNA normalization. All experiments were done in triplicate. The threshold cycle C_t value was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Mature *miR-29b* expression was determined by using TaqMan assays (Applied Biosystems) with *U6* snRNA as the internal reference control. The method to quantify mature miRNA was performed as described previously [24]. Highly target-specific stem–loop structure and reverse transcription primers were used for reverse transcription. After that, we used specific TaqMan hybridization probes for miRNA amplification, which allowed high specificity for mature *miR-29b* target and the formation of a reverse transcription primer/mature *miR-29b* chimaera, extending the 5′-end of the miRNA. RT-PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). Relative expression was performed as described previously using the $\Delta\Delta C_t$ method [25]. The expression of *miR-29b* was calculated using the equation $2^{-\Delta Ct}$, where $\Delta C_t = (C_{t\,miR\text{-}29b} - C_{t\,U6})$. The relative amount of *miR-29b* to internal control was calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_{t\,miR\text{-}29b} - C_{t\,U6})$. The sequences of forward and reverse primers used are listed in Table 2.

Western blot analysis

Lysates (50 μg) obtained from BeWo and JAR cells were electrophoresed by SDS/PAGE (12% gel) (Bio-Rad Laboratories) and electroblotted on to PVDF membranes (Hybond-P; GE Healthcare). After blocking with 5% (w/v) BSA in TBS (Tris-buffered saline)/Tween-20 (Bio-Rad Laboratories), the membranes were incubated with rabbit anti-human polyclonal antibodies against MCL-1, MMP2, integrin β1, FAK (focal adhesion kinase), anti-ERK (extracellular-signal-regulated kinase) 1/2, anti-(phospho-FAK), anti-(phospho-ERK1/2) and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (all from Cell Signaling Technology). ID (integrated density) values were then calculated using an AlphaImager 3400 (Alpha Innotech). These values were then normalized to the corresponding control. All experiments were repeated at least three times.

Determination of VEGFA levels by ELISA

The expression of VEGFA in the supernatant of trophoblast cell lines was determined using a VEGFA ELISA kit, according to the manufacturer's instructions (R&D Systems).

miRNA target prediction and GO (gene ontology)

Computer-based programs, including Targetscan (http://www.targetscan.org/), Pictar (http://pictar.bio.nyu.edu) and miRBase Targets (http://microrna.sanger.ac.uk), were used to predict potential target genes of *miR-29b*. The GO of the predicted targets was analysed using functional items via the GO website (http://www.geneontology.org). All gene definitions and functions were based on the National Institute of Health databases (http://www. ncbi.nlm.nih.gov/sites/entrez).

Transfection experiments

Overexpression or down-regulation of *miR-29b* expression in HTR-8/SVneo, BeWo and JAR cells was achieved by transfecting cells with pre-*miR-29b* (50 pmol) or anti-*miR-29b* (100 pmol) (Ambion) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. The corresponding scrambled negative control miRNA was used as the negative control. Cells were harvested by trypsinization 48 h post-transfection and were used for subsequent experiments.

Cell proliferation, cell cycle and apoptosis assays

Cell proliferation was determined using a CCK-8 kit (DojinDo). HTR-8/SVneo, BeWo and JAR cells were plated at 2.5×10^3 cells/well in 96-well plates and incubated overnight in medium supplemented with 10% FBS. After 48 h transfection, 10 μl of CCK-8 (cholecystokinin octapeptide) was added to each well and incubated for another 3 h. The absorbance values at 450 nm were measured on an ELx-800 Universal Microplate Reader (Biotek). For the apoptosis assay, cells were harvested, stained with PI (propidium iodide) and an anti-(annexin V) antibody and then analysed by FACS (Calibur; BD Biosciences). For the cell cycle experiments, the treated cells were harvested, washed once with PBS and fixed in 70% ethanol overnight. Staining of the DNA content was performed with 50 mg/ml PI and 1 mg/ml RNase A for 30 min. Analysis was performed with Cell Quest Pro software.

Cell-cycle modelling was performed with ModFit 3.0 software (Verity Software House).

Cell invasion assay

The invasion ability of HTR-8/SVneo and BeWo cells was determined by their ability to cross the 8 μm pores of a migration chamber that consists of transwells fitted with Millipore membranes (6.5 mm filters; Costar). Before cell seeding, inserts were coated with 50 μl of Growth Factor Reduced Matrigel (BD Biosciences). Cells were suspended in serum-free culture medium at a concentration of 4×10^5 cells/ml and then added to the upper chamber (at 4×10^4 cells/well). Simultaneously, 0.5 ml of culture medium with 10% FBS was added to the lower compartment, and the transwell-containing plates were incubated for 24 h in a 5% CO_2 atmosphere saturated with water. At the end of the incubation, cells that had entered the lower surface of the filter membrane were fixed with 90% ethanol for 30 min at room temperature (20 °C), washed three times with distilled water, and stained with 0.1% Crystal Violet in 0.1 M borate and 2% ethanol for 30 min at room temperature. Cells remaining on the upper surface of the filter membrane were gently scraped off with a cotton swab. Images of invaded cells were captured by a photomicroscope (BX51; Olympus). Cell invasion was quantified in a blinded manner by counting the number of the invaded cells on the lower surface of the membrane with five fields ($\times100$ magnification) per chamber. Experiments were performed three times in duplicates.

HTR-8/SVneo capillary tube and network formation assay on Matrigel

Growth Factor Reduced Matrigel (300 μl) in serum-free medium was added to 24-well plates and incubated for at least 1 h to gel (thick-layer Matrigel). HTR-8/SVneo cells (10^5 cells) were added to the pre-solidified Matrigel, which started the process of forming capillary tubes and networks. After incubation for 8 h, digital images ($\times100$ magnification) were taken from at least five different fields per well, and image analysis was undertaken using Image plus Pro software (Media Cybernetics). Quantification of the network complexity was achieved by measuring the total length of the tubes per mm^2.

Plasmid construct and luciferase analysis

The 3′-UTRs (untranslated regions) of *MCL1*, *VEGFA*, *MMP2* and *ITGB1* mRNA, which contain the target sites for *miR-29b*, were PCR-amplified and then introduced downstream of the luciferase reporter gene in the XbaI-cloning sites of the pGL3 control vector (Promega).

HTR-8/SVneo and BeWo cells were seeded on to 24-well plates 1 day before transfection. For *miR-29b* co-transfection, 200 ng of firefly luciferase and 20 ng of *Renilla* luciferase reporter plasmids were transiently transfected into the cells. After 48 h, the luciferase activity was measured using the Dual-luciferase assay kit (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity. All experiments were performed in triplicate.

Statistical analysis

Results are expressed as means $\pm$ S.E.M.. Statistical significance was assessed by ANOVA with Bonferroni's multiple comparison tests. The correlation between *miR-29b* expression and its target gene expression in the placenta from subjects with PE was analysed using Pearson correlation and linear regression analysis. Data for all experiments were analysed with Prism software (GraphPad). A statistical significance was set at $P < 0.05$. All experiments were repeated at least three times.

RESULTS

Prediction of target genes of *miR-29b* and their function network analysis

Previously, we have reported a significant up-regulation of *miR-29b* in the placentas of Chinese subjects with severe PE [14]. In order to understand the functions of *miR-29b* in PE, we first predicted its target genes using computer-based programs, including Targetscan, Pictar and miRBase. Using these bioinformatics approaches, we found that there were 851, 684 and 1152 *miR-29b*-target pairs in Targetscan, Pictar and miRBase respectively (results not shown). In addition, we investigated the biological functions of these target genes using GO. Interestingly, we observed enrichment for genes implicated in important cellular functions, such as proliferation, cell-cycle progression, apoptosis and migration (Figure 1). To date, the role of *miR-29b* in placenta trophoblast cells and the signalling pathways by which *miR-29b* exerts its function remain largely unknown. The combination of these factors motivated us to investigate whether *miR-29b* has any effect on human trophoblast cells.

miR-29b induces the apoptosis of trophoblast cells

First, we determined the basal expression of *miR-29b* in three trophoblast cell lines, namely HTR-8/SVneo, BeWo and JAR cells. After being normalized to that in JAR cells, the relative fold change of *miR-29b* in HTR-8/SVneo and BeWo cells was 28.75 and 11.06 respectively (see Supplementary Figure S1A at http://www.clinsci.org/cs/124/cs1240027add.htm). Moreover, we examined the transfection efficiency of *miR-29b* in the three cell lines and found that there was some variation among the cell lines (see Supplementary Figure S1B). Therefore we have adopted different strategies in the subsequent experiments according to the basal expression of *miR-29b* and the transfection efficiency in the trophoblast cell lines.

Next we investigated whether *miR-29b* had an effect on the apoptosis of trophoblast cells. Overexpression of *miR-29b* increased the apoptosis of HTR-8/SVneo cells 1.5- fold compared with the negative control ($P < 0.001$; Figure 2), whereas down-regulation of *miR-29b* inhibited this process ($P < 0.01$; Figure 2). We also interfered with the expression of *MCL1*, an anti-apoptotic member of the Bcl-2 family, which was predicted to be the target of *miR-29b* (results not shown) and has been shown to play a significant role in the survival of cancer cells [26]. The flow cytometry

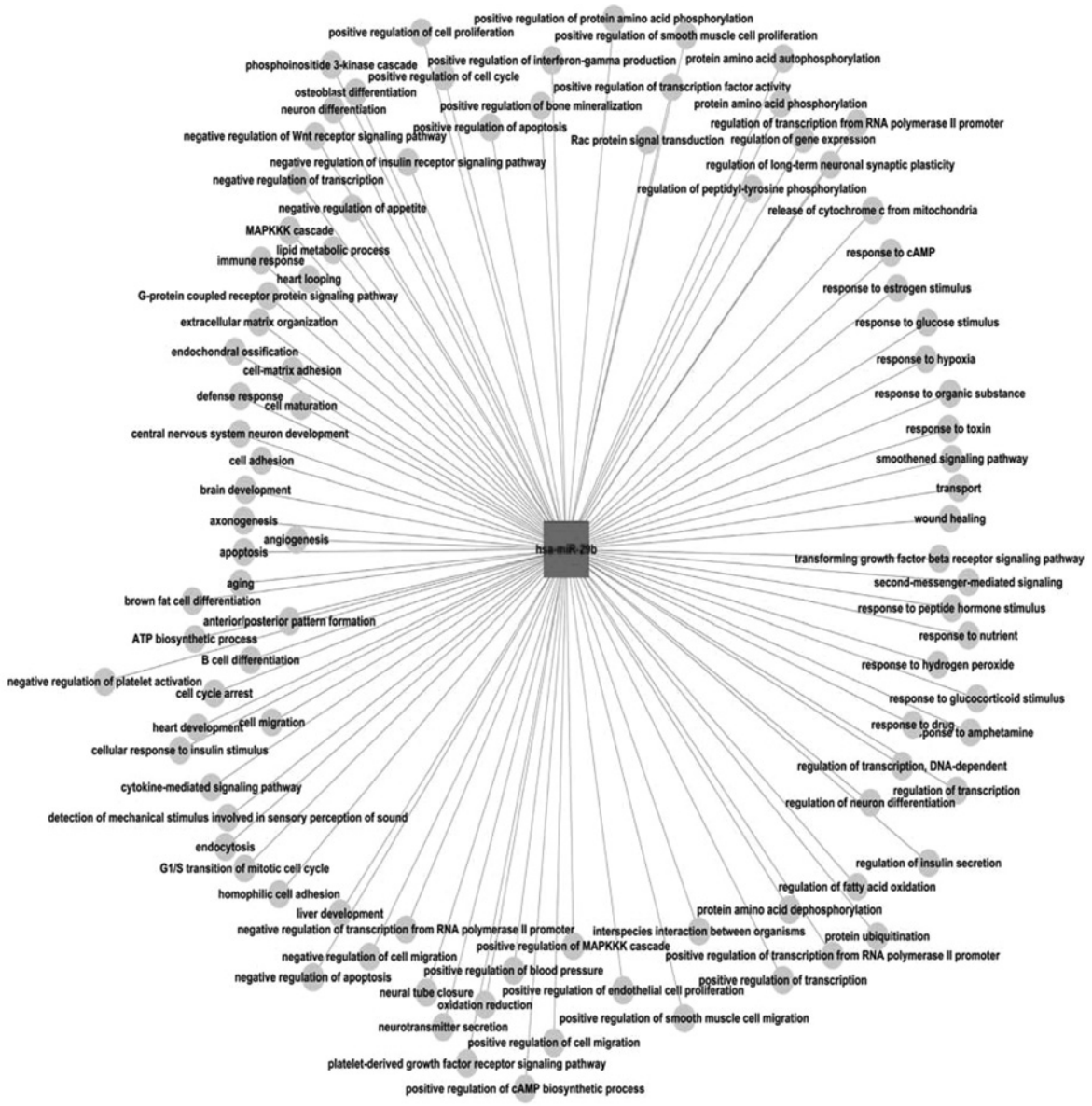

Figure 1 **Function network analysis of *miR-29b***

The network was generated through GO analysis, which was used to determine the probable biological function of the targets of *miR-29b*. The GO of the predicted targets was analysed using functional items via the GO website (http://www.geneontology.org). All gene definitions and functions were based on the National Institute of Health databases (http://www.ncbi.nlm.nih.gov/sites/entrez).

analysis showed that RNA interference of *MCL1* increased the apoptosis of trophoblast cells ($P < 0.001$; Figure 2). Similar results were also observed in BeWo cells (both $P < 0.001$; Figure 2). Taken together, these results suggest that *miR-29b* promotes the apoptosis of trophoblast cells, which might be partly due to the down-regulation of *MCL1* expression. We also investigated the effect of *miR-29b* on the proliferation and cell cycle of trophoblast cells. However, no significant change was observed on cell proliferation and cell cycle after up-regulating *miR-29b* (see Supplementary Figures S1C–S1E).

miR-29b inhibits the invasion of trophoblast cell lines and decreases capillary tube and network formation

It is well known that deficient trophoblast invasion of the placental bed spiral arterioles and altered trophoblast-mediated remodelling of the spiral arteries results in reduced uteroplacental perfusion and the onset of PE [2,3]. Therefore we examined the effect of *miR-29b* on the invasive capacity of trophoblast cells using Matrigel invasion assays. The results showed that overexpression of *miR-29b* markedly reduced the invasiveness of HTR-8/SVneo

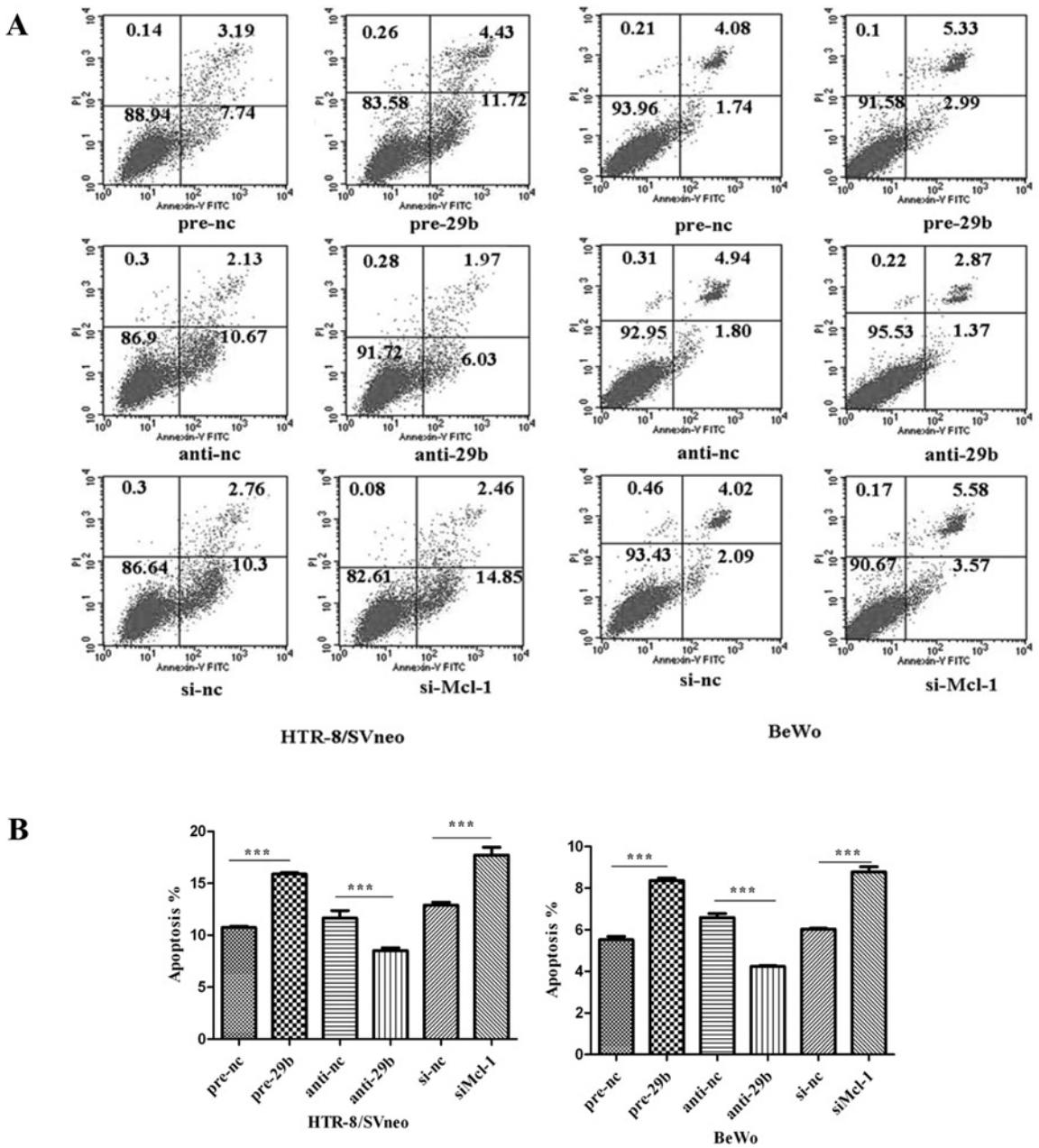

Figure 2 ***miR-29b* induces apoptosis in HTR-8/SVneo and BeWo cells**

(**A**) Annexin V/PI assays in HTR-8/Svneo (left-hand panels) and BeWo (right-hand panels) cells transfected with pre-*miR-29b* (pre-29b), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b), anti-negative control (anti-nc), short interfering RNA against MCL1 (si-MCL1) or short interfering RNA negative control (si-nc). After 48 h, annexin V assay was performed as described in the Materials and methods section. *y*-axis, cells stained with PI; *x*-axis, cells stained by annexin V–FITC. The results are shown as a percentage of apoptotic cells (annexin V-positive) and are representative of three independent experiments with similar results. (**B**) Percentage of apoptotic HTR-8/SVneo (left-hand panel) and BeWo (right-hand panel) cells. Values are means ± S.E.M. $^{***}P < 0.001$ using a Student's *t* test.

compared with the negative control. Meanwhile, knockdown of endogenous *miR-29b* promoted invasion (both $P < 0.001$; Figures 3A and 3B). Similar results were obtained in BeWo cells (Figures 3A and 3B). Thus these results indicate that *miR-29b* may be involved in the suppression of invasion of trophoblast cells.

A direct effect of *miR-29b* on angiogenesis has not been studied to date. As VEGFA, a key growth factor that modulates angiogenesis [27], was predicted to be targeted by *miR-29b* (results not shown), we have investigated the effect of *miR-29b* on angiogenesis of trophoblast cells. We chose the extravillous trophoblast cell line HTR-8/SVneo, which has an intrinsic capacity

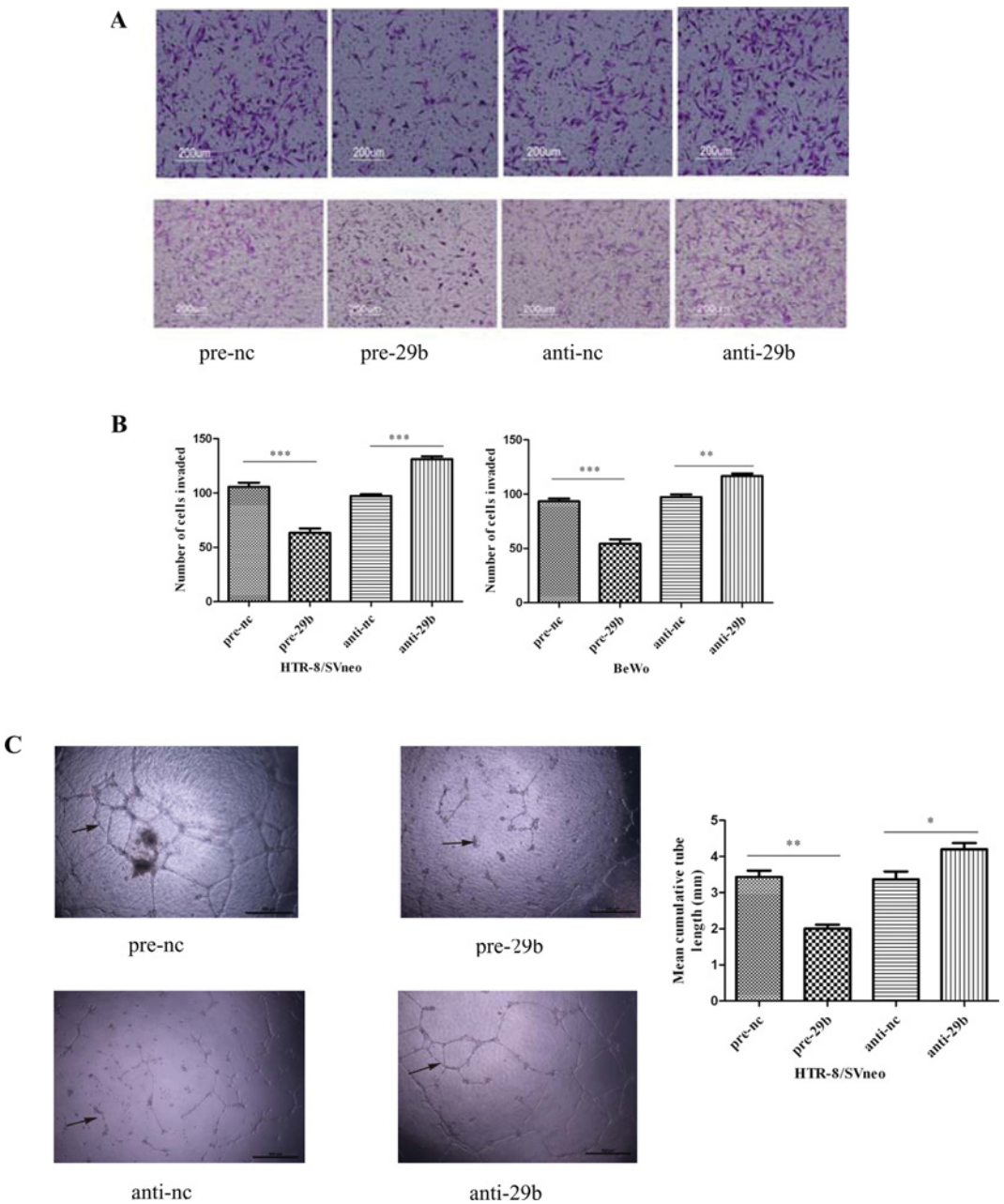

Figure 3 ***miR-29b* reduces the invasion and impairs capillary tube and network formation of trophoblast cells**

(**A**) Transwell analysis of HTR-8/SVneo (upper panel) and BeWo (lower panel) cells transfected with pre-*miR-29b* (pre-29b), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b), anti-negative control (anti-nc). The images are representative of three independent experiments. (**B**) The number of invaded cells was quantified using a microscope at ×100 magnification. Values are means ± S.E.M. from three experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$. (**C**) Tube formation of HTR-8/SVneo cells treated differently was photographed under a microscope at ×100 magnification (left-hand panels). The images are representative of three independent experiments. Mean tube length was quantified by image pro-plus software (right-hand panel). $^{*}P < 0.05$ and $^{**}P < 0.01$.

to form capillary tubes and networks when cells are cultured on thick-layer Matrigel. After 8 h of incubation, some of the capillary network 'arms' became disrupted and HTR8/SVneo cells became aggregated. Tube-like structures were defined as endothelial cord formations that were connected at both ends. We found that, after overexpression of *miR-29b*, the capillary tube and network formation of HTR-8/SVneo were prevented and the total length of tubes decreased by 60% ($P < 0.01$; Figure 3C). In contrast, neutralization of endogenous *miR-29b* increased capillary tube and network formation 1.4-fold ($P < 0.05$; Figure 3C). These results demonstrate that *miR-29b* is involved in the inhibition of angiogenesis of trophoblast cells.

miR-29b targets *MMP2, MCL1, VEGFA* and *ITGB1*

The findings described above prompted us to investigate the regulatory mechanism of *miR-29b*. To characterize the molecular basis of *miR-29b* in trophoblast cell function, we selected candidate genes based on (i) genes involved in the regulation of invasion, apoptosis and angiogenesis and (ii) putative *miR-29b* target genes using the online computer programs Targetscan, Pictar and miRBase Targets. This resulted in four candidate genes, namely *MMP2*, *MCL1*, *VEGFA* and *ITGB1*.

In order to determine whether *miR-29b* affected the expression of these genes in trophoblast cells, we first analysed their mRNA and protein expression levels after overexpression of *miR-29b*. Using quantitative RT-PCR, we found that the *MMP2*, *MCL1*, *VEGFA* and *ITGB1* mRNA levels were dramatically reduced after overexpression of *miR-29b* in HTR-8/SVneo, BeWo and JAR cells (both $P < 0.01$; Figure 4A, and Supplementary Figure S2 at http://www.clinsci.org/cs/124/cs1240027add.htm). Strikingly, the protein levels of MCL1, VEGFA, MMP2 and integrin $\beta 1$ were also substantially decreased after the overexpression of *miR-29b* in the trophoblast cell lines, as determined by Western blot analysis and ELISA (Figures 4B and 4C). Conversely, anti-*miR-29b*, by antagonizing endogenous *miR-29b*, enhanced the expression of the gene targets (both $P < 0.001$; Figures 4B and 4C).

Lastly, to test further whether *MCL1*, *MMP2*, *VEGFA* and *ITGB1* were direct targets of *miR-29b*, we searched predicted potential binding sites (Figure 5A) and constructed reporter plasmids containing the 3′-UTR of these genes. These reporter constructs were co-transfected with pre-*miR-29b* or anti-*miR-29b* in HTR-8/SVneo and BeWo cells. As shown in Figure 5(B), the results demonstrated that the increased expression of *miR-29b* significantly diminished luciferase activity (both $P < 0.001$; Figure 5B). Conversely, the decreased expression of *miR-29b* markedly enhanced the luciferase activity (both $P < 0.001$; Figure 5B). These results indicate that these genes are directly regulated by *miR-29b*.

Inverse correlation of *miR-29b* with *MCL1*, *MMP2*, *VEGFA* and *ITGB1* levels in placental tissues from PE

To confirm further the regulation of *MCL1*, *MMP2*, *VEGFA* and *ITGB1* by *miR-29b in vitro*, we first analysed the expression of *miR-29b* and these genes in placental tissues from 24 subjects with severe PE and 26 healthy controls. Consistent with our previous study [14], *miR-29b* was significantly increased in placental tissues from subjects with PE ($P < 0.001$; Figure 6A). Interestingly, the expression of *MCL1*, *MMP2*, *VEGFA* and *ITGB1* in the subjects with PE was markedly lower compared with the healthy controls ($P < 0.01$; Figure 6A). We next examined the correlation of *miR-29b* with these genes in our subjects. The Pearson correlation analysis indicated an inverse correlation between *miR-29b* and these genes (*miR-29b* and *MCL1* mRNA, $r = -0.6688$, $P < 0.001$; *miR-29b* and *MMP2* mRNA, $r = -0.8080$, $P < 0.001$; *miR-29b* and *VEGFA* mRNA, $r = -0.7190$, $P < 0.001$; *miR-29b* and *ITGB1* mRNA: $r = -0.7586$, $P < 0.01$) (Figure 6B).

miR-29b induces the dysregulation of FAK signalling in trophoblast cells

Additionally, in order to understand the pathway regulated by *miR-29b* in trophoblast cells, we analysed the FAK and ERK signalling pathways, which are involved in the invasion of trophobalst cells. Western blot analysis revealed that transfection of HTR-8/SVneo and BeWo cells with pre-*miR-29b* reduced the expression of phospho-FAK in both cell lines ($P < 0.01$; Figure 7), whereas phospho-ERK1/2 expression was unaffected. In contrast, cells transfected with anti-*miR-29b* had increased phospho-FAK expression ($P < 0.01$; Figure 7). This indicates that the overexpression of *miR-29b* leads to dysregulation of phospho-FAK signalling and suppression of MMP2 and integrin $\beta 1$.

DISCUSSION

miRNAs are emerging as major players in gene regulation and contribute to diverse biological processes. However, the molecular mechanisms by which miRNAs modulate the function of trophoblast cells are still unclear, especially the role of miRNAs in the pathogenesis of PE remain largely unknown. In the present study, we have found that the functional involvement of an up-regulation of *miR-29b* in dysregulating trophoblast cell invasion, survival and angiogenesis may lead to the onset of PE (Figure 6C).

Identification of biomarkers for PE diagnosis is of particular interest. Studies on miRNAs have offered the possibilities of developing a novel class of fetal nucleic acid markers in maternal plasma [28,29]. Moreover, abundantly and differentially expressed miRNA species in placental samples and in serum/plasma have been reported [11,12,14,30–35]. Future studies that examine early pregnancy *miR-29b* expression in placental samples (obtained during chorionic villi sampling procedures) or peripheral tissue (e.g. whole blood) with risk of PE could enhance our understanding of the pathogenesis of this disease and contribute to its diagnosis and management.

Trophoblast cells of the human placenta proliferate, migrate and invade the pregnant uterus in order to nourish the developing fetus, in a way that is imitated by malignant tumours [36–38]. Hence the normal trophoblast has been termed pesudomalignant. *miR-29b* has been identified as the best ‘hit’ in several experiments designed to detect miRNAs dysregulated in tumours [39–41]. However, no findings have reported the role of *miR-29b* in PE and trophoblast cells. Our present study identifies *MMP2*, *MCL1*, *VEGFA* and *ITGB1* as critical targets of *miR-29b* in trophoblast cells.

It has been reported in several studies that the Bcl-2 family member MCL1 is decreased in PE [42,43]. However, other investigators have suggested that increased apoptosis in PE might not be associated with significant alterations in Bcl-2 [44]. In our present study, we found the mRNA expression level of *MCL1* was decreased in placental tissues from subjects with PE compared with their control counterparts. Meanwhile, we have confirmed that *miR-29b* decreased the mRNA and protein expression of

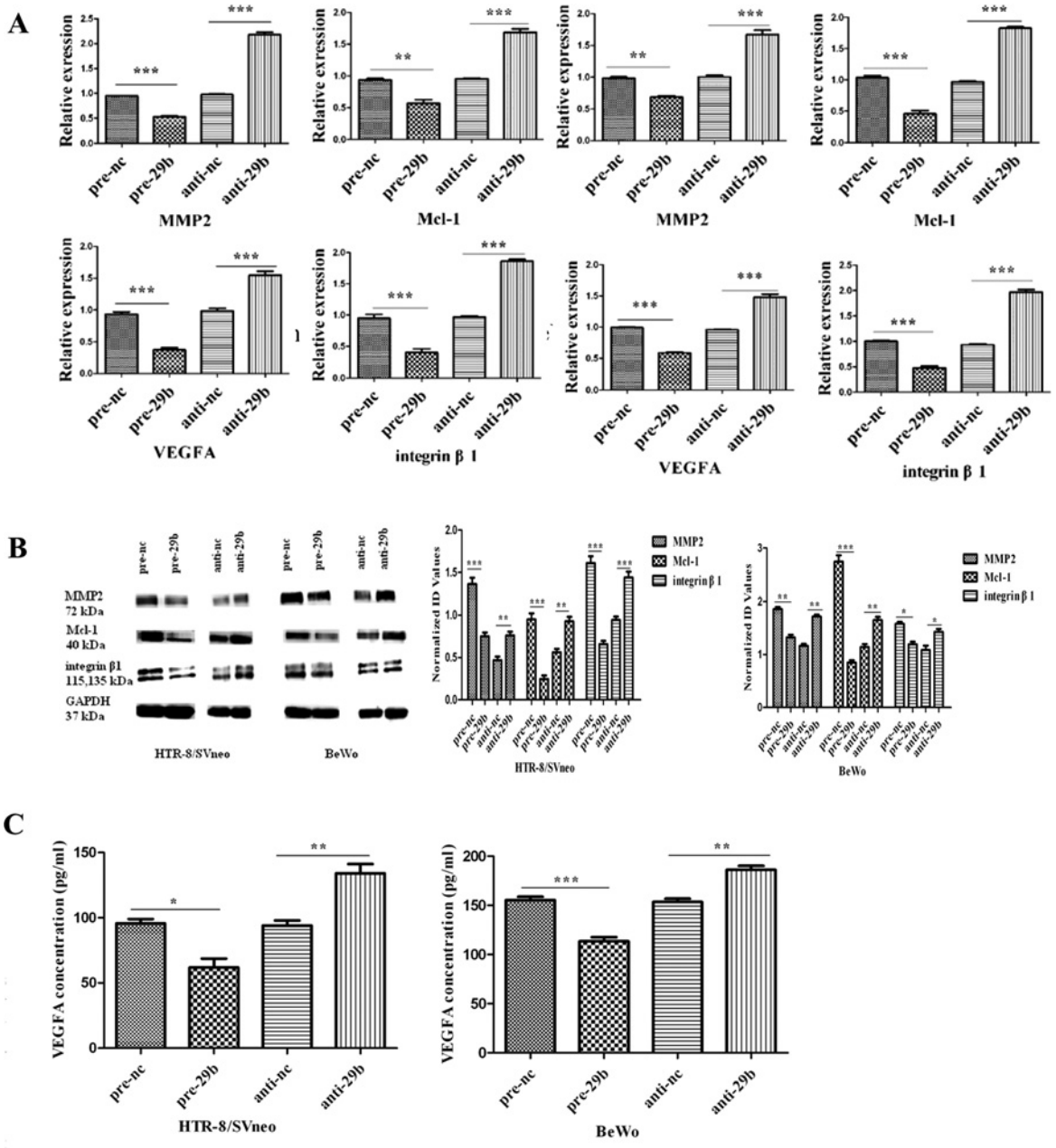

Figure 4 ***miR-29b* inhibits its target gene expression at the mRNA and protein levels**

(**A**) Quantitative RT-PCR was performed to determine the mRNA expression levels of *MMP2*, *MCL1*, *VEGFA* and *ITGB1* in HTR-8/SVneo and BeWo transfected with pre-*miR-29b* (pre-29b), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b) and anti-negative control (anti-nc). Values are means ± S.E.M. from three experiments performed in duplicate. (**B**) Western blot analysis of the protein expression of MMP2, MCL1 and integrin β1 performed on total cell extracts from HTR-8/SVneo and BeWo cells transfected with pre-*miR-29b* (pre-29b), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b) and anti-negative control (anti-nc). GAPDH was used as a loading control. The protein bands were quantified and normalized to GAPDH. Quantification of the protein expression is shown in the middle and right-hand panels as means ± S.E.M. (**C**) ELISA of VEGFA levels in the supernatant of serum-starved HTR-8/SVneo and BeWo cells transfected with pre-*miR-29b* (pre-29b), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b) and anti-negative control (anti-nc). Values are means ± S.E.M. from three experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

MCL1 in trophoblast cells lines, and *miR-29b* or small interfering RNA against *MCL1* can induce trophoblast cell apoptosis. All of these findings indicate that *MCL1* plays an important role in trophoblast cell survival and turnover, and that *miR-29b* may contribute to the increased apoptosis of trophoblast cells in PE via down-regulation of *MCL1*.

It has been demonstrated that human trophoblast invasiveness *in vitro* depends on the production of MMP2 [45]. In addition,

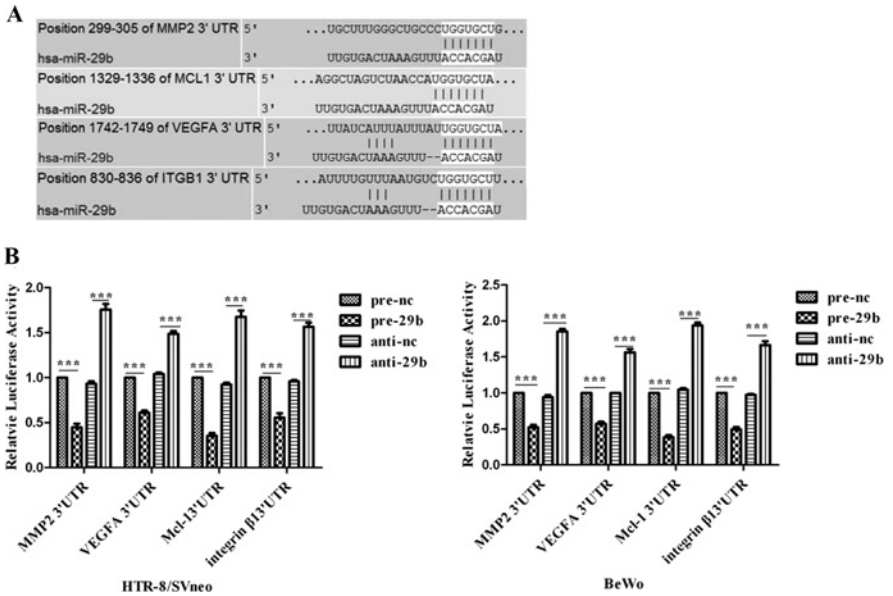

Figure 5 ***MCL1*, *MMP2*, *VEGFA* and *ITGB1* are direct targets of *miR-29b***

(**A**) Predicted interaction between the *miR-29b* seed and the seed matches on human *VEGFA*, *MMP2*, *MCL1* and *ITGB1* 3′-UTR mRNAs, as determined with the software Targetscan. Seed regions are highlighted in grey. (**B**) Analysis of luciferase activity in HTR-8/SVneo and BeWo cells. Cells were co-transfected with pre-*miR-29b* (*pre-29b*) (50 pmol) or anti-*miR-29bi* (*anti-29b*) (100 pmol), pRL-TK and the firefly luciferase reporter comprising the 3′-UTR of the these putative target genes respectively. pRL-TK expressing *Renilla* luciferase was co-transfected as an internal control to correct the differences in both transfection and harvest efficiencies. The firefly luciferase activity of each sample was normalized to the *Renilla* luciferase activity. Values are means ± S.E.M. from three experiments performed in duplicate. $^{***}P < 0.001$.

during trophobalst invasion, trophoblast cells undergo variation in integrin phenotype, acquiring integrins $\alpha 5\beta 1$ and $\alpha 1\beta 1$ [46]. In the present study, we observed that the overexpression of *miR-29b* reduced trophoblast cell invasion via down-regulating the levels of its targets *MMP2* and *ITGB1*, and that the expression of *MMP2* and *ITGB1* mRNA was decreased in placental tissue from subjects with PE. Taken together, many other molecules involved in the regulation of normal human trophoblast cell invasion besides *miR-29b* will be revealed in the future, which will contribute to the understanding of the mechanisms underlying PE and possible prevention.

VEGF is a positive regulator of angiogenesis and plays a crucial role in the growth of vascular endothelial cells, the production of blood vessels and the promotion of vessel permeability [47]. Previous studies have elucidated that the hypoxia-driven disruption of the angiogenic balance involving VEGF and sFLT-1 (soluble Fms-like tyrosine kinase-1) might contribute to some of the maternal symptoms of PE [48]. In the present study, for the first time, we found that *miR-29b* had a binding site in the 3′-UTR of *VEGFA* mRNA and inhibited tubular network formation, partly via decreased secretion of VEGFA in HTR-8/SVneo cells. However, tube network formation of HTR-8/SVneo cells on Matrigel occurs as a consequence of a number of necessary biological events, including cell migration, proliferation, cell–cell junction formation and cell elongation. It is speculated that other targets of *miR-29b* may participate in angiogenesis.

Multiple growth factors expressed at the fetal–maternal interface are involved in the regulation of trophoblast migration and invasion through ERK and FAK signalling [49]. However, the molecular mechanisms governing invasion of human trophoblasts remain largely elusive. In trophoblasts, ERKs were shown to regulate the EGF (epidermal growth factor)-dependent induction of MMP2, indicating that proteinases crucial for trophoblast invasion are targets of this particular signalling pathway [50]. Moreover, phosphorylation of Tyr^{397} on FAK is a critical component of the signalling pathway that mediates cytotrophoblast migration/invasion [51]. Our present findings show that *miR-29b* may play an essential role in trophobalst invasion though diminishing the activation of FAK phosphorylation, although the precise mechanism for the *miR-29b*-mediated regulation of FAK remains to be determined.

Published findings on the role of miRNAs in trophoblast cells is surprisingly sparse. Lee et al. [13] studied the role of miRNAs in trophoblast and found that iron–sulfur cluster protein [ISCU (iron–sulfur cluster scaffold homologue)] down-regulation by *miR-210* perturbing trophoblast iron metabolism was associated with defective placentation. Luo et al. [52] reported that *microRNA-378a-5p* promoted trophoblast cell survival, migration and invasion by targeting Nodal. In the present study, we have demonstrated the effects and possible mechanisms of *miR-29b* on trophoblast function *in vitro* and analysed further the expression of *miR-29b* and its targets *in vivo* in placenta tissues from subjects with PE. In summary, these findings suggest a role for *miR-29b* in the regulation of altered placental gene expression in PE. Whether other miRNA family members also affect trophoblast cell apoptosis and invasion remains to be investigated.

An important issue that remains to be addressed is why *miR-29b* is up-regulated in PE. Chang et al. [53] have reported that

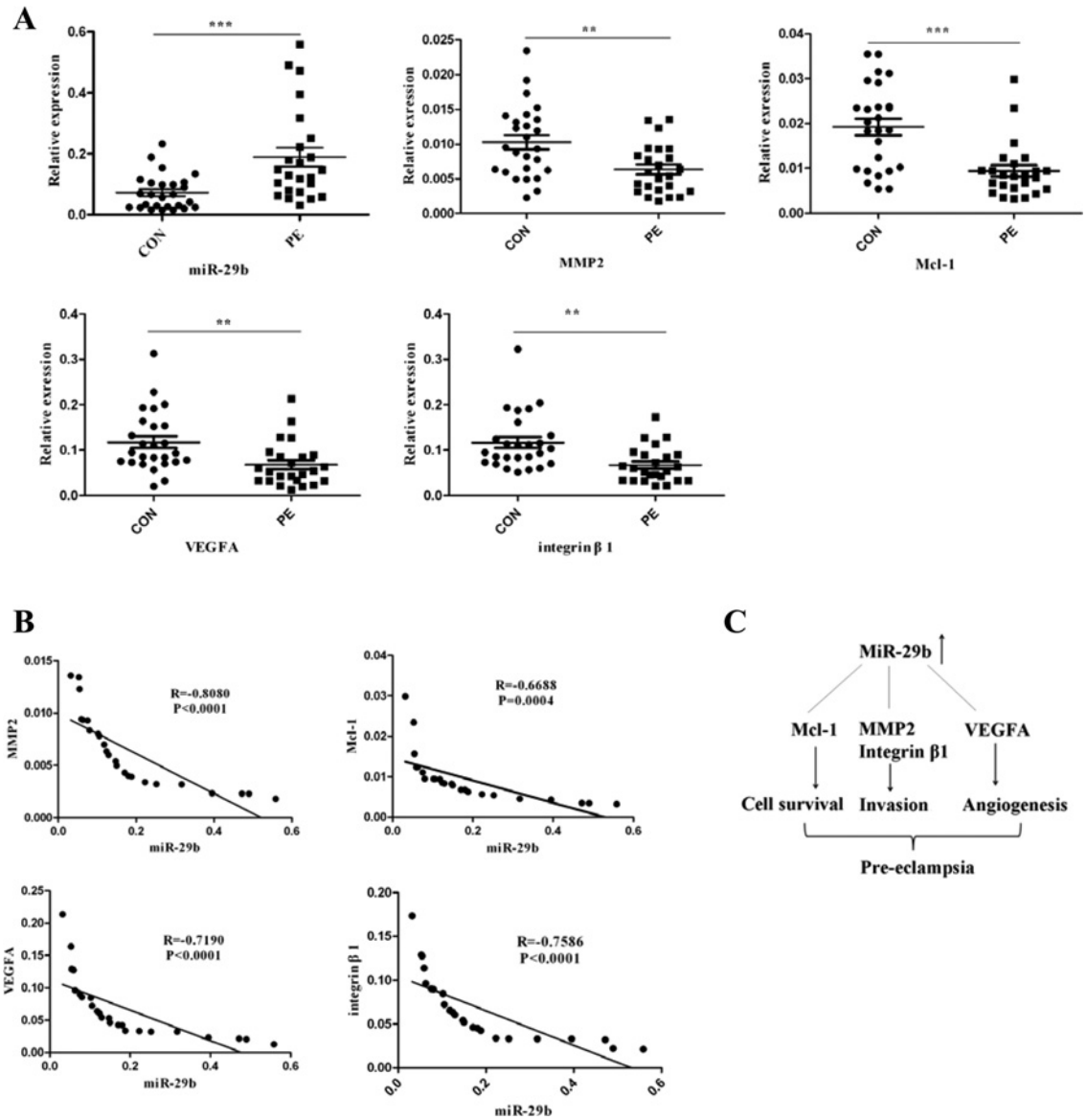

Figure 6 Significant inverse correlation of *miR-29b* and *MCL1*, *MMP2*, *VEGFA* and *ITGB1* in placental tissues from subjects with PE

(**A**) Endogenous expression levels of *MCL1*, *MMP2*, *VEGFA*, *ITGB1* and *miR-29b* in placental samples from subjects with PE ($n = 24$) and those with a normal pregnancy (CON) ($n = 26$) were assessed with quantitative RT-PCR. (**B**) Inverse correlation between endogenous *miR-29b* levels and *MCL1*, *MMP2*, *VEGFA* and *ITGB1* mRNA levels in PE patients ($n = 24$) determined by quantitative RT-PCR. Statistical analysis was performed using Pearson's correlation and linear regression analysis. R, regression coefficient. (**C**) Schematic diagram showing the hypothetical role of *miR-29b* in pathogenesis of PE through inhibition of invasion and angiogenesis of trophoblast cells and promotion of apoptosis of trophoblast cells.

c-Myc was shown to contribute to *miR-29* repression. Meanwhile, another group [21] has found that *miR-29* was repressed by NF-κB (nuclear factor κB) acting through YY1 (Yin Yang 1) and the Polycomb group. Taken together, several factors may interact with the regulatory region of *miR-29b*, including promoters of the *miR-29b* gene, transcription-factor-binding proteins, chromosomal structures or epigenetic factors. Therefore in the future it will be essential to identify other mechanisms of up-regulation.

Conclusions

The results of the present study have suggested that the up-regulation of *miR-29b* expression may contribute to the onset of PE through repression of trophoblast cell invasion and angiogenesis and enhancement of trophoblast cell apoptosis. In addition, its target genes, *MMP2*, *MCL1*, *VEGFA* and *ITGB1*, appear to be involved in these processes. In summary, understanding the regulation of genes by *miR-29b* would provide new insights into the pathogenesis of PE, and *miR-29b* could be employed as a new

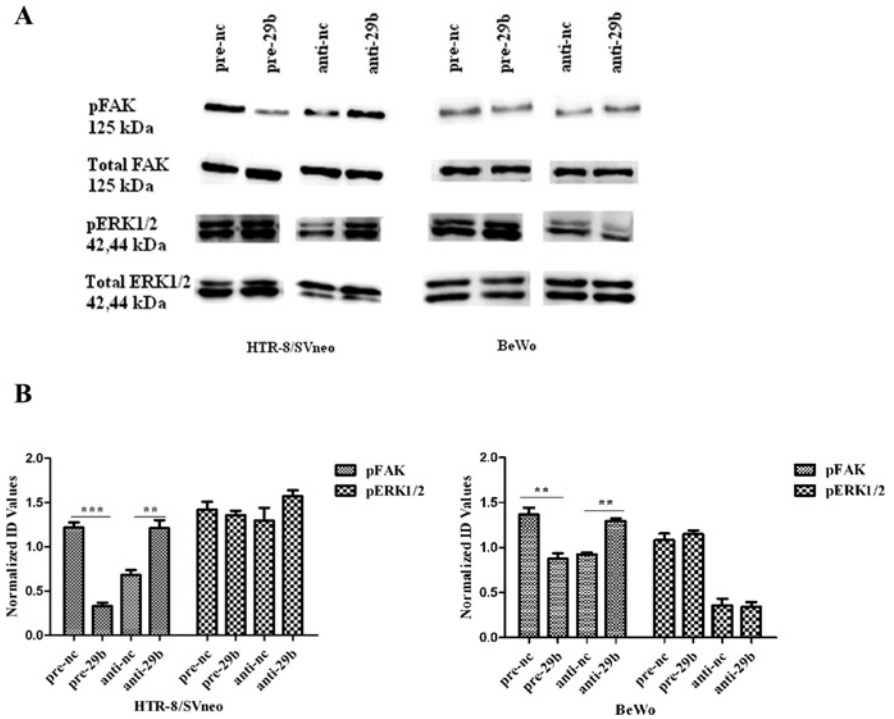

Figure 7 ***miR-29b* modulates FAK signalling in trophoblast cells**

(**A**) HTR-8/SVneo and BeWo cells were transfected with pre-*miR-29b* (*pre-29b*), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b) and anti-negative control (anti-nc) for 48 h and then the protein levels of phospho-ERK1/2 (pERK1/2), phospho-FAK (pFAK), total ERK1/2 and total FAK were detected by Western blot analysis. (**B**) The protein bands were quantified and normalized to total ERK1/2 and total FAK respectively. Values are means ± S.E.M. from three experiments performed in duplicate. $^{**}P < 0.01$ and $^{***}P < 0.001$.

prognostic marker and/or as an effective therapeutic target for PE.

CLINICAL PERSPECTIVES

- *miR-29b*, an miRNA that has a role in the regulation of cell proliferation, differentiation and apoptosis, is differentially expressed in PE, but its role in trophoblast cells and in the pathogenesis of the disease remain unknown.
- In the present study, we report that the up-regulation of *miR-29b* in placenta may contribute to the onset of PE through the repression of trophoblast cell invasion and angiogensis, and enhancement of trophoblast cell apoptosis. In addition, its target genes, including *MMP2*, *MCL1*, *VEGFA* and *ITGB1*, may be involved in these processes.
- Thus new insights into the involvement of *miR-29b* in the pathogenesis of PE have been provided and may open a new window for therapeutic intervention in the disease. *miR-29b* could be employed as a new prognostic marker and/or as an effective therapeutic target for PE.

AUTHOR CONTRIBUTION

Pengfei Li participated in the *miR-29b*-related cell experiments, statistical and bioinformatics analysis, and writing the paper; Wei Guo participated in placental tissue collection and processing, and analysis and interpretation of the data; Leilei Du performed the quantitative RT-PCR of *miR-29b* and provided technical support; Junli Zhao performed the quantitative RT-PCR of the target genes of *miR-29b*; Yaping Wang performed the clinical analysis; Liu Liu performed the plasmid construct and luciferase analysis; Yali Hu supervised the study; and Yayi Hou provided the study concept and designed the experiments.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

microRNA-29b contributes to pre-eclampsia through its effects on apoptosis, invasion and angiogenesis of trophoblast cells

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See the following pages for Supplementary Figures S1 and S2.

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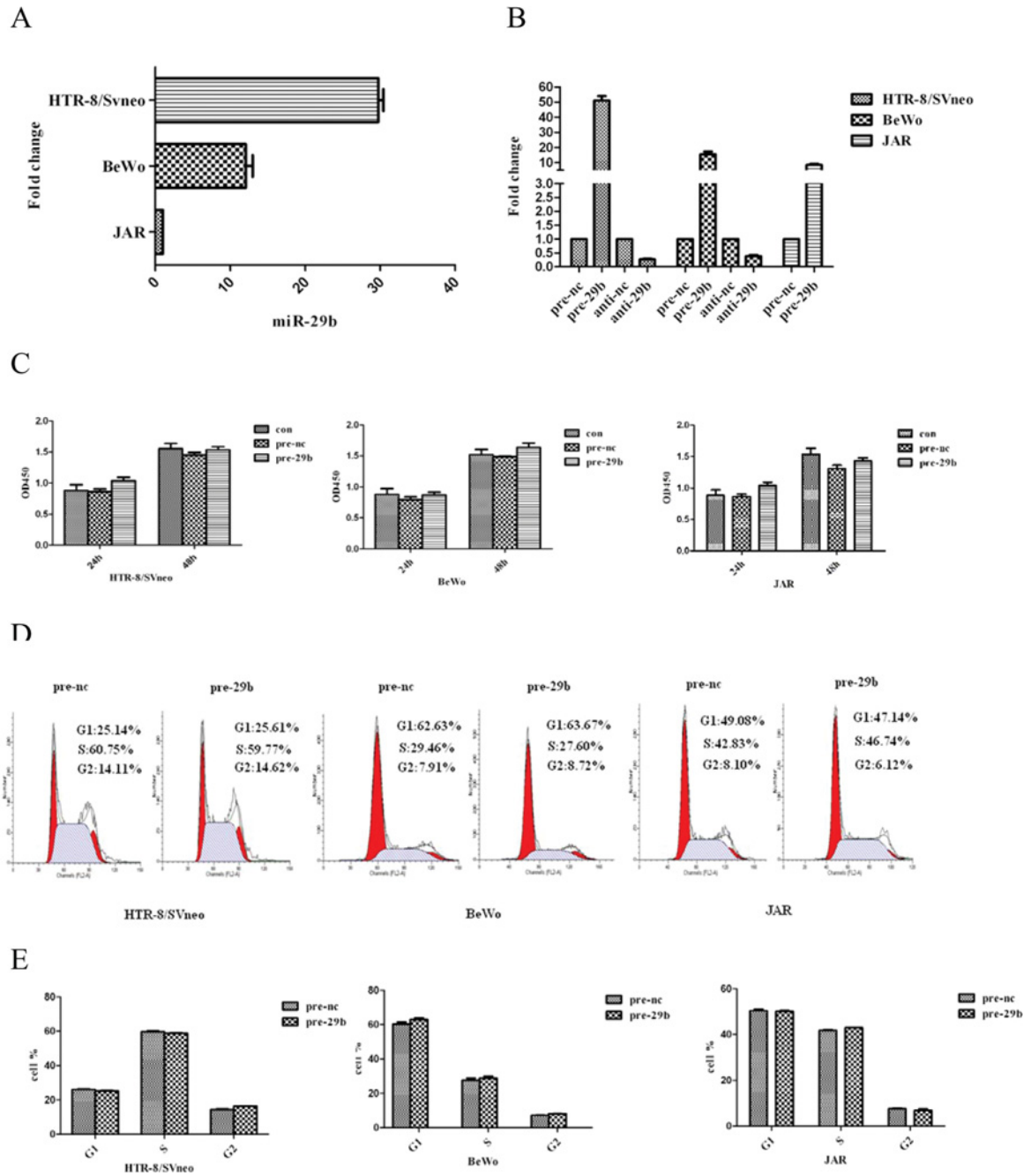

Figure S1 **m*iR-29b* does not affect the proliferation and cell-cycle distribution of trophoblast cells**

(**A**) The baseline expression level of *miR-29b* in human trophoblast cell lines HTR-8/Svneo, BeWo and JAR was examined by quantitative RT-PCR. Experiments were performed three times. (**B**) The transfection efficiency of *miR-29b* in the trophobalst cell lines. HTR-8/SVneo, BeWo and JAR cells were transfected with pre-*miR-29b* (pre-29b), pre-negative control (pre-nc), anti-*miR-29bi* (anti-29b) and anti-negative control (anti-nc). After 48 h, the expression level of *miR-29b* was determined by quantitative RT-PCR. (**C**) HTR-8/SVneo, BeWo and JAR were transfected with pre-*miR-29b* (pre-29b) and pre-negative control (pre-nc) in triplicate. At the same times on days 1 and 2 post-transfection, CCK8 was added, according to the manufacturer's instructions. After a further 3 h in culture at 37°C in an incubator, the absorbance values (OD) at 450 nm were measured on a plate reader. (**D**) Cell-cycle distribution of HTR-8/SVneo, BeWo and JAR. Cells were harvested after 48 h of transfection and subsequently assayed for their DNA content by flow cytometry. Representative traces are shown. The *x*-axis represents DNA content, whereas the *y*-axis represents the count scale. (**E**) Quantification of the cells in different phases of the cell cycle. Values are means ± S.E.M. from three independent experiments. (**E**) A statistical analysis is shown. Con, control.

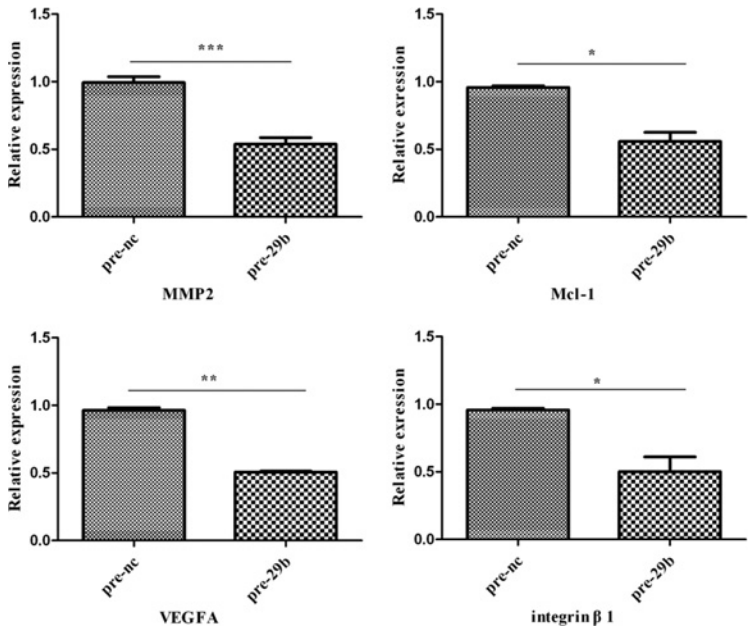

Figure S2 Overexpression of *miR-29b* inhibits the mRNA expression levels of its target genes

Quantitative RT-PCR analysis of the mRNA expression of *VEGFA*, *MMP2*, *MCL1* and *ITGB* in JAR cells treated with pre-*miR-29b* (pre-29b) and pre-negative control (pre-nc) after 48 h. The results are means ± S.E.M. from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

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