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Original Article

Involvement of p38/MMP-9/TIMP-1 in premature rupture of the human fetal membranes

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Abstract: Background: Premature rupture of the membrane (PROM) is a major cause of preterm birth, and the mechanisms still not fully defined. However, the mechanisms for PROM still not fully defined. This study investigated the roles of p38 in regulation the expression of MMP-9 and TIMP-1 in fetal membranes of patients with preterm premature rupture of membrane (PPROM) and full-term premature rupture of membrane (FPROM). Methods: We observed the expression of MMP-9, p38 and TIMP-1 in fetal membranes between the patients with PPROM and FPROM. To investigate the roles of p38, we down-regulated p38 expression using siRNA in primary human amniotic epithelial (HAE) cells. Results: In fetal membranes of PPROM patients, the expression of MMP-9 and p38 were significantly increased, whereas the expression of TIMP-1 were decreased compared to the FPROM patients. *In vitro*, knockdown the expression of p38 significantly down-regulated the expression of MMP-9, and up-regulated the expression of TIMP-1. Conclusion: This study demonstrates that p38 may play an important role in primary human amniotic epithelial cells by regulating the expression of MMP-9 and TIMP-1.

Keywords: p38, premature rupture of the membrane, TIMP-1, MMP-9

Introduction

Premature rupture of membranes (PROM) is characterized by rupture of fetal membranes before the onset of labor. Preterm premature rupture of membranes (PPROM) is the rupture of fetal membranes before 37 weeks' gestation, whereas full-term premature rupture of membranes (FPROM) refers to the rupture of the membranes after or with the onset of labor [1, 2]. Neonatal morbidity and mortality become a major health struggle mostly due to the prematurity. About 4% of pregnant women have PPROM, which is related to early births results in approximately 20% of all perinatal deaths [1-3]. Of note, PPROM poses as the leading single etiological factor of preterm delivery [4].

The fetal membranes contain epithelial, mesenchyme cells and extracellular matrix (ECM) [5]. ECM remodeling plays an important role in the processes of preterm including fetal membrane rupture [5, 6]. Matrix metalloproteinases (MMPs) as a kind of zinc-dependent enzymes, exerts an important function in regulating the degradation of most of the ECM and basement membrane components. Moreover, MMPs' involvement in multiple biological activities such as cell migration, cell differentiation, cell apoptosis, wound healing, and angiogenesis has been demonstrated recently [7, 8]. The study on the interactions among several MMPs such as MMP-9 and MMP-2 unraveled the critical regulatory role in the breakdown of collagens and the rupture of fetal membrane [9]. Tissue inhibitors of metalloproteinases (TIMPs) belong to naturally occurring inhibitors of MMPs, which perform as a potential modulator to control the functions of MMPs in many pathological process [7]. As a glycoprotein, TIMP-1 is secreted and formed a complex with MMP-9 [10]. In preterm labor, MMP-9 are found in human placenta, human fetal membranes and amniotic fluid. The expression of MMP-9 is

increased, whereas the levels of TIMP-1 is decreased [6, 11-13]. Anincrease in MMP-2 and MMP-9 levels as well as the decreased TIMP-1 in the fetal membranes and amnioticfluid are reported to be associated with term labor [6, 11-13]. However, the difference between the expressions of levels of MMP-9 and TIMP-1 levels in preterm premature rupture of membrane (PPROM) and full-term premature rupture of membrane (FPROM) is not clear. As p38 is involved in many biological process and diseases [14], for instance, MAPK is essential for controlling the proteolytic enzymes that degrade the basement membrane [14, 15] Of note, given that p38 are important for regulating MMP-9 and TIMP-1 activities [15], there is no direct information on the status p38 levels in PPROM and FPROM patient. Nonetheless, the roles of p38 in regulating primary HAE cells properties are also unclear. Thus, in the present study, we compared the expression of MMP-9, TIMP-1 as well as p38 levels in human patients with PPROM and FPROM.

Materials and methods

Participants

Participants were recruited after written informed consent under a protocol approved by the Committee on Human Research at the Qianfoshan Hospital Affiliated to Shandong University. All samples in our study were from placentas of single pregnancy. Placental tissues from PPROM (n=20), FPROM (n=15), and normal-term births (n=20) were obtained from individuals at Qianfoshan Hospital.

Tissue collection

Fetal membranes were collected from participants with normal birth after spontaneous onset of labor and artificial rupture of the membranes (38 weeks with no prior history of PTB or PPROM), FPRPOM (38 weeks), or PPROM (both <34 weeks). In this study, the diagnosis of membrane rupture was made by a positive Nitrazine test result and/or positive pooling on speculum examination and oligohydramnios on ultrasound examination. Several aliquots of tissue were collected randomly from the maternal side of the placenta. The tissues were snap frozen in liquid nitrogen and stored at -80°C before use.

Tissue processing for immunostaining

Tissue sections were cut into 3 to 5 μm, mounted on positively charged slides (Fisher Scientific, Pittsburgh, USA), and dried in a slide to ensure adherence to the slides. Sections were deparaffinized in xylene and then rehydrated through a series of graded alcohols followed by rinse in distilled water. Endogenous peroxides were quenched by 0.3% H_2O_2 in methanol. The levels of MMP-9, TIMP-2 and p38 were visualized by an immunofluorescent technique. The slides were then sequentially blocked in 5% BSA in PBST for 30 minutes and incubated with rabbit anti- MMP-9, TIMP-2 and p38 (1:100) (Abcam, USA) overnight at 4°C. The bound antibodies were tracked with biotinylated goat anti-rabbit secondary antibody (Abcam, USA) for 30 minutes and horseradish peroxidase-labeled streptavidin for 30 minutes. The bound horseradish peroxidase was assayed with 3, 3-N-diaminobenzidine tertrahydrochloride (DAB) (Abcam, USA). The sections were re-stained with hematoxylin and the images were recorded on an Olympus microscope (Olympus Corp, Japan).

Western blotting analysis

Denatured protein samples were resolved on SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocking with non-fat milk, membrane was incubated overnight at 4°C with antibodies including MMP-9, TIMP-1, p38, p-p38 and GAPDH (Abcam, USA) followed by incubation with the anti-rabbit HRP-conjugated secondary antibodies (Santa Cruz, Billerica, MA, USA). Chemiluminescence detection was performed using ECL advance Western blotting detection reagents (GE healthcare, Little Chalfont, Buckinghamshire, UK). The relative expression of MMP-9, TIMP-1, p38 was quantified by image J software.

Preparation and characterization of human amniotic epithelial cell

Cultures of human amniotic epithelial (HAE) cells were prepared as described previously [16-18]. Briefly, human amniotic membrane was mechanically peeled from chorion of a placenta obtained from an uncomplicated elective Caesarean section with the informed consent of each donor patient. The HAE cell layer was thoroughly scraped out from the underlying tis-

Table 1. Primers information for PCR used in this study

Gene	Gene accession	Primer sequence
MMP-9	NM_004994.2	Forward: TTCAGGGAGACGCCCATTTC
		Reverse: AAACCGAGTTGGAACCACGA
TIMP-1	NM_003254.2	Forward: GTTGGCTGTGAGGAATGCAC
		Reverse: AAACCGAGTTGGAACCACGA
p38	NM_001315.2	Forward:CGTGTTGCAGATCCAGACCA
		Reverse: GCCAGAATGCAGCCTACAGA
GAPDH	NM_001256799.2	Forward: ACACCCACTCCTCCACCTTT
		Reverse: TTACTCCTTGGAGGCCATGT

Table 2. Demographic and Gestational Characteristics of Studied Patients

Characteristic	Control (n=20)	FPROM (n=15)	PPROM (n=20)
Maternal age (years)	25 ± 6	26 ± 5	27 ± 4
Gestational age (weeks, days)	38 w ± 3 d	38 w ± 3 d	32 w ± 5 d
Marital status	Married	Married	Married
Ethnicity	Yellow	Yellow	Yellow
Smoked during pregnancy	0	0	0

Data are presented as means ± SD.

sues such as the spongy and fibroblast layers. The HAE cell layer was then treated with 0.125% trypsin three times each for 20 min to obtain dissociated HAE cells. The cells were cultured in RPMI-1640 (GIBCO) medium containing 10% fetal calf serum (GIBCO) at 37°C in 95% air/5% CO_2 humidified atmosphere. The culture medium was changed every 3 days. Human Amniotic Epithelial Cells were then staining for their specific markers such as CK-19. In addition, cell surface marker such as CD9, CD10, CD29, CD38, CD73, cytokeratin, CD34, CD44 and cytokeratin (BD-Bioscience, San Diego, CA) examined by flow cytometer analysis as described previously [18]. Briefly, after HAE cells were staining with either FITC- or PE-labeled antibodies. The expression of CD9, CD10, CD29, CD38, CD73, cytokeratin, CD34 and CD44 were examined by flow cytometer (BD Biosciences, USA).

Cell Immunofluorescence staining of biomarkers for human amniotic epithelial cells

Briefly, cells seeding on confocal dishes were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 and then blocked with 5% normal goat serum. The cells were then incubated with anti-CK-19 (Abcam, USA) antibodies in 1% BSA at 4°C overnight, and detected by Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Abcam, USA). The cell nuclei were stained by 4'-6-diamidino-2-phenylindole (DAPI) for 15 minutes, and the immunofluorescence images were acquired on Zeiss fluorescence microscopy (Carl Zeiss, Germany).

Real-time PCR analysis

The mRNA levels of MMP-9, TIMP-2 and p38 were detected by Real-time PCR. Briefly, total RNA was extracted using an RNA Extraction Agent (TaKaRa Bio, Shiga, Japan) following manufacturer instructions. cDNA was synthesized using a TaKaRa Bio Reverse Transcription kit. Real-time PCR was performed using the SYBR Green kit of the TaKaRa Bio Reaction System according to the manufacturer's instructions. The primers information were showed in **Table 1**.

Assessment of cell viability and growth

Cell viability was determined by the CCK-8 quantitative colorimetric assay, capable of detecting viable cells. The cells were seeded on a 96-well plate and transfected with P38 siRNAs. siRNA 1 Sense 5'-AUUUUACCCAAUAUGGAACCUdTdT-3', Anti-sense 5'-GUUCCAUAUUGGGUAAAAUCUdTdT-3'; siRNA 2 Sense 5'-ACACGUAACCCCGUUUUUGUGdTdT-3', Anti-sense 5'-CAAAAACGGGGUUACGUGUGGdTdT-3'; siRNA 3 Sense 5'-AAUCACAUUUUCAUGUUUCAUdTdT-3', Anti-sense 5'-GAAACAUGAAAAUGUGAUUGGdTdT-3'; vehicle NT siRNA Sense 5'-CAGUCGCGUUUGCGACUGGdTdT-3', Anti-sense 5'-CCAGUCGCAAACGCGACUGdTdT-3' with Lipofectamin RNAiMAX (Invitrogen) for 72 hr, then cell viability was detected at various times (0, 24, 48, and 72 hr). Thereafter the medium was changed and incubated with CCK-8 (Dojindo Molecular Technologies, Gaithers burg, MD, USA) for 4 h. The cell viability then measured spectrophotometrically at 450 nm with a reference wavelength of 600 nm.

Flow cytometric analysis of cell apoptosis

After the cells were transfected with p38 siRNA or vehicle NT siRNA with Lipofectamin RNAiMAX

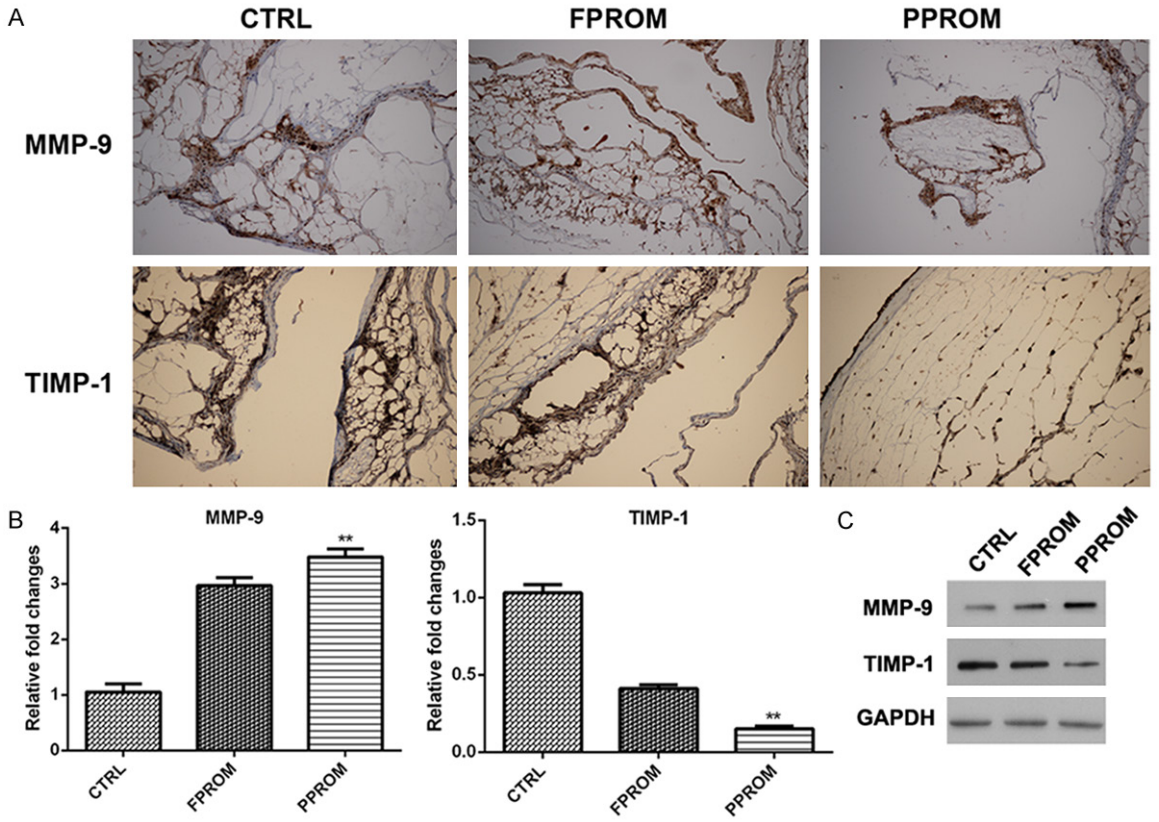

Figure 1. The expression of MMP-9 and TIMP-1 in different experiment groups. A. Representative images to show the differences of MMP-9 and TIMP-1 in fetal human membranes from each cohort. B. Real-time PCR detected the expression of MMP-9 and TIMP-1 in different experiment groups. C. Western blotting detected the expression of MMP-9 and TIMP-1 in different experiment groups. CTRL: full-term normal delivery experiment group. FPROM: full-term premature rupture of the membranes groups; PPROM: preterm premature rupture of membranes group. **, $P<0.01$ compared with PPROM group.

(Invitrogen) for 72, cells ($1x10^6$) were freshly harvested and suspended in a 1:1 (v/v) mixture of PBS and 0.2 M Na_2HPO_4-0.1 M citric acid (pH 7.5). Following the fixation with ice-cold ethanol at 4°C for 1 h, the cells were resuspended in an Annexin V binding buffer and then incubated in a buffer containing 200 ng/ml Annexin V-FITC conjugates (Sigma, MO, USA) at room temperature for 15 min. Subsequently, the cells were stained with PI (Sigma, MO, USA) (300 ng/ml) for 10 min. The stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, USA).

Analysis of cell cycle progression

After cells were transfected with p38 siRNA or vehicle NT siRNA with Lipofectamin RNAiMAX (Invitrogen) for 72 hr, cells were trypsinized, harvested, and fixed in 1 ml 80% cold ethanol. Then, cells were centrifuged and the cell pellets were resuspended in 500 μl propidium iodine (10 μg/ml) containing 300 μg/ml RNase (Sigma, MO, USA). Then cells were incubated on ice for 30 min. Cell cycle distribution was recorded using FACScaliberflow cytometer (BD Biosciences, USA).

Gelatin zymography

After 24 hours of cells culture in serum-free RPMI-1640, the supernatant was collected after centrifugation at 2000 g for 10 min. According to the cell counting, the protein concentration is determined to be consistent. Samples were mixed in an SDS-polyacrylamide gel electrophoresis loading buffer applied to a SDS/9% polyacrylamide gels containing 1 mg/mL gelatin (Bio-Rad, USA) and separated by electrophorosis. Subsequently, the gels were incubated overnight at 37°C in zymography buffer (50 mmol/L Tris, pH7.5, and 10 mmol/L

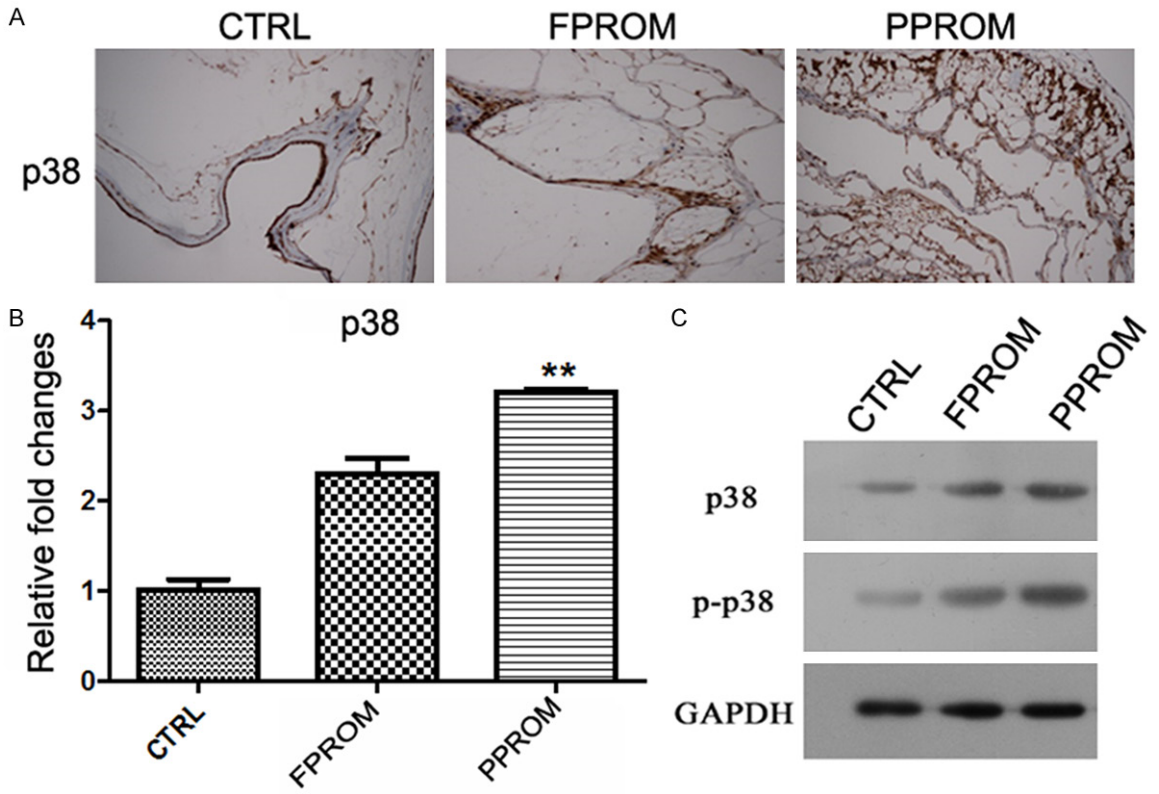

Figure 2. Expression of p38 in different experiment groups. A. Representative images to show the differences of p38 in fetal human membranes from each cohort. B. Real-time PCR detected the expression of p38 in different experiment groups. C. Western blotting detected the expression of p38 in different experiment groups. CTRL: full-term normal delivery women. FPROM: full-term premature rupture of the membranes group; PPROM: preterm premature rupture of membranes group. **, P<0.01 compared with PPROM group.

$CaCl_2$) and stained with Coomassie brilliant blue (Sigma-Aldrich, USA). Gelatinolytic activity was visualized as clear areas of lysis in the gel. Densitometric analysis was performed by using NIH Image software (NIH, USA).

Statistics analysis

Statistical analysis was performed by SPSS19.0 (SPSS Inc, USA). Data were expressed as means ± standard deviation (SD). All statistical comparisons between the groups were identified by using one-way ANOVA analysis followed by paired Student's t-tests, where appropriate. The bar graphs were produced using GraphPad Prism 5.02 (GraphPad Software, USA). Differences at $P<0.05$ and $P<0.01$ were considered statistically significant.

Results

Participants and pregnancy outcomes

Demographic details of the study participants are provided in **Table 2**. We examined 55 tissue samples from three different groups, namely, control, FPROM and PPROM group. Maternal age, gestational age, marital status, ethnicity and number of cigarettes smoked during pregnancy were recorded. Gestational ages were similar between the control group and FPROM group. Cigarette smokers from this study were excluded to our term group to avoid any confounding effects.

Expression of MMP-9 and TIMP-1 human fetal membranes by immunostaining analysis

To examine the levels of MMP-9 and TIMP-1 in protein levels of different groups of human fetal membranes, we employed by immunostaining analysis. As showed in **Figure 1A**, in control group (women with term labor and vaginal delivery after an uncomplicated pregnancy), only a small number of positive cells also detected. Whereas the expression of MMP-9 was significantly increased in patient of PPROM and FPROM groups. Notably, the expression of MMP-9 in PPROM groups was also significantly

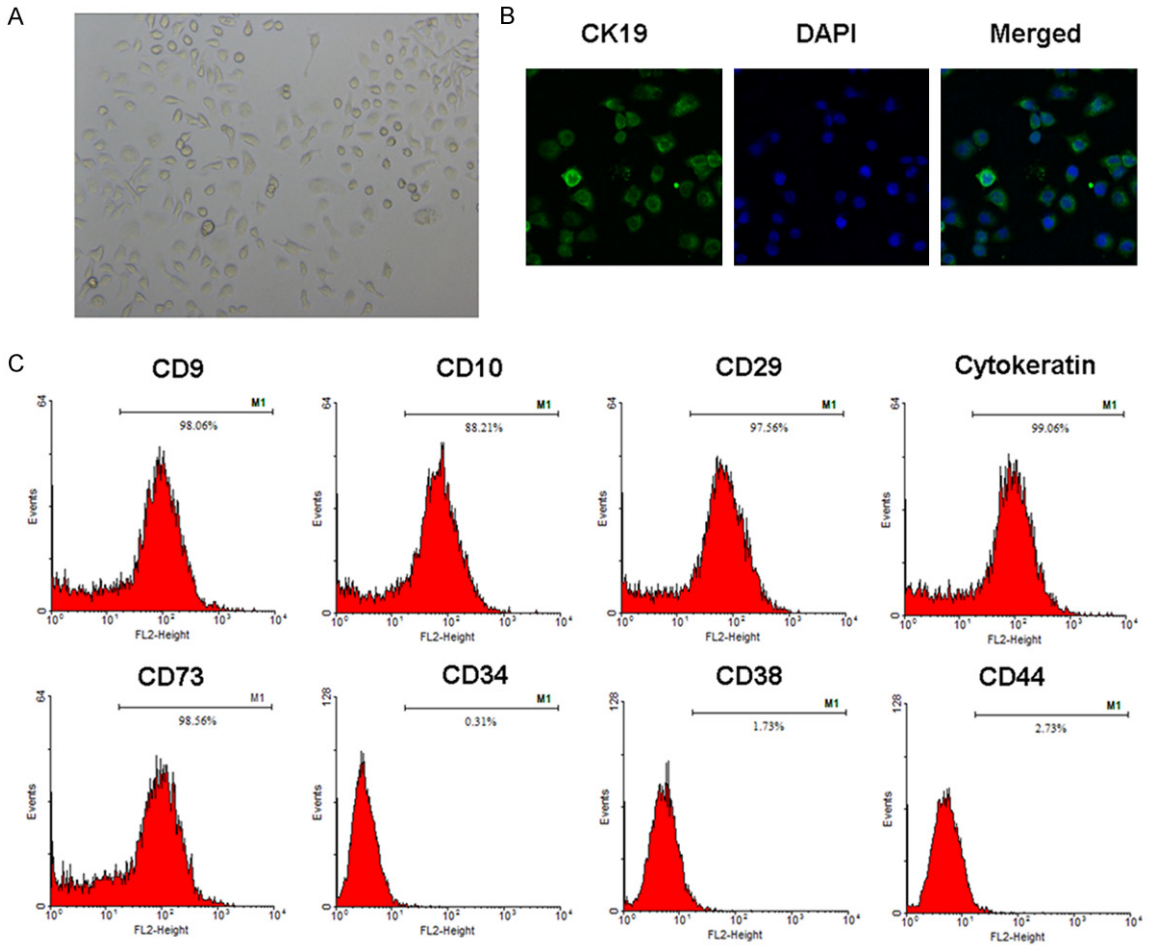

Figure 3. Characterization of HAE cells. A. Representative image of HAE cells observed under invert microscope. B. Immunostaing detect the expression of CK-19 in HAE cells. C. Flow cytometer examined the expression of CD9, CD10, CD29, C73 cytokeratin, CD34, CD38 and CD44 in HAE cells.

increased compared to FPROM group. Moreover, the expression of MMP-9 inhibitor TIMP-1 in both PPROM and FPROM groups was decreased compared to control group. The levels of TIMP-1 in PPROM groups were significantly lower than FPROM.

Expression of MMP-9 and TIMP-1 human fetal membranes

To further confirm whether the changes in the levels of MMP-9 and TIMP-1 also occurred in mRNA levels and protein levels. Real-time PCR and Western blotting technologies were used to examine the expression of MMP-9 and TIMP-1 in different groups. As showed in **Figure 1B** and **1C**, the expression of MMP-9 were significantly up-regulated in both PPROM and FPROM groups compared to control group. Importantly, the expression of MMP-9 in PPROM group was significantly high than that in FPROM group. We also detected the expression of TIMP-1 in different experimental groups. As showed in **Figure 1B** and **1C**, the expression of TIMP-1 in both PPROM and FPROM groups were dramatically decreased compared to control group. It was to note that the levels of TIMP-1 in PPROM group were significantly lower than FPROM group.

Expression of p38 in human fetal membranes

To examine whether MAPK kinase p38 levels were changed inhuman fetal membranes of PROM patients. We firstly used immunostaining technology examined the expression of p38 in different groups. As showed in **Figure 2A**, the expression of p38 was significantly increased in patient of PPROM and FPROM groups compared to control group. Notably, the expression

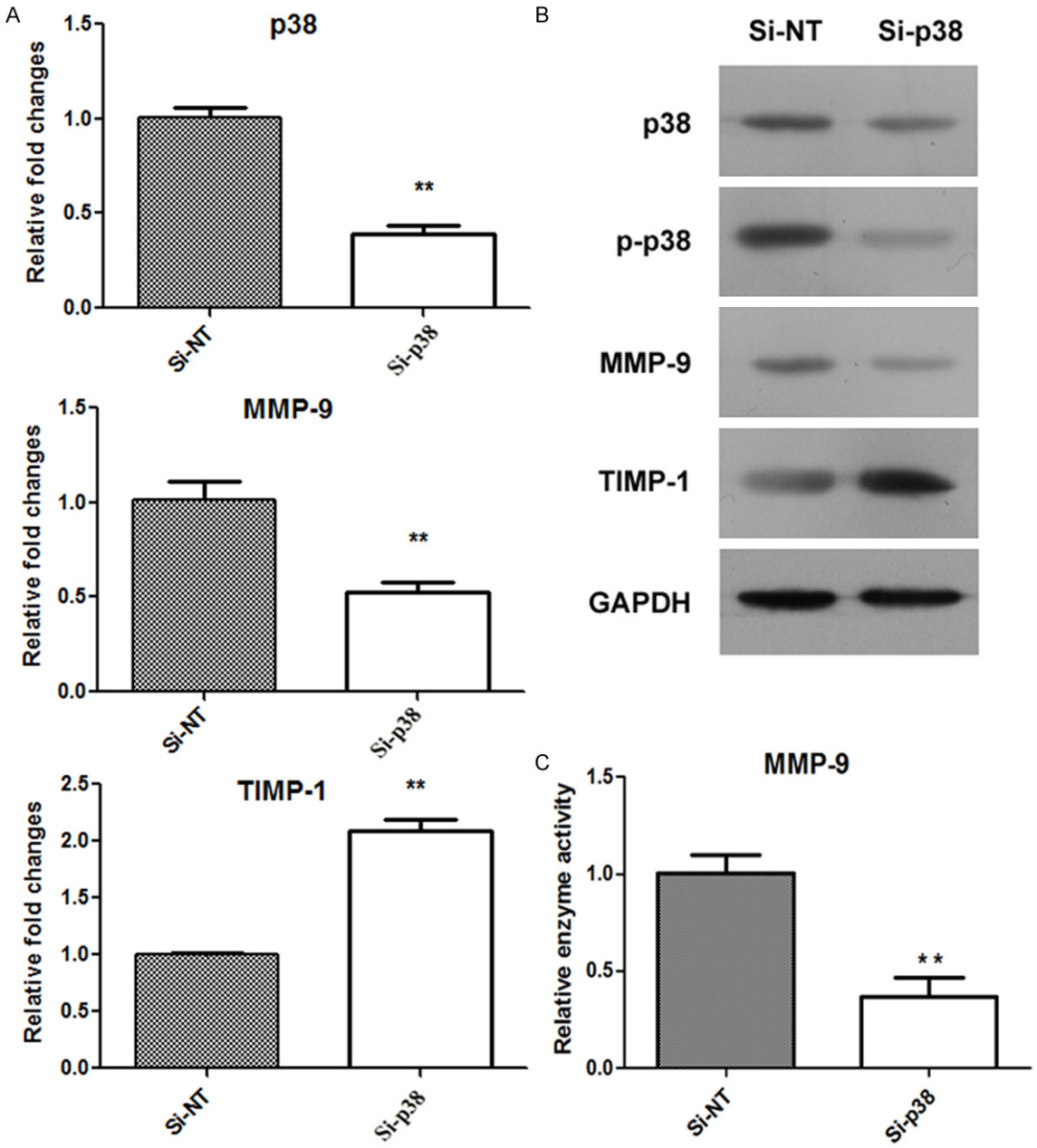

Figure 4. Effects of p38 knockdown on the expression of MMP-9 and TIMP-1 in HAE cells. A. Effect of knockdown the expression of p38 on the expression of p38, MMP-9, and TIMP-1 detected by Real-time PCR analysis. B. Effect of knockdown the expression of p38 on the expression of p38, MMP-9, and TIMP-1 detected by Western blotting analysis. C. Relative enzyme activity of MMP-9. **, P<0.01 compared CTRL group.

of p38 in PPROM groups was also significantly higher than FPROM group. Secondly, Real-time PCR and Western blotting was used to detect the expression of p38 of different group. As showed in **Figure 2B** and **2C**, in PPROM and FRPOM groups, the levels of p38 were dramatically up-regulated. The levels of p38 in PPROM group also significantly higher than FPROM group. Importantly, p38 was found to be most abundant in amnion epithelium, chorion laeve trophoblasts, and placental syncytiotrophoblasts.

Characterization of human amniotic epithelial (HAE) cells

To characterize of HAE cells, we firstly investigated its morphologically under invert micro-

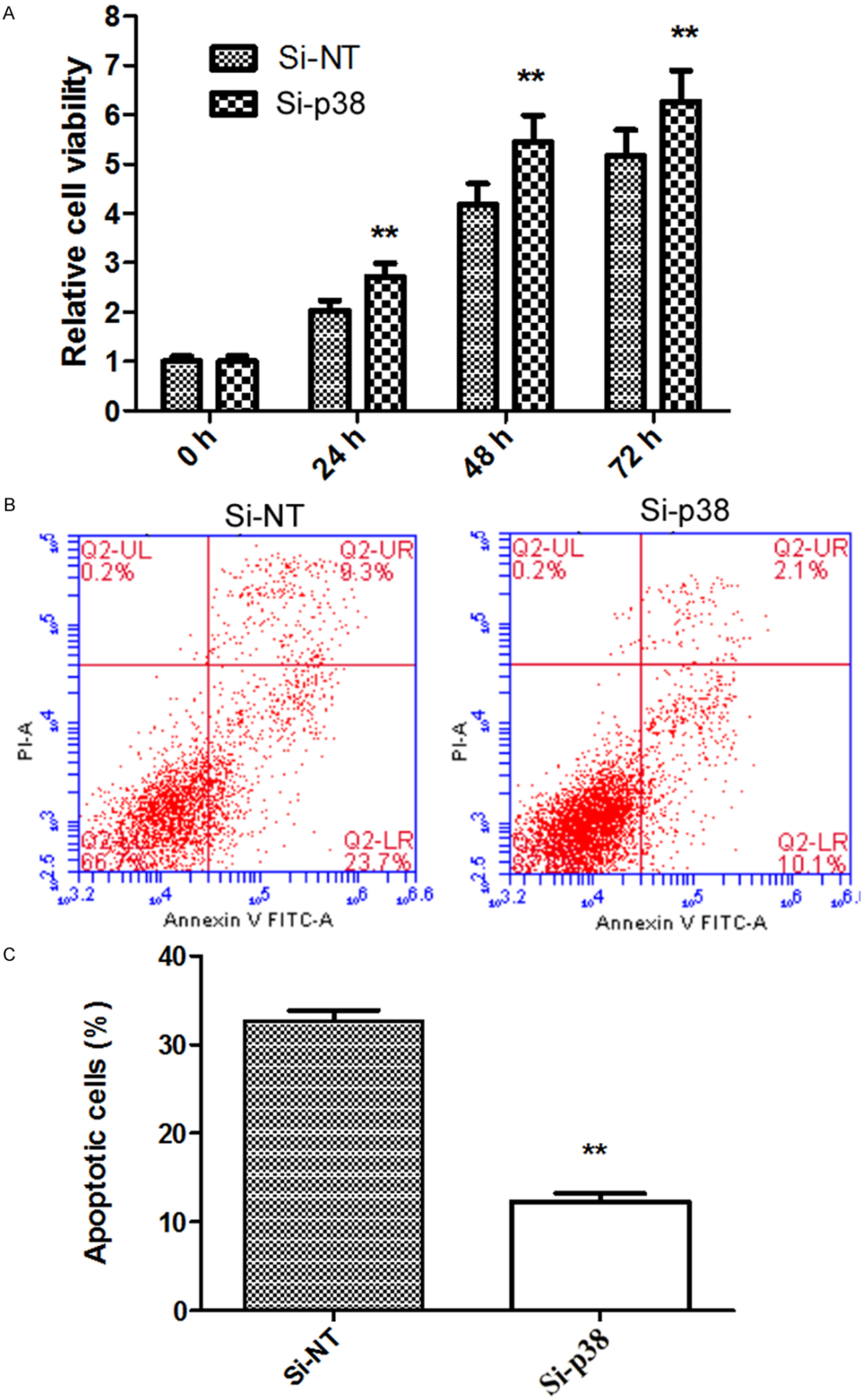
A
Relative cell viability
Si-NT
Si-p38
0 h
24 h
48 h
72 h
**
B
Si-NT
Si-p38
Q2-UL
0.2%
Q2-UR
8.3%
Q2-LR
23.7%
Q2-UL
0.2%
Q2-UR
2.1%
Q2-LR
10.1%
PI-A
Annexin V FITC-A
C
Apoptotic cells (%)
Si-NT
Si-p38

Figure 5. Effects of p38 knockdown on cell proliferation and cell apoptosis. A. Effect of knockdown the expression of p38 on cell proliferation detected by CCK-8 assay. B. Effect of knockdown the expression of p38 on cell apoptosis examined by Annexin V-FITC/PI double staining. C. Quantification of B. **, P<0.01 compared CTRL group.

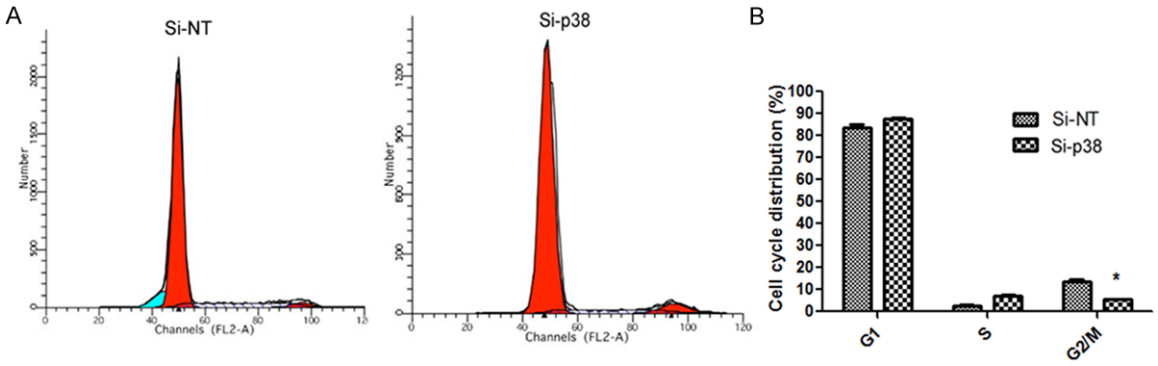

Figure 6. Effect of p38 knockdown on cell cycle progression. A. Representative images of knockdown the expression of p38 on HAE cells cell cycle progression. B. Quantification data in A.

scope, as showed in **Figure 3A**, after 72 hr of culture, the cells were flattened with fibroblastic-like morphology. Next, we staining of the cells with their specific markers such as CK-19. As showed in **Figure 3B**, about 98% of cells were positive for the expression of CK-19. Finally, flow cytometer analysis was used to detect the expression of several marker of HAE cells. AS showed in **Figure 3C**, HAE cells were positive for the expression ofCD9, CD10, CD29, CD73, cytokeratina and negative for the expression of CD34, CD38, CD44 [18]. These results suggest that our isolated HAE cells were appropriate for further experiments.

Knockdown the expression of p38 on the expression of MMP-9 and TIMP-1 in HAE cells

To examine the effect of p38 on regulation the expression of MMP-9 and TIMP-1 level, we knockdown the expression of p38 HAE cells with RNAi, as showed in **Figure 4**, Real-time PCR and Western blotting results showed that transfected of p38 siRNA significantly decreased the expression of p38. Moreover, knockdown the expression of p38 significantly decreased the expression of MMP-9 and increased the expression of TIMP-1 in both mRNA and protein levels. Gelatin zymography and densitometric analysis also confirmed that the relative enzyme activity of MMP-9 was impaired due to the reduction of p38. These results suggest that p38 may play an important role in regulating the expression of MMP-9 and TIMP-1 in HAE cells.

Knockdown the expression of p38 increases cell proliferation and reduces cell apoptosis

To examine the roles of p38 in HAE cells, we first knockdown the expression of P38 and then examined the effects of knockdown the expression of p38 on cell proliferation at various duration (0, 24, 48 and 72 hr). As showed in **Figure 5A**, knockdown p38 significantly increased cell proliferation in a time dependent manner. Moreover, knockdown the expression of p38 also significantly reduces cell apoptosis (**Figure 5B** and **5C**) as reflected by Annexin-V-FITC/PI staining analysis.

Knockdown the expression of p38 on cell cycle progression

The effect of Knockdown the expression of p38 on cell cycle was evaluated using flow cytometric analysis. After knockdown the expression of p38, as showed in **Figure 6A** and **6B**, cells in the G2/M population decreased from about 14.5% to 5.8% compared to control. The decreased of cell population at the G2/M phase was accompanied by an increase in the cell population of G1 phase.

Discussion

PROM is a major pregnancy complication that can result in adverse neonatal and perinatal outcomes [19]. About half of preterm birth cases are related with PROM [19]. The inflammatory responses and the produced cytokines

such as TNF-α, IL-6, IL-1, IL-8, prostaglandin are of great importance for cervical changes and stimulation of myometrial contractions [20-23]. While toxic agents or infection may lead to the initiation of cell apoptosis pathway and contribute to PROM [24, 25]. Of note, MMPs and TIMPs is associated with PROM and preterm birth [6, 24, 26].

Cumulative findings exhibited that multiple factors for the pregnancy complications (such as smoker, toxic agent or infectious or other) lead to PROM directly or indirectly via the MMP system activities, suggesting a central role of MMPs in preterm birth [27]. Indeed, MMP-9 and TIMP-1 levels have been reported to be associated with preterm lab and PPROM [6, 10, 11, 13]. Consistent with previously reports [6, 10, 11, 13], our study also presented that the up-regulated the expression of MMP-9 and down-regulated the expression of TIMP-1 in FPROM patients significantly compared with control group. Notably, the levels of MMP-9 in FPROM group also significantly decreased compared with PPROM group, whereas the expression of TIMP-1 in FPROM group dramatically increased compared with PPROM group. Intriguingly, in immunohistochemical staining of MMP-9, the expressions from control group, FPROM and PPROM groups showed no significant difference, which may be due to the deactivation of MMP-9 proenzyme. However, the distribution of MMP-9 in fetal membranes of extracellular matrix (ECM) in FPROM and PPROM groups were more extensive than that in control group, while the degradation of the extracellular matrix of membranes was severer than that in control group. These results suggest that MMP-9 and TIMP-1 may play an important role in regulating regulation of PROM.

P38 is stress responsive kinase against a broad range of cellular stimuli and cytokines. It plays an important regulatory role in a variety of biological process such as cell proliferation, cell growth and cell differentiation [28, 29], which leads to several diseases [30, 31]. Recent evidence unraveled its involvement as a mediator in cell survival in response to DNA damage, besides the role of the induction of apoptosis [32]. Though p38 are reported to regulate MMP-9 and TIMP-2 activities in other disease status (such as cancer) [33], the roles of p38 in PPROM are not clear. In this study, we found that in the patients of PPROM and FPROM, the expression of p38 in both protein and mRNA levels are dramatically increased compared with control group, indicating that the changes of MMP-1 and TIMP-1 in PROM may occur via p38. We thus proposed that the elevated levels of p38 imbalanced the expressions of MMP-9 and TIMP-1, which aggravated extracellular matrix degradation and weakened the fetal membranes that eventually caused PROM.

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an important role in several diseases. Previous observations showed that matrix metalloproteinases MMP-2 and MMP-9, and matrix metalloproteinase inhibitors TIMP-1 and TIMP-2 mRNAs were synthesized in diseased valves, manifesting that they may contribute to matrix remodelling in valvular disease [34]. Recent progresses also presented that excessive peritoneal fluid TIMP-1 negatively impacts ovarian function via matrix metalloproteinase (MMP)-dependent and/or MMP-independent actions pathways [35]. In three well-characterized subfamilies of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases (ERKs) function in the control of cell division. The c-Jun amino-terminal kinases (JNKs) are critical regulators of transcription. While the p38 MAPKs are activated by inflammatory cytokines and environmental stresses and may contribute to diseases like asthma and autoimmunity [36, 37]. It has been demonstrated that the treatment which involved a p38 inhibitor (SB203580) resulted in the synergistic reduction of MMP-2 and MMP-9 expression and then increase of TIMP-1 and TIMP-2 expression. Experimental evidence on fetal brain astrocytes characterized that p38 modulated expression of tumor necrosis factor-related apoptosis-inducing ligand induced by interferon-gamma [37]. The study on cell motility and migration showed that p38 signaling pathway has effect on glioma cells invasion and metastasis [38, 39]. Similarly, to further examine the roles of p38 in regulation of MMP-9 and TIMP-1 in HAE cells, we knockdown the expression of p38 in primary HAE cells and found the increasing expression of TIMP-1. CCK-8 assay also performed that HAE cell proliferation was a significantly enhanced in a time-dependent manner. As demonstrated by studies, p38-interacting protein

(p38IP) regulated G2/M progression. By using RNA interference to knock down p38IP, it has been indicated that the GCN5-SAGA complex is required for G2/M progression, mainly on the grounds that p38IP favors the acetylation of alpha-tubulin by preventing the degradation of GCN5, in turn promoting the formation of the mitotic [40]. Likewise, the knockdown of p38 caused cells decreased in the G2/M phase compared with control, accompanied by an increase of cell population in the G1 phase of the cell cycle. Together, these results indicated that the inhibition of p38 promotes HAE cells growth and proliferation as well as cell cycle progression.

In summary, we found that the increased in the expression of MMP-9 and the decreased in the expression of TIMP-1 in PPROM and FPROM patients compared with control group. The levels of MMP-9 and TIMP-1 in FPROM group and PPROM group also have significantly different. Moreover, the changes in the expression of MMP-9 and TIMP-1 levels were accompanied by the up-regulated the expression of p38. In an *in vitro* HAE cell culture model, we found p38 is essential for regulating the expression MMP-9 and TIMP-1 levels. Importantly, p38 also plays a key role in controlling cell proliferation, cell apoptosis and cell cycle progression in HAE cells. Collectively, our results indicate that p38/MMP-9/TIMP-1 pathway may be implicated in PROM. These results implicate the importance of p38 in PROM.

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Disclosure of conflict of interest

None.

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