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Conditioned Medium from Human Decidual Stromal Cells has a Concentration-dependent Effect on Trophoblast Cell Invasion

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ABSTRACT

The regulation of trophoblast cell invasion is a crucial aspect of implantation and placental development. Evidence indicates that the uterine microenvironment exerts important influence over trophoblast cell invasion. However, the precise effect of decidual cells on trophoblast cell invasion remained unidentified. In the present study, a cell line representative of normal human trophoblast (B6Tert) was used to examine the effect of decidual stromal cell conditioned media (DSCM) on trophoblast cell invasion. *In vitro* assay showed the concentration-dependent effect of DSCM on B6Tert cell invasion. RT-PCR and gelatin zymography demonstrated that DSCM evidently produced an effect on the mRNA expression and proenzyme production of MMP-2 in a dose-dependent manner, but exerted no effect on mRNA expression and proenzyme production of MMP-9. The data indicates that the decidual microenvironment may exert the key control for trophoblast cell invasion mainly through influencing MMP-2 expression.

1. Introduction

Adequate invasion of the placental trophoblast cells is necessary for implantation and placentation in the establishment of successful pregnancy. During normal pregnancy, cytotrophoblasts cross the placental–maternal bridges and invade the maternal deciduas, stimulating remodeling of the arterial wall such that the artery dilates, the supply of nutrients to the growing fetus is enhanced and made independent of the influence of maternal vasoconstrictors [1]. Cytotrophoblasts are invasive because of their ability to secrete matrix metalloproteinases (MMPs), a multigene family of Zn^{2+}-dependent endopeptidases that degrade the decidual extracellular matrix (ECM) and basement membrane components. Two MMPs secreted by cytotrophoblasts, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) can degrade collagen IV, the main component of the basement membranes, and are therefore regarded as the key enzymes in the invasive process [2].

Unlike tumour invasion, trophoblast cell invasion is under highly control such that the invasion remains confined to the inner one-third of the myometrial segments and only last into the 5th month of pregnancy in women. The overinvasion and inadequate invasion can lead to such pathologies of pregnancy as choriocarcinoma and preeclampsia, respectively [3,4]. Evidence indicates that the uterine microenvironment exerts important influence over trophoblast cell invasion, the conditioned media from first trimester human decidual cells could suppress invasion of trophoblast cells [5]. However, little is known about the mechanisms responsible for human trophoblast cell invasion provided by the decidual tissue of the uterus, and the precise effect of decidual cells on trophoblast cell invasion remained unidentified.

In the present study, we use B6Tert cell line, a cytotrophoblast cell line derived from human normal placental tissues in early pregnancy and has been proven to possess similar endocrine functions to those of the primary cultured human cytotrophoblast in the first trimester [6,7] to investigate the effect of decidua-conditioned medium on trophoblast cell invasion. The production of MMP-2 and MMP-9 was also examined to elucidate the mechanisms of decidua function in human trophoblasts.

2. Methods

2.1. Tissue collection

Human decidual tissues were obtained from legal abortions (6–8 weeks of gestational age), with the approval of the Local Ethical Committee and the consent of the participating patients who were admitted to Department of Obstetrics and Gynecology, Tangdu Hospital in Xi'an and Haidian Hospital in Beijing, China. All the patients accepted no special medical treatment before termination of pregnancy, and the decidual tissues were pathologically normal. Tissues were collected into

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Dulbecco Modified Eagle medium (DMEM; Gibco, BRL, USA) and then transported to the laboratory and processed as described below.

2.2. Decidual stromal cell culture

Decidual stromal cells were isolated as previously described [8] with the following modifications. Briefly, decidual tissues were washed with DMEM, minced finely and digested with DMEM containing 0.1% collagenase TypeIA, 0.02% deoxyribonuclease type I (Gibco, BRL, USA) in a 37 °C shaking water bath for 1 h, and was subjected to consecutive filtration through 150 μm, 80 μm mesh, respectively. Stromal cells were collected and applied to Percoll gradient centrifugation. Cell viability was >90% using trypan blue exclusion. Immunohistochemical localization of vimentin was carried out to confirm the isolated cells.

Stromal cells were plated in 60-mm^2 culture dishes (3×10^5 cells/dish) and cultured in 10% FBS DMEM media supplemented with antibiotics and estradiol (10^{-9} mol/L) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. On day 2, fresh 10% FBS DMEM medium was applied with estradiol (E2, 10^{-9} mol/L) and progesterone (P, 10^{-9} mol/L) to maintain the decidualization. Once 80% confluent, cells were washed twice with sterile PBS and subjected to 24 h in serum-free medium (0.2% BSA DMEM) supplemented with E2 and P. Then the sex steroids of conditioned media were removed by charcoal-dextran treatment [9] to generate charcoal-dextran treated decidual stromal cell conditioned media (DSCM). The fresh serum-free medium (0.2% BSA DMEM) supplemented with E2 and P was served as a control after the charcoal-dextran treatment (medium control). *In vitro* decidualization of the stromal culture was confirmed by PRL production and by morphologic assessment.

2.3. Culture of human B6Tert cell line and experimental design

B6Tert cell line is an immortalized normal human cytotrophoblast cell line (6–8 weeks gestation) transfected with htert gene. The B6Tert cells were cultured as previously described [6,7]. In brief, the cells were cultured in serum-free FD medium (F-12: DMEM, 1:1; Gibco, BRL, USA) with supplement of 10 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.1% BSA, and 2 mM glutamine (Sigma, USA), and kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The culture media were refreshed every 1–2 days. Subculture at a ratio of 1:3 was performed with routine trypsinization every 5 days. All studies were performed using B6Tert cells plated in 60-mm^2 culture dishes at a density of 1×10^5 cells and grown to 80% confluence. At least 24 h before DSCM treatment, EGF was withdrawn from the culture medium.

The DSCM treatment was performed under the following experimental conditions: a) B6Tert cells cultured in FD medium, untreated control (B6 alone); b) B6Tert cells cultured in FD medium containing 5% medium control (B6 + 5% control) or 5% DSCM (B6 + 5% DSCM); c) B6 + 10% control, B6 + 10% DSCM; d) B6 + 20% control, B6 + 20% DSCM. After a 24 h incubation period, B6Tert cells were used for Transwell invasion assay, RT-PCR analysis and gelatin zymography.

2.4. Transwell invasion assay

In vitro cellular invasion was assayed by determining the ability of cells to invade a synthetic basement membrane. Briefly, B6Tert cells were plated at 2×10^4 cells in transwell inserts (8-μm pore size; Costar, Cambridge, MA, USA) pre-coated with TypeIcollagen (ColI; 80 μg/ml; Cell Matrix TypeIA, Institute of Biochemistry, Osaka, Japan), and incubated with FD medium supplemented with or without DSCM. Lower chambers were loaded with the same medium. The cells were incubated at 37 °C and allowed to invade through the collagen barrier for 24 h. Following incubation, the invading cells were fixed with 4% PFA and stained with hematoxylin, non-invading cells were removed using a cotton swab. Cell invasion index was determined by counting the number of stained cells in 10 randomly selected non-overlapping fields of the membranes using a light microscope.

2.5. RT-PCR analysis

Total RNA was isolated from the cultured B6Tert cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration of total RNA was measured on a spectrophotometer (Jenway Ltd, Dunmow, Essex, UK). RNA integrity was confirmed by electrophoresis in a 1.5% agarose denaturing gel and 1 μg of the total RNA was subsequently reverse transcribed into cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (MBI, Fermentas, USA). The primers for PCR were designed according to specific cDNA sequences in NCBI database. The primer sequences and the reaction conditions are shown in Table 1. A 25 μl PCR master-mix was prepared as follows: 1 μl RT products, 200 μmol/l dNTPs, 2 mmol/l $MgCl_2$, 1 IU Taq polymerase, and 10 pmol of each primer. The amplification was ensured within the exponential phase of PCR by preliminary experiments. PCR products were subjected to electrophoresis on agarose gels and the relative densities of *MMP* genes normalized with *GAPDH* were analyzed using the Image-Pro Plus (software version 6.0; Media Cybernetics, Silver Spring, USA).

Table 1
The Primer sequences and reaction conditions for RT-PCR.

Gene	Primer Sequences (F: forward; R: reverse)	Annealing temperature (°C)	Cycle (n)
MMP-2	F: CACCTACACCAAGAACTTCC R: AACACAGCCTTCTCCTCCTG	58	28
MMP-9	F: CCCTTCTACGGCCACTACTGTG R: GCACTGCAGGATGTCATAG	58	30
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	55	23

2.6. Gelatin zymography

B6Tert cells were lysed with lysis buffer (62.5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate – SDS, 10% glycerol) on ice for 30 min as previously described [10,11]. The supernatant was collected after centrifugation. Protein estimation was carried out using Bradford method, and 8 μg of protein per lane was separated by 10% SDS-polyacry1-amide gel electrophoresis (SDS-PAGE) containing 1 mg/ml gelatin. After electrophoresis, the gel was washed at room temperature for 1 h in 2.5% Triton X-100, 50 mM Tris–HCl (pH 7.5), and then incubated at 37 °C overnight in a buffer containing 150 mM NaCl, 5 mM CaCl, and 50 mM Tris–HCl (pH 7.6). The gel was subsequently stained with 0.1% (w/v) Coomassie Brilliant Blue R-250, and destained in 10% (v/v) methanol and 5% (v/v) glacial acetic acid. The results were analyzed using the Image-Pro Plus.

2.7. Statistical analysis

All values are presented as the mean ± SD of three individual experiments done in triplicate. Comparison of the values between groups was performed using one-way ANOVA by SPSS 11.0 software and a P value of <0.05 was taken to be statistically.

3. Results

3.1. Effect of DSCM on B6Tert cell invasion

The invasion assay was performed to understand the effect of DSCM on B6Tert cell invasion. Fig. 1 depicts graphically the results of invasion assay which showed that DSCM produced an effect on B6Tert cell invasion in a dose-dependent manner. B6Tert cells cultured in FD medium containing 5% DSCM significantly increased the invasion of cytotrophoblast cells and the cell invasion indice was approximately 145.5% compared to untreated control ($P < 0.05$). However, adding 20% DSCM to the medium significantly decreased the penetration ability of B6Tert cells (cell invasion index: 65.4%, $P < 0.05$) but did not block it completely. Adding 10% DSCM did not alter the invasive capacity of B6Tert cells compared to untreated control. There was no difference in invasion between medium control groups (B6 + control) and untreated control (B6Tert alone).

3.2. Effect of DSCM on MMP-2 and MMP-9 production in B6Tert cells

We determined the change of *MMP-2* and *MMP-9* mRNA production in B6Tert cells using RT-PCR analysis. Relative expression of *MMP* gene was calculated by normalizing with *GAPDH* mRNA expression using densitometric quantitation. The mean level of *MMP-2* gene expression was changed by DSCM in a dose-dependent fashion (Fig. 2), increased by 5% DSCM (1.35 ± 0.13, ±SD) whereas decreased by 20% DSCM (0.30 ± 0.11, ±SD) compared to untreated control (0.84 ± 0.12, ±SD, $P < 0.05$). There was no difference of *MMP-2* gene expression between other groups and untreated control. No effects of DSCM on *MMP-9* mRNA productions were detected (data not shown).

To determine whether the mRNA levels correlated with the levels of proMMP-2 and proMMP-9 activity, we measured the proMMP-2 and proMMP-9 activity in B6Tert cells by gelatin zymography. Similar results were obtained as shown in Fig. 3 and the proMMP-2 activity was changed by DSCM in a dose-dependent

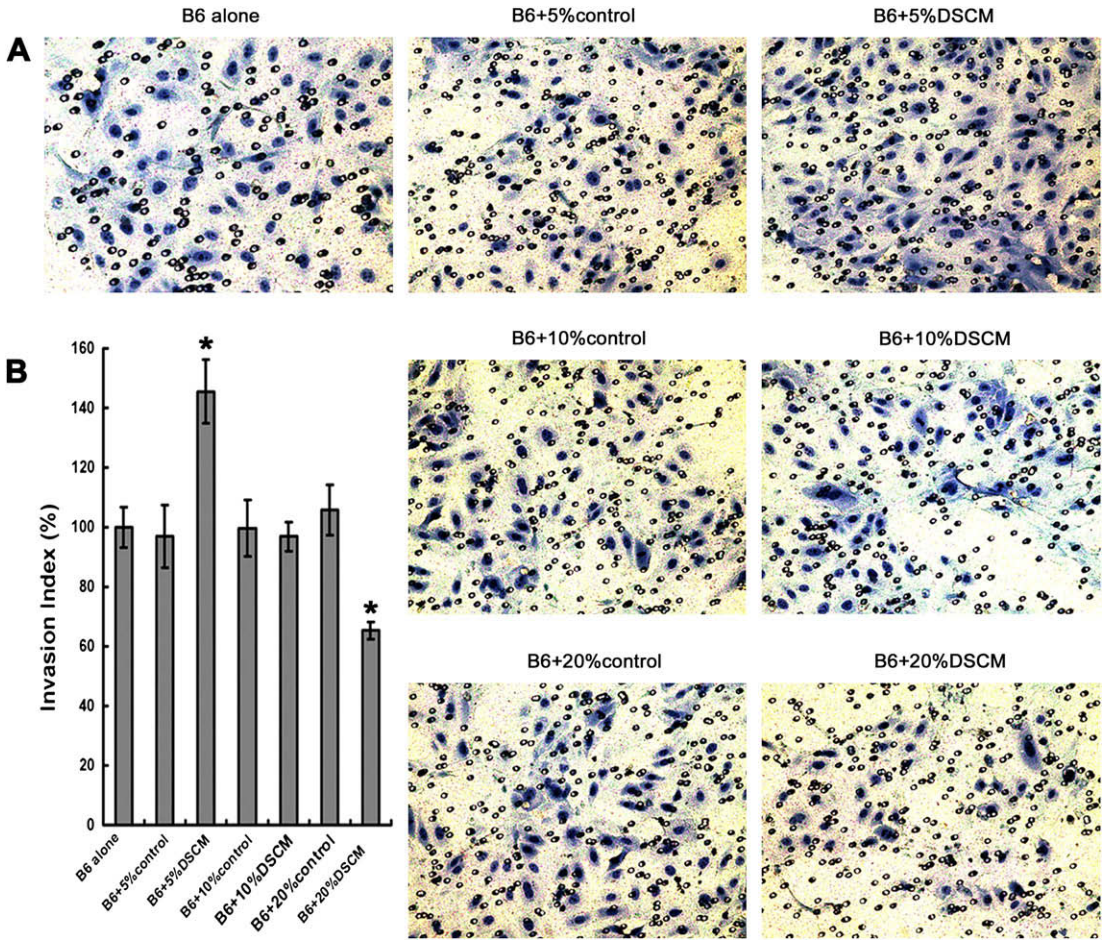

Fig. 1. Concentration-dependent effect of decidual stromal cell conditioned media (DSCM) on B6Tert cell invasion. (A) The B6Tert cells were cultured in the presence or absence of increasing concentrations (5–20% as indicated) of DSCM or the control medium, and their invasive capacities analyzed by invasion assay. (B) Statistical analysis by ANOVA was performed according to three independent experiments and the value was presented as mean ± SD. (*Compared with the B6 alone group, $P < 0.05$).

manner. No effects of DSCM on proMMP-9 activity were detected (data not shown).

4. Discussion

The present study demonstrated the concentration-dependent effect of DSCM on invasion and MMP-2 production of B6Tert cells. We found that addition of DSCM at high concentrations suppressed invasion and MMP-2 production of B6Tert cells, whereas addition of DSCM at low concentrations increased invasion and MMP-2 production of B6Tert cells. B6Tert cell line used in our study was previously established by Wang et al. [6] as the *in vitro* cell model for the investigation of trophoblastic behaviour. B6Tert has been characterized as a cytotrophoblast cell line derived from human normal placenta villi, and proven to produce various biomarkers of normal extravillous cytotrophoblasts (EVTs) during the early weeks of gestation. The production of MMPs and their invasive abilities *in vitro* shown in our study further indicate that the B6Tert cell line may be an ideal model to investigate invasion function in human trophoblasts.

Using the invasion assay, we found DSCM produced an effect on B6Tert cell invasion in a dose-dependent manner. Our observations extend the findings by Graham et al. [5], who showed the conditioned media from first trimester human decidual cells suppress invasion of trophoblast cells, besides this inhibitory effect, we found also that DSCM stimulates invasion of trophoblast cells. The decidual cells used by Graham et al. were composed of epithelial cells, stromal cells and different subsets of leukocytes, the inhibitory effect of decidual cells found by them may be partly due to different amount of leukocytes present in the conditioned medium. The present study used one decidual cell type to investigate the regulatory role of decidual stromal cells on trophoblast cells, and the results indicated that the decidual microenvironment may provide the key control for the invading degree of trophoblast. Both choriocarcinoma and preeclampsia may be refractory to this control and associated with aberrant trophoblast cell invasion.

MMPs have been considered critical for trophoblast cell invasion. To unravel the mechanisms of the concentration-dependent effect of DSCM on trophoblast cell invasion, we determined the *MMP* mRNA production and proMMP activity using RT-PCR analysis and gelatin zymography, respectively. Several studies showed that both isolated human decidual stromal cells and human endometrium could produce MMPs [12,13], the MMPs secretion produced by decidual stromal cells may exist in the culture media of treated groups, so we used B6Tert cell protein instead of culture media in this study to investigate MMP activity of trophoblastic cells by gelatin zymography. Our results demonstrated that both *MMP-2* mRNA production and proMMP-2 activity were changed by DSCM in a dose-dependent fashion whereas no effects of DSCM on MMP-9 production were detected. These results suggested that the concentration-dependent effect of DSCM on trophoblast cell invasion may act, at least in part, through effects on expression and activity of MMP-2, the invasive ability of cytotrophoblasts may be regulated mainly by MMP-2. The results correlate well with

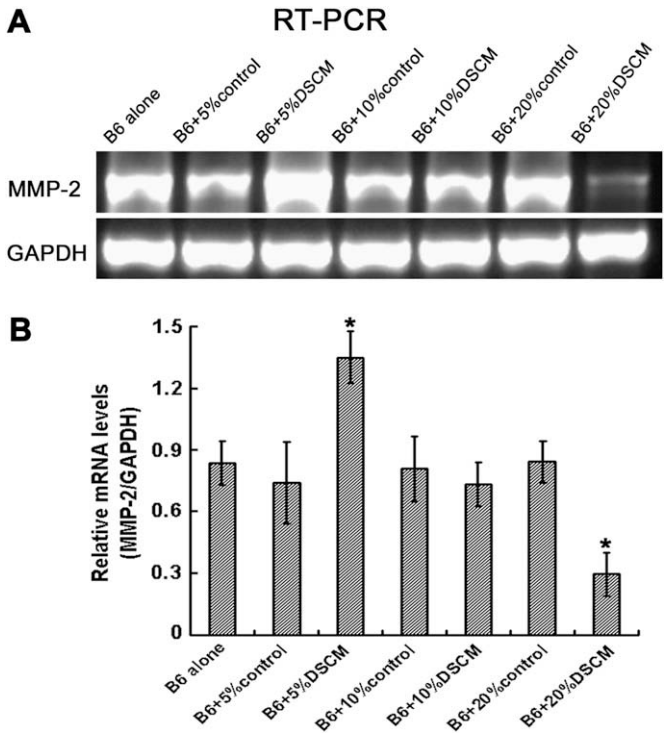

Fig. 2. Semi-quantitative RT-PCR to manifest the dose-dependent regulation on *MMP-2* mRNA expression by decidual stromal cell conditioned media (DSCM) in B6Tert cells. (A) RT-PCR analysis revealing *MMP-2* mRNA levels in B6Tert cells cultured in the presence or absence of increasing doses of DSCM or the control medium as indicated. (B) Statistical analysis by ANOVA for RT-PCR analysis according to three independent experiments. The values of *MMP-2* were normalized with that of *GAPDH* and the relative amount was presented as mean ± SD. (*Compared with the B6 alone group, $P < 0.05$).

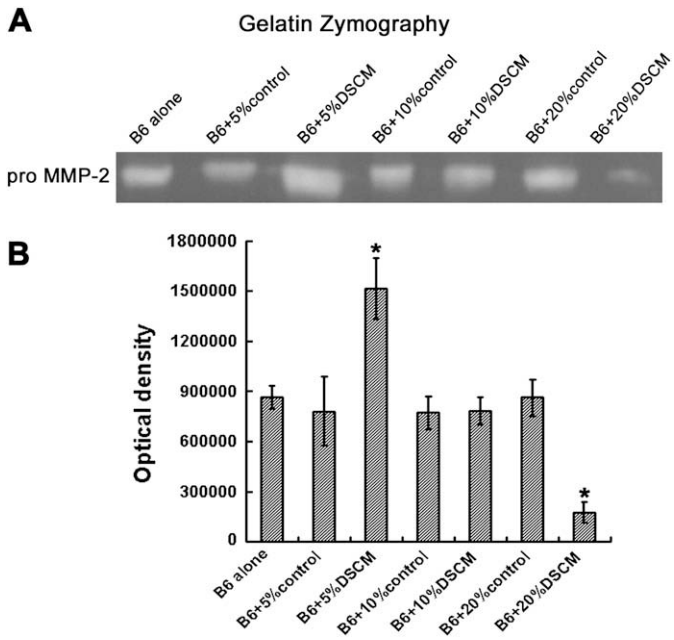

Fig. 3. Gelatin zymography to measure proMMP-2 activity in B6Tert cells cultured in the presence or absence of increasing doses of decidual stromal cell conditioned media (DSCM) or the control medium. (A) A typical result of gelatin zymography revealing the dose-dependent effect of DSCM on proMMP-2 activity. (B) Densitometric analysis of gelatin zymography. Statistical analysis was performed by ANOVA according to three independent experiments, and the values of proMMP-2 are presented as ±SD. (*Compared with the B6 alone group, $P < 0.05$).

a previous report: Isaka et al. [14] found that MMP-2 was located in invasive EVTs in first trimester placenta, whereas MMP-9 was located in the non-invasive villous cytotrophoblasts, which suggests that MMP-2 may play a more important role in the process of trophoblastic invasion than MMP-9. Also in keeping with our findings, Staun-Ram et al. [15] divided first trimester trophoblasts into two groups: 6–8 weeks (early) and 9–12 w (late) of gestation, and found that in the early trophoblasts, MMP-2 is the main gelatinase and the key enzyme in trophoblast cell invasion, thereafter in late trophoblasts, both MMP-2 and MMP-9 participate in trophoblast cell invasion. Therefore, it is likely that MMP-2 is more critical for human trophoblastic cell invasion in the early trophoblasts. In contrast with our results, some studies have found MMP-9 to be the key enzyme in the trophoblast invasion *in vitro* [16,17]. The reason for this discrepancy is not known, but may be due to the dynamic expression of MMPs during the first trimester. The choice of cell line may also contribute to a difference in results. In addition, MMPs secretion could be induced by PKA (protein kinase A), PKC (protein kinase C) and PTK (protein tyrosine kinase) pathways, and different signal pathways are likely not to affect the same enzymes, thereby may result in varying dominant enzymes [15]. Further studies are necessary to clarify the cell signaling pathways involved in the regulation of MMPs by DSCM.

The regulation of trophoblast cell invasion is a crucial aspect of implantation and placental development. Further studies are necessary to elucidate the precise regulation of trophoblast cell invasion. There is an intimate relationship between trophoblasts and the maternal deciduas throughout the process of implantation and early placentation. The decidual cells are believed to produce factors that fulfill paracrine, nutritional, and immunoregulatory functions during pregnancy [18,19]. Some studies have shown that decidual stromal cells produce factors such as interleukin-1β, tumour necrosis factor-α and insulin-like growth factor binding protein-1 [20,21], which up-regulate MMP-2 expression in trophoblasts [20,22,23]; as well as factors such as tissue-specific inhibitor of matrix metalloproteinase and transforming growth factor-β1 [13,24], which down-regulate MMP-2 expression [20]. Taken together, we propose that DSCM may regulate trophoblast cell invasion mainly through influencing MMP-2 expression and/or activity, as well as influencing interactions between MMP-2 and other factors secreted by decidual stromal cells. However, further investigation is needed to clarify the roles of these factors secreted by decidual stromal cells in trophoblast cell invasion.

In summary, this study elucidated that DSCM produce a concentration-dependent effect on invasion and MMP-2 production of cytotrophoblasts. Our findings provide further evidence that the uterine microenvironment exerts the key control for trophoblast cell invasion.

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