

Original Article

CXCL12 controls over-invasion of trophoblasts via up-regulating CD82 expression in DSCs at maternal-fetal interface of human early pregnancy in a paracrine manner

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Abstract: Tetraspanin CD82 has been identified as a potential contributor to controlling trophoblast invasiveness in human first-trimester pregnancy. However, it is unclear how the regulation of CD82 expression at maternal-fetal interface. The present study is to investigate the effect of the trophoblast-derived CXCL12 on CD82 expression in decidual stromal cells (DSCs) that in turn controls trophoblast cell invasiveness. In-cell Western was used to evaluate the expression of CD82 in DSCs. A co-culture model was established to investigate the reciprocal interaction between trophoblasts and DSCs via CXCL12/CXCR4 and CD82 expression. We found that both anti-CXCL12 and anti-CXCR4 neutralizing antibody can eliminate increase of CD82 expression in DSCs induced by the trophoblasts supernatant. Moreover, the invasiveness of trophoblasts pre-treated with anti-CXCR4 neutralizing antibody was significantly decreased. Interestingly, when DSCs were pre-treated with anti-CXCR4 neutralizing antibody, the trophoblasts invasiveness in the co-culture was enhanced, and thus anti-CXCR4 neutralizing antibody can reverse the decrease of trophoblasts invasiveness induced by CD82. The trophoblast cell-derived CXCL12 does not only increase the invasiveness in an autocrine manner, but also control the over-invasion of trophoblasts through promoting CD82 expression in DSCs in a paracrine manner, which maintains a physiological balance of human trophoblasts invasiveness via the cross-talk between trophoblasts and DSCs.

Keywords: CXCL12, CD82, trophoblasts, DSCs, invasiveness

Introduction

Trophoblast cells display a very unique capability that of invasion into decidual tissue that is similar to tumor cells. The imposing difference is that trophoblast cell invasion is temporally and locally controlled in contrast to the unlimited tumor cells invasion [1]. However, dysfunction between the interaction of trophoblasts with the mother-derived decidual cells or impaired trophoblast cell invasion has been demonstrated in association with not only fetal intrauterine growth restriction and pre-eclampsia, but also first-trimester and late-term miscarriage [2-5].

Among the various types of mother-derived cells at the maternal-fetal interface, decidual stromal cells are major cellular component in human

deciduas. It involved in a series of immune regulations such as production of cytokines, especially, a main source of the specific extracellular matrix (ECM) during pregnancy, indicating that the DSCs might be an important regulator on trophoblasts migration and invasion [6, 7]. In addition, a lot of tumor metastasis suppressors are expressed in DSCs, such as CD82 and non-metastatic gene 23-H1 (NME1, also known as nm23-H1) [8]. Our previous research has found that DSCs-expressed CD82 can promote the tissue inhibitor of metalloproteinases 1 (TIMP1) expression, and further control the invasiveness of trophoblast in human first-trimester pregnancy, but the mechanism underlining that has not been elucidated [9].

Local cytokines at the maternal-fetal interface are produced either by trophoblasts themselves

or the other component cells [10]. These cytokines are often able to control trophoblast behavior in some way. The relationship of chemokines to pregnancy has been investigated by more and more researchers [11, 12]. It has been demonstrated that first-trimester human trophoblast secrete CXCL12 that not only induces trophoblast proliferation [13] and invasion [14] in an autocrine manner, but also recruits CD56^{bright}CD16⁻ NK cells into deciduas in a paracrine manner [15-17]. Moreover, DSCs and trophoblasts in the early pregnancy co-secrete CCL2 and co-express its receptor CCR2 [18, 19]. These chemokines might play an important role in both immune and non-immune functions at the maternal-fetal interface during human pregnancy.

To better understand the mechanisms in maternal-fetal dialogue, we first evaluated the effect of trophoblast cell-derived CXCL12 on the CD82 expression in DSCs by in-cell Western. Thereafter, a co-culture model was established to investigate their potential regulating role in the interaction between DSCs and trophoblasts by matrigel invasion assay.

Materials and methods

Human placental tissue collection, cell isolation and culture

All procedures involving participants in this study were approved by Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University, and all subjects have completed an informed consent to collect tissue samples.

Decidual (n=10) and placental tissues (n=10) were from elective termination of the first-trimester pregnancies (gestational age, 6-8 weeks) for no medical reason, or unexplained miscarriage (gestational age, 6-8 weeks). The tissues from the first-trimester pregnancy or miscarriages were put immediately into ice-cold Dulbecco's modified Eagle's medium (DMEM high D-glucose; Gibco Grand Island, NY, USA), transported to the laboratory within 30 min after surgery, and washed with Hank's balanced salt solution for isolation of DSCs and trophoblast cells.

The DSCs (n=6) were isolated according to the previous methods [18]. The decidual tissues

were dissected free of trophoblast, and washed in Ca²⁺Mg²⁺-free phosphate-buffered saline (PBS) and minced. The minced tissues were left in a solution of 0.25% trypsin/0.025% EDTA (Invitrogen, USA) for 10 min at 37°C for four times. The enzymatic reaction was stopped by adding cold DMEM high D-glucose medium with 20% fetal calf serum (GIBCO). The suspension was filtered through sterile gauzes (pore diameter sizes: 100, 300 and 400 mesh), and the filtered suspension was centrifuged at 400×g for 10min. The supernatant was discarded, and the cell pellet was suspended in PBS solution and centrifuged on a discontinuous gradient of 20, 40 and 50% Percoll (Amersham USA) for 20min at 800×g. The cells were recovered from the 20/40% interface containing mainly DSCs, and suspended with 10% fetal bovine serum (FBS) in medium (GIBCO). After cultured for 30min, the non-adherent DSCs were recovered free of leukocytes.

The villous tissues (n=6) were treated for trophoblast isolation according to our previous method [20]. The obtained placental tissues were pooled, and digested by 0.25% trypsin and 0.02% DNase type I (Sigma, Saint Louis, Missouri, USA) at 37°C with gentle agitation for 5min, and then was subjected further to four cycles of 10 min digestion. The trypsinized cell suspensions was filtered through sterile gauzes (pore diameter sizes: 100, 300 and 400 mesh), and the filtered suspension was centrifuged at 400×g for 10min. After the supernatant was discarded, the cell pellet was suspended in DMEM with high D-glucose, and then carefully layered over a discontinuous Percoll gradient (50% to 20% in 10% increments), and centrifuged for 20min at 800×g. The cells sedimenting at densities between 1.048 and 1.062 were collected and washed with DMEM supplemented with 20% heat-inactivated FBS, and then, the cells were incubated in a 6-well plate coated with Matrigel (BD Biosciences, USA) in 5% CO₂ at 37°C.

Immunostaining

For immunocytochemical staining, DSCs and trophoblast cells from different patients growing on coverslips were cultured for 48h. The coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature, washed in PBS and permeabilized for 10 min with 0.25% Triton-100 in PBS. The cells were then incubated with 1%

BSA in PBS/Tween (PBST) for 30 min to block non-specific binding of antibodies. The anti-human vimentin monoclonal antibody (Sino-America Co. Ltd) as markers for DSCs, and HLA-G (Applied Biosystems, USA) and cytokeratin-7 antibodies (Zymed Laboratories, USA) as markers for trophoblast cells were then added. The cells were incubated with primary antibody or mouse IgG isotypic control (Sino-America Co. Ltd) overnight at 4°C, and then incubated with a peroxidase-conjugated secondary antibody for 60 min at 37°C. The slides were stained with DAB, and counterstained with haematoxylin. The experiments were repeated three times from 6 different patients.

For immunohistochemistry, paraffin sections (5 mm) of human decidua and villi from the early pregnancy were dehydrated in Tris-buffered saline (TBS), and incubated with 3% hydrogen peroxide and 1% bovine serum albumin (BSA)/TBS to block endogenous peroxidase. The samples were then incubated with mouse anti-human CD82 antibody (1:50, SC-17752, Santa Cruz Biotechnology, USA), mouse anti-human CXCR4 antibody (15ug/ml, R&D Systems, Abingdon, UK), goat anti-human CCR2 antibody (1:200, ab1668, Abcam, Cambridge, UK), or mouse IgG isotype (for CD82 and CXCR4 groups) (Sino-America Co. Ltd), or goat IgG isotype (for CCR2 group) (Sino-America Co. Ltd) overnight at 4°C in a humidity chamber. After washing three times with TBS, the sections were overlaid with peroxidase-conjugated anti-mouse IgG (SP-9002, Golden Bridge International, Inc, USA) or anti-goat IgG (PV-9003, Golden Bridge International, Inc, USA), and the reaction was developed with 3,3-diaminobenzidine (DAB), and counterstained with haematoxylin. The experiments were repeated three times with 6 different patients.

In-cell Western

According to the description by Egorina [21], we used a set-up assay called in-cell Western to determine the in-cell protein expression level of CD82. The procedure was as follows: DSCs in 96-well plate were incubated with either trophoblasts supernatant, or recombinant CCL2 (R&D Systems, Abingdon, UK) at the concentration from 10 to 500ng/ml, or anti-CXCL12 neutralizing antibody (2-50ug/ml) (R&D Systems, Abingdon, UK), or anti-CXCR4 neutralizing antibody (0.8-20ug/ml) (R&D Systems, Abingdon,

UK), or anti-CCL2 neutralizing antibody (0.2-5ug/ml) (R&D Systems, Abingdon, UK), or various concentrations of RS102895 (a CCR2 antagonist, 0-500 ng/ml, Sigma, USA) for 48h, and then cells immediately fixed with 4% formaldehyde in PBS for 20min at room temperature. After washing with 0.1% Triton, these cells were blocked by add 150ul LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska, USA) for 90min at room temperature, and then incubated with mouse anti-human CD82 antibody (20ug/ml, SC-17752, Santa Cruz Biotechnology, USA) and with rabbit anti-human actin antibody (1:100, Santa Cruz Biotechnology, USA) as control. After overnight treatment at 4°C, the wells were incubated with the appropriate secondary, IRDye™700DX-conjugated affinity purified (red fluorescence) anti-mouse and IRDye™800DX-conjugated affinity purified (green fluorescence) anti-rabbit, fluorescence antibody recommended by the manufacturer (Rockland, Inc, Gibertsville, PA, USA). This procedure was carried out in the dark. Images of target gene were obtained using the Odyssey Infrared Imaging System (LI-COR Biosciences German version of Ltd.). The protein expression level was calculated as the ratio of the intensity of target gene to that of actin. The experiments were carried out in triplicate, and repeated three times.

Enzyme-linked immunosorbent assay for determination of CXCL12 and CCL2

In order to analyze the CXCL12 and CCL2 secretion levels in the primary DSCs or trophoblasts (1×10^5 cells/well), we collected the supernatant of these two cells that were seeded in 24-well plates for 24, 48 or 72h. Thereafter, the culture supernatants were harvested, centrifuged to remove cellular debris, and stored at -80°C until being assayed by enzyme-linked immunosorbent assay (ELISA). The CXCL12 and CCL2 concentrations in the supernatant were quantified by ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Each experiment was carried out in triplicate, and repeated three times

CD82 silence in DSCs

For siRNA transfection, the primary DSCs were seeded in 96-well plates. When cells had reached confluency, medium was changed to OPTIMEM (Invitrogen, USA). The short interfering RNA (siRNA) oligonucleotides targeting

CD82 (set of three oligonucleotides; Stealth Select RNAi; Invitrogen) and Lipofectamine™ 2000 (Invitrogen, USA) were mixed in OPTIMEM, and then added to the cells at room temperature with nontargeting siRNA oligonucleotides as negative control. After 6h incubation, the cells were incubated in DMEM for further 72h in 5% CO₂ at 37°C, and the gene knockdown was confirmed by in-cell Western.

Matrigel invasion assay

The invasion of trophoblast cells across the Matrigel was evaluated objectively in invasion chamber, based on our previous procedure [22]. Briefly, the cells inserts (8mm pore size, 6.5mm diameter, Corning, USA) coated with 15-25ul matrigel were placed in a 24-well plate. The CD82-silenced DSCs (1×10^5) in the lower chamber and or trophoblast cells (1×10^5) in the upper chamber were pre-treated with or without anti-CXCR4 neutralizing antibody (4ug/ml) for 4h, then was formed a indirect co-culture unit. The cells were then incubated at 37°C for 48h. The inserts were removed, washed in PBS and the non-invading cells together with the matrigel were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in methanol for 10min at room temperature and stained with hematoxylin. The result was observed under Olympus BX51+DP70 microscope (Olympus, Tokyo, Japan). The cells migrated to the lower surfaces were counted in full fields at a magnification of x 200. Each experiment was carried out in triplicate, and repeated three times.

Statistics

All values were shown in the mean \pm SE. Data were analyzed using one-way analysis of variance and least significant difference (equal variances assumed), or Tamhane's test (equal variances not assumed) was used post hoc for multiple comparisons with Statistical Package for the Social Sciences software version 11.5. Differences were considered as statistically significant at $P < 0.05$.

Results

The primary trophoblasts up-regulates CD82 expression in DSCs through secreting soluble molecules

In the present study, we first characterized the

purity of primary human trophoblasts and DSCs by immunocytochemistry. The trophoblast cells were almost all stained for CK7 and HLA-G, whereas were not found stained with anti-vimentin antibody (**Figure 1A**). The purity of the isolated trophoblast cells was >95%. On the contrary, almost all vimentin-positive DSCs were negative for CK7 and HLA-G (**Figure 1A**).

Thereafter, we used immunohistochemistry and in-cell Western to detect CD82 expression at human maternal-fetal interface in the early pregnancy, as shown in **Figure 1B**, only did human decidua and DSCs express CD82, but villi and trophoblast cells were negative for CD82.

In order to testify whether trophoblasts can regulate CD82 expression in DSCs, we collected the supernatant of trophoblasts after cultured for 72h, and then added to primary DSCs at the concentration ratio of 10:1, 1:1 and 1:10, respectively. After 48h, it was shown in in-cell Western that trophoblasts could enhance CD82 expression in DSCs in a dose dependent manner (**Figure 1B**, $P < 0.05$). It may be concluded that trophoblast cells up-regulate the CD82 expression in DSC via producing some soluble molecules and further control the invasiveness of themselves.

The trophoblast cell-derived CXCL12 up-regulates CD82 expression in DSCs

Many transcription factors are involved in CD82 transcription regulation [23], especially NF- κ B. NF- κ B P50 subunit was found to bind to an upstream region of the CD82 gene transcription start site [24]. The over-expression or activation of NF- κ B elevates the mRNA and protein levels of CD82 in murine pro-B cells [25], and various human adenocarcinoma cell lines [26].

As we have known previously, human trophoblast cells can secrete various chemokines, such as CXCL12 and CCL2, which are also involved in regulating the invasion of trophoblasts [14, 18]. Moreover, CXCL12 has been reported to activate a variety of signal pathways, including NF- κ B in pre-B cell lines [27], and induce p65 nuclear translocation in mesoangioblasts [28]. To explore the effect of trophoblasts on the CD82 expression in DSCs, we first evaluated the expression of CXCL12/CXCR4 and CCL2/CCR2 in trophoblasts and DSCs, respectively, in the early pregnancy. It is showed in **Figure 2A** that both primary trophoblasts and DSCs se-

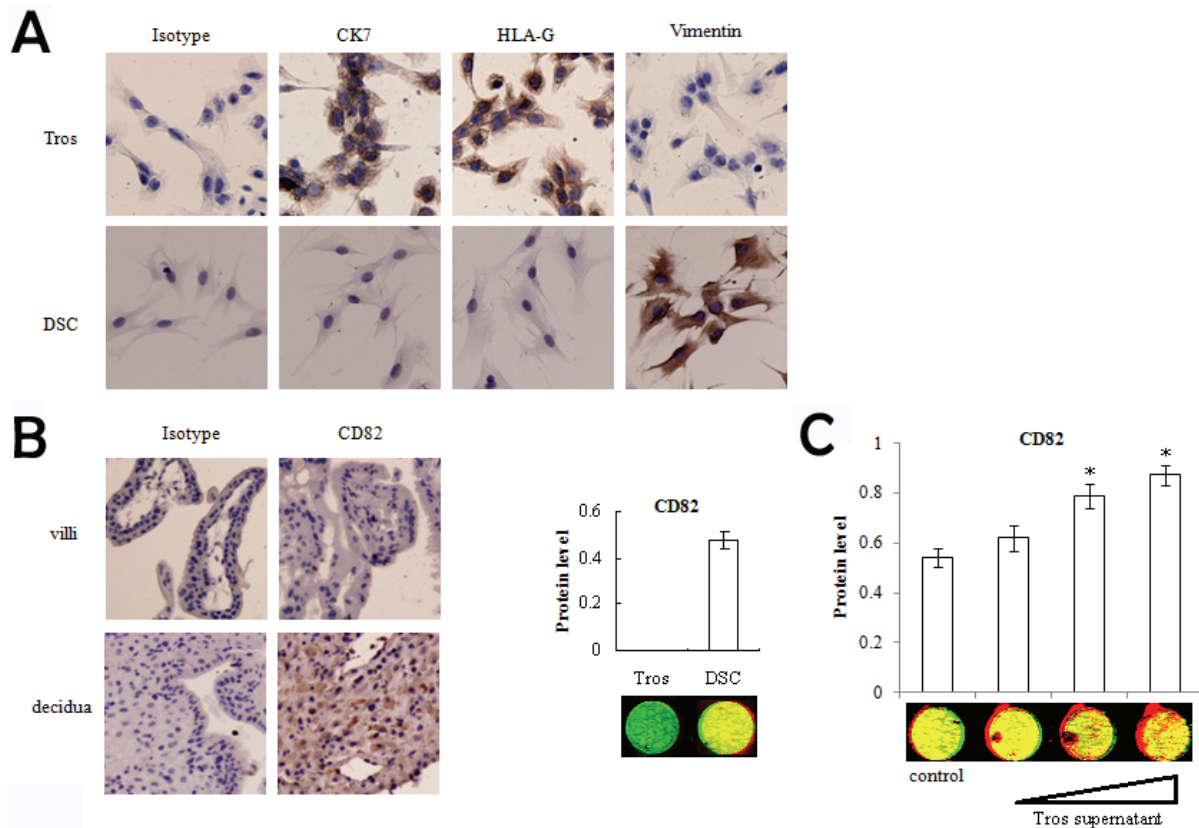


Figure 1. The primary trophoblast cells up-regulate CD82 expression in DSCs through secreting soluble molecules. **(A)** Characterization of human first-trimester trophoblasts and DSCs by immunocytochemistry. Trophoblast cells migrated together, but were not fused when cultured *in vitro* with matrigel that were stained strongly by anti-CK7 and anti-HLA-G monoclonal antibody (mAb), not by anti-vimentin mAb. Decidual stromal cells (DSCs) were positive for vimentin and negative for CK7 and HLA-G. Magnification: $\times 200$. **(B)** Immunohistochemistry and in-cell Western were used to analyze CD82 expression in the paraffin-embedded tissues, primary trophoblasts and DSCs, respectively. CD82 is only expressed in DSCs and not in trophoblast cells of human first-trimester pregnancy. Magnification: $\times 200$. CD82 (red); actin antibody (mAb), not by anti-vimentin mAb. Decidual stromal cells (DSCs) were positive for vimentin and negative for CK7 and HLA-G. Magnification: $\times 200$. **(C)** Immunohistochemistry and in-cell Western were used to analyze CD82 expression in the paraffin-embedded tissues, primary trophoblasts and DSCs, respectively. CD82 is only expressed in DSCs and not in trophoblast cells of human first-trimester pregnancy. Magnification: $\times 200$. CD82 (red); actin (green). **(C)** The primary DSCs were treated with trophoblasts supernatant for 48h (the ratio of DMEM-High Glucose medium to trophoblasts supernatant was 10:1, 1:1 and 1:10, respectively). In-cell Western indicates that trophoblasts can increase CD82 expression in DSCs. CD82 (red); actin (green). Results were highly reproducible in three independent experiments. Tros: Trophoblast cells. Error bars depict the standard error of the mean. * $P < 0.05$ compared to the control.

crete CXCL12 (Figure 2A, left) and CCL2 (Figure 2A, right), but the secretion of CXCL12 in trophoblasts was much more than that of DSCs. According to the results of immunohistochemistry, both trophoblasts and DSCs express CXCR4, the receptor of CXCL12, and the receptor of CCL2, CCR2 (Figure 2B).

Thereafter, we used in-cell Western to investigate the effect of CXCL12 and CCL2 on CD82

expression in DSCs, and found that the expression of CD82 in DSCs was obviously increased by recombinant human CXCL12 with increase of concentrations (Figure 2C, left, $P < 0.05$), but recombinant human CCL2 could not change the CD82 expression (Figure 2C, right, $P > 0.05$). We found further that the increase of CD82 expression in DSCs induced by trophoblasts supernatant was completely abolished by anti-CXCL12 (Figure 2D, left, $P < 0.05$ or $P < 0.01$) or

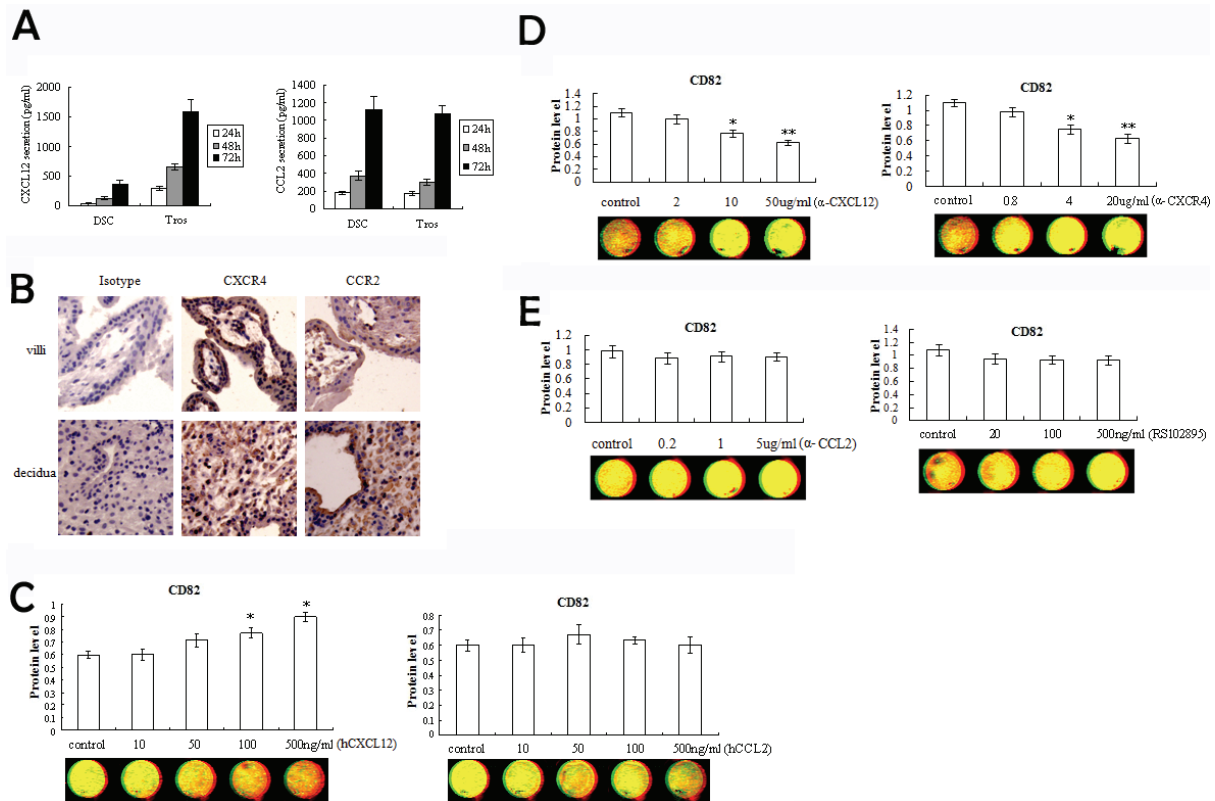


Figure 2. CXCL12 secreted by trophoblasts promotes CD82 expression in DSCs. (A) CXCL12 and CCL2 secretion in the primary trophoblasts and DSCs at 24, 48 and 72h, respectively. (B) The paraffin-embedded villi and decidua were analyzed by immunohistochemistry. The results show that both trophoblasts and DSCs express CXCR4 and CCR2. Magnification: $\times 200$. (C) DSCs were incubated with recombinant human CXCL12 or CCL2 for 48h, then in-cell Western demonstrates that CXCL12 can up-regulate CD82 expression in DSCs in a dose-dependent manner, but not by CCL2. CD82 (red); actin (green). In-cell Western was used to investigate CD82 expression in DSCs treated with trophoblasts supernatant and different concentration of anti-CXCL12 neutralizing antibody, anti-CXCR4 neutralizing antibody (D), anti-CCL2 neutralizing antibody or RS102895 (a CCR2 antagonist) (E) for 48h. The results show that both anti-CXCL12 neutralizing antibody and anti-CXCR4 neutralizing antibody can reverse the increase of CD82 expression induced by trophoblasts supernatant. CD82 (red); actin (green). These pictures are representatives of three individual experiments. Error bars depict the standard error of the mean. * $P < 0.05$, ** $P < 0.01$ compared to the control.

anti-CXCR4 (Figure 2D, right, $P < 0.05$ or $P < 0.01$) neutralizing antibody. However, anti-CCL2 neutralizing antibody or RS102895 (a CCR2 antagonist) couldn't participate in CD82 expression regulation by the trophoblast cell-derived supernatant (Figure 2E, $P > 0.05$), which suggests that trophoblasts up-regulate expression of CD82 in DSCs mainly via secreting CXCL12 rather than CCL2.

The trophoblast-derived CXCL12 regulates the invasiveness of trophoblasts through autocrine/paracrine mechanisms

To testify whether the trophoblast-derived

CXCL12 regulates invasion through promoting CD82 expression in DSCs, we first silenced CD82 expression in DSCs (Figure 3A, $P < 0.01$), then used recombinant human CXCL12 to treat the transfected DSCs (the procedure for Figure 3C), or anti-CXCR4 neutralizing antibody to pre-treat the siRNA transfected DSCs and/or trophoblasts (the procedure for Figure 3D) for 4h, and further constructed the indirect co-culture unit for another 48h (Figure 3B). As shown in Figure 3C, the invasiveness of trophoblasts in co-culture unit was markedly enhanced after CD82 silence in DSCs ($P < 0.01$). However, the invasiveness of trophoblasts was decreased when they were co-cultured with the CXCL12-

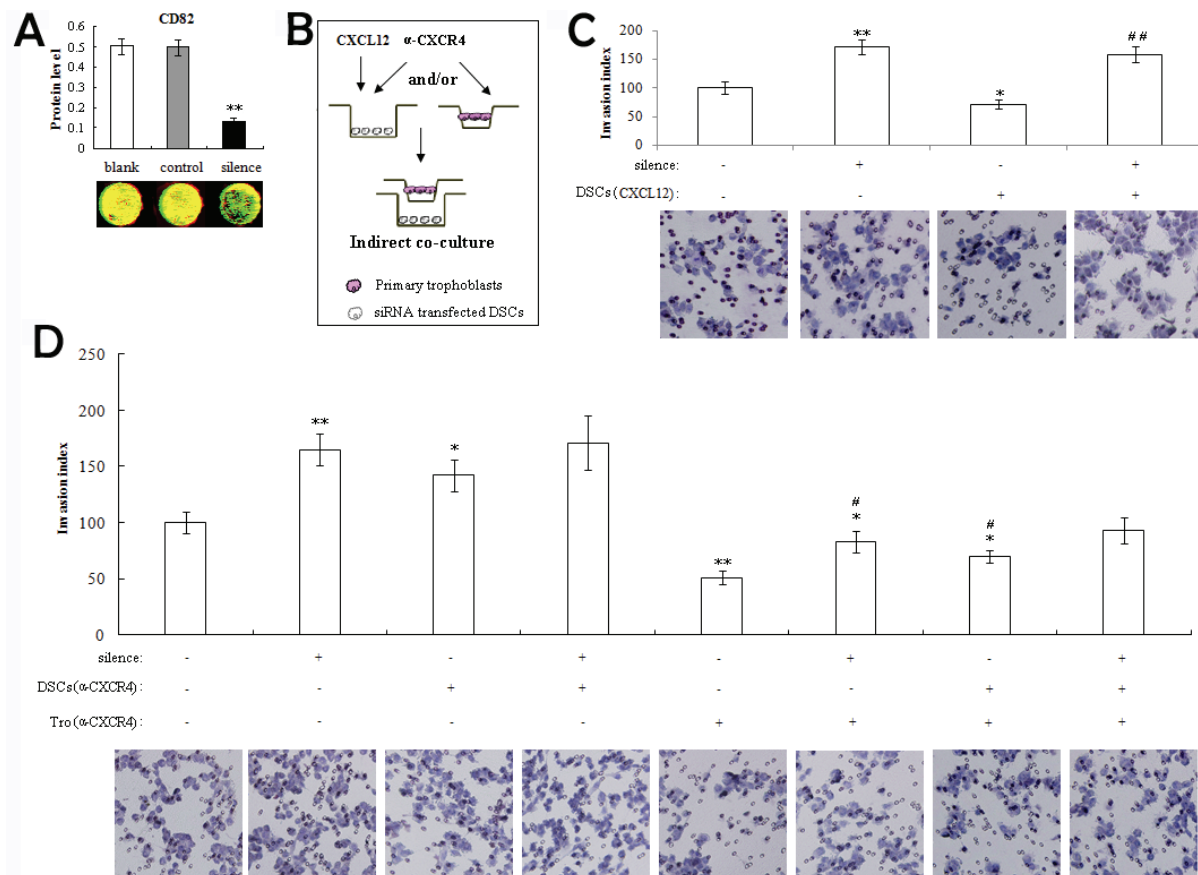


Figure 3. The trophoblast-derived CXCL12 regulates the invasiveness of trophoblasts through autocrine/paracrine mechanisms. (A) DSCs were silenced for CD82 for 72h, and then the efficiency was demonstrated by in-cell Western. CD82 (red); actin (green). (B) We treated siRNA transfected DSCs with recombinant CXCL12 (C), or pre-treated transfected DSCs and/or trophoblasts with anti-CXCR4 neutralizing antibody (D) for 4h, and then constructed the indirect co-culture unit. After 48h, the Matrigel invasion assay was used to detect the trophoblasts invasion in the co-culture unit. The results show that CXCL12 secreted by trophoblasts in early pregnancy can promote the invasiveness of themselves in autocrine manner, on the other hand, can control the trophoblasts invasiveness through up-regulating CD82 expression in DSCs. Magnification: $\times 200$. Results are highly reproducible in three independent experiments. blank: no Transfection; control: the nontargeting siRNA oligonucleotides; silence: CD82 was knocked down. DSCs (CXCL12): DSCs were dealt with recombinant CXCL12; DSCs (α -CXCR4): DSCs were pre-treated with anti-CXCR4 neutralizing antibody; Tro(α -CXCR4): Trophoblasts were pre-treated with anti-CXCR4 neutralizing antibody. Error bars depict the standard error of the mean. * $P < 0.05$, ** $P < 0.01$ compared to the si-neg control. # $P < 0.05$ compared to si-negative control and anti-CXCR4 neutralizing antibody pre-treated DSCs.

pulsed DSCs ($P < 0.05$). As shown in **Figure 3D**, the invasiveness of the pre-treated trophoblasts with anti-CXCR4 neutralizing antibody was significantly weakened (**Figure 3D**, $P < 0.01$), but anti-CXCR4 neutralizing antibody could not change the trophoblasts invasion regulated by CD82. Surprisingly, we found that the trophoblasts invasiveness in co-culture unit was increased when the siRNA transfected DSCs were pre-treated with anti-CXCR4 neutralizing antibody

(**Figure 3D**, $P < 0.05$), and anti-CXCR4 neutralizing antibody could abolish the decrease of trophoblasts invasion induced by DSCs-expressed CD82. In addition, when the primary trophoblasts and the siRNA transfected DSCs were pre-treated with anti-CXCR4 neutralizing antibody, the invasion of trophoblasts in co-culture unit was decreased, but was higher than that of the pre-treated trophoblasts (**Figure 3D**, $P < 0.05$), which suggests that the trophoblast

cell-derived CXCL12 can promote the invasion of themselves in an autocrine manner, on the other hand, can suppress the trophoblasts invasion through up-regulating CD82 expression in DSCs in a paracrine manner in human early pregnancy.

Discussion

As a key component of human placenta, trophoblast is the only embryo-derived cell that interacts directly with the mother-derived cells in decidua. Trophoblast invasion is a series of tightly controlled program that is pivotal to implantation and placentation. Excessive or insufficient invasion of trophoblasts has been demonstrated as highly associated to pregnancy failure [2-4]. Trophoblasts invasion is regulated by cross-talking of paracrine and autocrine regulation between the trophoblast cells and DSCs at maternal-fetal interface [29]. Our previous research has demonstrated that tumor metastasis suppressor gene CD82 is expressed in the primary DSCs, but not in the primary trophoblasts which up-regulates TIMP1 expression in DSCs and controls the invasiveness of trophoblast cells through suppressing integrin β 1/MAPK/ERK1/2 signal pathway. In this present study, we have found that trophoblasts can promote CD82 expression in DSCs through secreting some molecules, so CD82 might be a media of the cross-talking between trophoblasts and DSCs at maternal-fetal interface in humans.

Chemokines are a sort of small molecular cytokine that is involved in a series of physiology and pathological biological events, including chemotaxis, cellular proliferation, differentiation, apoptosis, hematopoiesis, angiogenesis, pro-tumor and anti-tumor, and inflammatory disease [30-34]. It has been demonstrated that human first-trimester trophoblasts can secrete a lot of chemokines, especially CXCL12 [13-16] and CCL2 [19], which induce maternal-fetal tolerance, and improve the biologic functions of trophoblast cells, including proliferation and or invasion. In the present work, we have found that DSCs can highly express CXCR4, CXCL12 receptor, and CCR2, CCL2 receptor. In addition, CXCL12 can activate NF- κ B [27], and induce p65 nuclear translocation [28], which plays an important role in enhancing CD82 transcription. Therefore, we propose that the trophoblast-derived CXCL12 and/or CCL2 might be involved in CD82 expression regulation.

To verify our speculation, we first investigated the effect of recombinant human CXCL12 and CCL2 on CD82 expression in DSCs. Unlike CXCL12 that are able to up-regulate CD82 expression, exogenous CCL2 exhibited no detectable effect on the CD82 expression. Moreover, unlike neutralizing antibody to CXCL12 or CXCR4, neutralizing antibody to CCL2 or CCR2 antagonist failed to block the increase of CD82 expression induced by trophoblasts supernatant, suggesting trophoblasts promote CD82 expression of DSCs in paracrine manner mainly through secreting CXCL12. Therefore, this action was independent of CCL2/CCR2 pathway.

Our previous work has shown that CXCL12 increases trophoblasts invasion in autocrine manner through promoting MMP2 and MMP9 expression [14]. To better understand the role of CXCL12/CXCR4 interaction on CD82 expression in DSCs, we established an indirectly co-culture model. As we predicted, after co-culture with the CD82-silenced DSCs, the trophoblasts invasion increased. However, if these siRNA transfected DSCs were pre-treated with anti-CXCR4 neutralizing antibody before co-culture, the invasiveness of trophoblasts was obviously increased, but also the decrease of trophoblasts invasion induced by DSCs-derived CD82 can be abolished. But this result is different from the direct co-culture results in our previous work [14], which suggests that trophoblasts up-regulate CD82 expression of DSCs through secreting CXCL12, at the same time, possibly reduce CD82 expression via expressing some cell surface molecule and or cell-cell contact effect. In addition, if DSCs and trophoblasts are simultaneously pre-treated with anti-CXCR4 neutralizing antibody, trophoblast invasiveness is impaired, and stronger than the trophoblasts pre-treatment, which suggests that the trophoblast cell-derived CXCL12 promotes the cross-talk between trophoblasts and DSCs through up-regulating CD82 expression, which provides a precise two-way regulation on trophoblasts invasiveness.

In conclusion, as shown in **Figure 4**, human first-trimester trophoblasts promote their own invasiveness and MMP expression in an autocrine manner via CXCL12/CXCR4 axis.¹⁴ Our present study has further demonstrated that the trophoblast cell-derived CXCL12 can also up-regulate CD82 expression in DSCs in a paracrine manner that in turn controls tro-

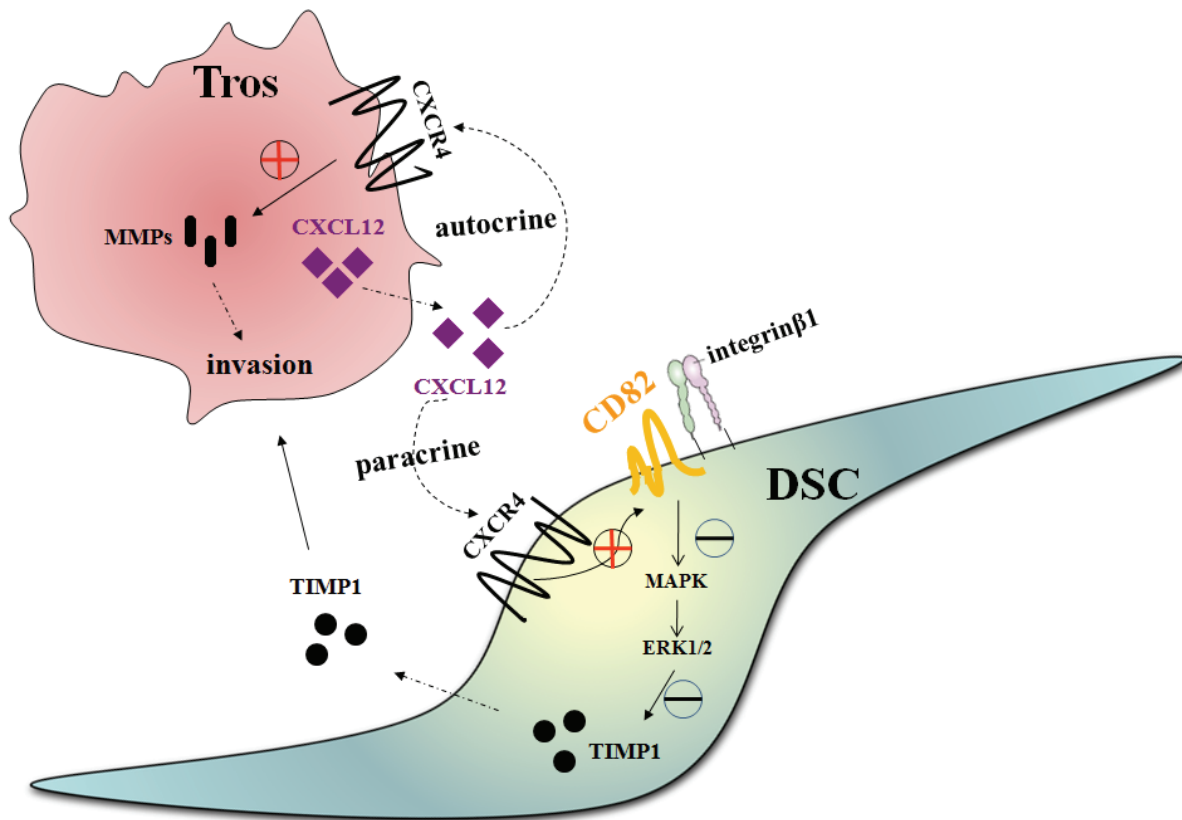


Figure 4. Schematic roles of CD82 and CXCL12 in the regulation of trophoblasts invasiveness. On the one hand, The CXCL12 secreted by trophoblasts promotes MMP2 and MMP9 expression, and further increases trophoblasts invasion in an autocrine manner. On the other hand, trophoblast cell-derived CXCL12 up-regulates CD82 expression in DSCs in a paracrine manner, which improves TIMP1 expression in DSCs and controls the trophoblasts over-invasion via suppressing integrin β 1/MAPK/ERK1/2 signal pathway. Therefore, CXCL12 can maintain optimal invasion of trophoblast in the early pregnancy, and take part in maintenance of normal pregnancy.

phoblast over-invasion through suppressing integrin β 1/MAPK/ERK1/2 signal pathway, which suggests that CXCL12 plays an important role in the appropriate invasion of trophoblasts at maternal-fetal interface in humans.

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References

- [1] Fitzgerald JS, Poehlmann TG, Schleussner E, Markert UR. Trophoblast invasion: the role of intracellular cytokine signaling via signal transducer and activator of transcription 3 (STAT3). *Hum Reprod Update*. 2008; 14(4):335-44.
- [2] Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biol Reprod*. 2003; 69(1):1-7.
- [3] Bose P, Kadyrov M, Goldin R, Hahn S, Backos M, Regan L, Huppertz B. Aberrations of early

- trophoblast differentiation predispose to pregnancy failure: lessons from the anti-phospholipid syndrome. *Placenta*. 2006; 27 (8):869–75.
- [4] Torry DS, Labarrere CA, McIntyre JA. Uteroplacental vascular involvement in recurrent spontaneous abortion. *Curr Opin Obstet Gynecol*. 1998; 10(5):379–82.
- [5] Kimatrai M, Oliver C, Abadia-Molina AC, Garcia-Pacheco JM, Olivares EG. Contractile activity of human decidua stromal cells. *J Clin Endocrinol Metab*. 2003; 88(2):844–9.
- [6] Aplin JD, Charlton AK, Ayad S. An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell Tissue Res*. 1988; 253(1):231–240.
- [7] Zhu HH, Huang JR, Mazela J, Elias J, Tseng L. Progesterone stimulates the biosynthesis of fibronectin and accumulation of fibronectin mRNA in human endometrial stromal cells. *Hum Reprod*. 1992; 7(2):141–6.
- [8] Xie KM, Hou XF, Li MQ, Li DJ. NME1 at the human maternal-fetal interface downregulates titin expression and invasiveness of trophoblast cells via MAPK pathway in early pregnancy. *Reproduction*. 2010; 139(4):799–808.
- [9] Li MQ, Hou XF, Shao J, Tang CL, Li DJ. The DSCs-expressed CD82 controls the invasiveness of trophoblast cells via integrin β 1/MAPK/MAPK3/1 signaling pathway in human first-trimester pregnancy. *Biol Reprod*. 2010; 82 (5):968–79.
- [10] Makrigiannakis A, Minas V, Kalantaridou SN, Nikas G, Chrousos GP. Hormonal and cytokine regulation of early implantation. *Trends Endocrinol Metab*. 2006; 17(5):178–85.
- [11] Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update*. 2005; 11(6):613–30.
- [12] Hannan NJ, Salamonsen LA. Role of chemokines in the endometrium and in embryo implantation. *Curr Opin Obstet Gynecol*. 2007; 19 (3):266–72.
- [13] Wu X, Li DJ, Yuan MM, Zhu Y, Wang MY. The expression of CXCR4/CXCL12 in first-trimester human trophoblast cells. *Biol Reprod*. 2004; 70 (6):1877–85.
- [14] Zhou WH, Du MR, Dong L, Yu J, Li DJ. Chemokine CXCL12 promotes the cross-talk between trophoblasts and decidua stromal cells in human first-trimester pregnancy. *Hum Reprod*. 2008; 23(2):2669–79.
- [15] Wu X, Jin LP, Yuan MM, Zhu Y, Wang MY, Li DJ. Human first-trimester trophoblast cells recruit CD56brightCD16- NK cells into decidua by way of expressing and secreting of CXCL12/stromal cell-derived factor 1. *J Immunol*. 2005; 175 (1):61–8.
- [16] Hanna J, Wald O, Goldman-Wohl D, Prus D, Markel G, Gazit R, Katz G, Haimov-Kochman R, Fujii N, Yagel S, Peled A, Mandelboim O. CXCL12 expression by invasive trophoblasts induces the specific migration of CD16- human natural killer cells. *Blood*. 2003; 102(5):1569–77.
- [17] Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, Bossi F, Mocci C, Sarazani F, Tedesco F, Santoni A, Gismondi A. Recruitment of circulating NK cells through decidua tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood*. 2008; 111(6):3108–15.
- [18] He YY, Du MR, Guo PF, He XJ, Zhou WH, Zhu XY, Li DJ. Regulation of C-C motif chemokine ligand 2 and its receptor in human decidua stromal cells by pregnancy-associated hormones in early gestation. *Hum Reprod*. 2007; 22 (10):2733–42.
- [19] Chen SU, Chou CH, Chao KH, Lee H, Lin CW, Lu HF, Yang YS. Lysophosphatidic acid up-regulates expression of growth-regulated oncogene- α , interleukin-8, and monocyte chemoattractant protein-1 in human first-trimester trophoblasts: possible roles in angiogenesis and immune regulation. *Endocrinology*. 2010; 151(1):369–79.
- [20] Zhou WH, Du MR, Dong L, Zhu XY, Yang JY, He YY, Li DJ. Cyclosporin A increases expression of matrix metalloproteinase 9 and 2 and invasiveness in vitro of the first-trimester human trophoblast cell via the mitogen-activated protein kinase pathway. *Hum Reprod*. 2007; 22 (10):2743–50.
- [21] Egorina EM, Sovershaev MA, Osterud B. In-cell Western assay: a new approach to visualize tissue factor in human monocytes. *J Thromb Haemost*. 2006; 4(3):614–20.
- [22] Yoshizaki T, Sato H, Maruyama Y, Muroto S, Furukawa M, Park CS, Seiki M. Increased expression of membrane type 1-matrix metalloproteinase in head and neck carcinoma. *Cancer*. 1997; 79(1):139–44.
- [23] Liu WM, Zhang XA. KAI1/CD82, a tumor metastasis suppressor. *Cancer Letters*. 2006; 240 (2):183–94.
- [24] Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG. Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF- κ B and beta-amyloid precursor protein. *Cell*. 2002; 110(1):55–67.
- [25] Li J, Peet GW, Balzarano D, Li X, Massa P, Barton RW. Novel NEMO/I κ B kinase and NF- κ B target genes at the pre-B to immature B cell transition. *J Biol Chem*. 2001; 276 (21):18579–90.
- [26] Shinohara T, Miki T, Nishimura N, Nohihara H, Hamada H, Mukaida N, Sone S. Nuclear factor κ B-dependent expression of metastasis suppressor KAI1/CD82 gene in lung cancer cell lines expressing mutant p53. *Cancer Res*. 2001; 61(2):673–8.
- [27] Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang

- YM, Qin SX, Newman W, Groopman JE. The alpha-chemokine, stromal cell-derived factor-1 alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J Biol Chem*. 1998; 273(36):23169-75.
- [28] Palumbo R, Galvez BG, Pusterla T, De Marchis F, Cossu G, Marcu KB, Bianchi ME. Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF- κ B activation. *J Cell Biol*. 2007; 179(1):33-40.
- [29] Elsebeth SR, Eliezer S. Human trophoblast function during the implantation process. *Reprod Biol Endocrinol*. 2005; 3:56-67.
- [30] Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol*. 2000; 18:217-42.
- [31] Yoshie O. Role of chemokines in trafficking of lymphocytes and dendritic cells. *Int J Hematol*. 2000; 72(4):399-407.
- [32] Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity*. 2000; 12(2):121-7.
- [33] Mackay CR. Chemokines: immunology's high impact factors. *Nat Immunol*. 2001; 2(2):95-101.
- [34] Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer*. 2004; 4(7):540-550.