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# The DSCs-Expressed CD82 Controls the Invasiveness of Trophoblast Cells via Integrinbeta1/MAPK/MAPK3/1 Signaling Pathway in Human First-Trimester Pregnancy[1]

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## ABSTRACT

**CD82 is recognized as a wide-spectrum tumor metastasis suppressor that inhibits cancer cell motility and invasiveness. At the human maternal-fetal interface, the decidua is believed to effectively limit the inappropriate invasion of trophoblasts. Here we have found the transcription and translation of CD82 in decidual stromal cells (DSCs), whereas trophoblast cells do not express CD82. The in-cell Western analysis reveals attenuation of CD82 translation in DSCs by human chorionic gonadotropin (hCG), but not by estrogen or progesterone. It is demonstrated that silencing of CD82 by RNA interference increases integrinβ1, decreases TIMP1 expression in DSCs, and promotes the invasion of the first-trimester human trophoblasts in the coculture. Moreover, U0126, or anti-integrinβ1 neutralizing antibody, reverses the decreased TIMP1 expression and the increased invasiveness of trophoblast cells, and the antibody also inhibits the MAPK3/1 phosphorylation induced by CD82 silence. After transfection with CD82, the invasive index of BeWo cells decreases significantly with TIMP1 increase. The results above indicate that the DSCs-expressed CD82 up-regulates the expression of TIMP1 in an autocrine manner and inhibits the invasiveness of human first-trimester trophoblast cells partly through the integrinβ1/MAPK/MAPK3/1 signaling pathway. Furthermore, we have found that the mRNA and protein level of CD82 in decidua of the miscarriage is significantly higher than that of the normal early pregnancy, which implies that the abnormal higher CD82 expression in decidua restricts appropriate invasion of trophoblasts that leads to early pregnancy wastage.**

*CD82, deciduas, DSCs, integrinβ1, invasion, MAPK/MAPK3/1, placenta, signal transduction, trophoblast cells*

## INTRODUCTION

Implantation of human conceptus involves invasion of trophoblast cells into the uterine epithelium and the underlying stroma, which undergo a complex process of proliferation, migration, and differentiation. A typical feature of placentation in humans is the high-intensity invasion of trophoblasts in order to gain access to the maternal circulation during the first trimester [1]. An impaired endovascular trophoblast invasion has been confirmed to be associated not only with pre-eclampsia—fetal intrauterine growth restriction—but also human first-trimester or late miscarriage [2–4].

Trophoblast cells display the unique capability to physiologicallyinvade the surrounding tissue, similar to tumors [5]. Trophoblast and tumor cells share the same biochemical mediators: the matrix metallopoteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) [5, 6]. MMPs, such as MMP9 and MMP2, are critical for extracellular matrix (ECM) degradation and the invasion of the trophoblast. Moreover, MMPs are also involved in cell-cell communication via cell surface proteins to support adhesion and migration [7]. Therefore, as cytotrophoblast cells differentiate, they change their repertoire of cell adhesion molecules, cadherins, and integrins. Aberration in the levels and proteolytic activities of MMP9 and MMP2 is one of the important factors affecting placentation and spiral artery remodeling [8]. However, as opposed to malignant invasion, trophoblastic invasion during implantation and placentation is stringently controlled both in space and time. The decidua forms a dense cellular matrix believed to generate a local cytokine environment that promotes trophoblast attachment and acts as a physical barrier limiting trophoblast overinvasion [9, 10]. In addition, decidual cells express TIMPs, extracelluar matrix proteins, and adhesion molecules that directly control invasion of the trophoblast cells [11, 12].

The *CD82* gene (kangai1) encodes a 267-amino acid protein that contains four putative transmembrane domains. It is originally identified based on its function as a metastasis suppressor gene. CD82 plays an important role in inhibiting cancer cell motility, invasion, and metastasis, and thus inhibits the formation of tumor metastases without affecting tumor growth [13–18]. An increasing body of evidence shows that CD82 inhibits cell motility through regulating an associated protein, such as integrin [19–22], epidermal growth factor receptor (EGFR) [23], and duffy antigen receptor for chemokines (DARC) [24]. Gellersen et al. [25] found that the expression of CD82 in decidual cells at the human maternal-fetal interface is involved in decidual transformation from human endometrial stromal cells (ESCs).

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The present study is designed to examine the role of CD82 at the human maternal-fetal interface and its potential implication in the control of trophoblast invasion and the unexplained miscarriage. We first investigated the effect of CD82 on the invasion of trophoblast cells and the expression of MMP2, MMP9, TIMP1, TIMP2, and titin through integrinβ1 and MAPK/MAPK3/1 signaling pathways in human decidual stromal cells (DSCs) and BeWo cells. Furthermore, hormonal regulation of CD82 expression in DSCs was also observed.

## MATERIALS AND METHODS

### Tissue Collection and Cell Culture

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

Decidual and placental tissues were from elective termination of a first-trimester pregnancy (gestational age 6–8 wk) for no medical reason or from an unexplained miscarriage in the first trimester. The tissues from first-trimester pregnancies were immediately put into ice-cold Dulbecco modified Eagle medium (DMEM high D-glucose; Gibco), transported to the laboratory within 30 min after surgery, and washed in Hanks balanced salt solution for isolation of DSCs and trophoblast cells.

The DSCs were isolated according to the previous methods [26, 27]. The decidual tissues were treated carefully, free of trophoblasts, washed in $Ca2^{+}Mg^{2+}$-free PBS, and minced. The minced tissues were left in PBS with 0.25% (wt/vol) trypsin and 0.025% (wt/vol) ethylenediaminetetraacetic acid (Invitrogen) four times for 10 min each at 37°C. The enzymatic reaction was stopped by adding cold DMEM high D-glucose medium with 20% (vol/vol) fetal calf serum (Gibco). The suspension was filtered through sterile gauze (pore diameter sizes 100, 300, and 400 mesh), and the filtered suspension was centrifuged at 400 × *g* for 10 min. The supernatant was discarded, and the cell pellet was suspended in PBS solution and centrifuged on a discontinuous gradient of 20%, 40%, and 50% (vol/vol) Percoll (Amersham) for 20 min at 800 × *g*. The cells were recovered from the 20%/40% (vol/vol) interface containing mainly DSCs and suspended with 10% (vol/vol) fetal bovine serum (FBS) in medium (Gibco). After being cultured for 30 min, the nonadherent DSCs were recovered free of leukocytes. Immunocytochemistry showed >98% vimentin-positive and cytokeratin-negative cells (i.e., mesenchymal cells).

The villous tissues were treated for trophoblast isolation according to our previous method [28, 29]. The obtained placental tissues were pooled and digested by 0.25% (wt/vol) tryspin and 0.02% (wt/vol) DNase type I (Sigma) at 37°C with gentle agitation for 5 min, followed by four cycles of 10-min digestion. The trypsinized cell suspension was filtered through sterile gauzes (pore diameter sizes 100, 300, and 400 mesh), and the filtered suspension was centrifuged at 400 × *g* for 10 min. After the supernatant was discarded, the cell pellet was suspended in DMEM with high D-glucose, carefully layered over a discontinuous Percoll Gradient (50% to 20%, in 10% steps; vol/vol), and centrifuged for 20 min at 800 × *g*. The cells, which were sedimenting at densities between 1.048 and 1.062, were collected and washed with DMEM supplemented with 20% (vol/vol) heat-inactivated FBS and then incubated in a six-well plate coated with Matrigel (BD Biosciences) in 5% $CO_2$ at 37°C. This method resulted in a 95% purity in trophoblast cells. The BeWo choriocarcinoma cell line was purchased from the Chinese Center for Type Culture Collection (CCTCC) and maintained as monolayers in Kaighn Modification of Ham F-12 Medium (Sigma) supplemented with 10% (vol/vol) FBS under standard culture conditions of 5% $CO_2$ in air at 37°C, with medium renewal every 2–3 days.

### Immunostaining

For immunohistochemistry, paraffin sections (5 mm) of human decidua and villi from unexplained miscarriage or early pregnancy termination were dehydrated in Tris-buffered saline (TBS) and incubated with hydrogen peroxide and 1% bovine serum albumin (BSA)/TBS to block endogenous peroxidase. The samples were then incubated with murine anti-human vimentin monoclonal antibody (1:100; ZA0511; Dingguo), cytokeratin-7 antibody (1:100; 18–0234; Zymed Laboratories), anti-human CD82 antibody (1:50; SC-17752; Santa Cruz Biotechnology), or mouse IgG isotype overnight at 4°C in a humid chamber. After washing three times with TBS, the sections were overlaid with peroxidase-conjugated goat anti-mouse IgG (SP-9002; Golden Bridge International, Inc.), and the reaction was developed with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin. The experiments were repeated five times.

For immunocytochemical staining, DSCs, trophoblast cells, and BeWo cells growing on coverslips were cultured for 48 h. The coverslips were fixed in 4% (vol/vol) paraformaldehyde for 20 min at room temperature, washed in PBS, and permeabilized for 10 min with 0.25% (vol/vol) Triton-100 in PBS. The cells were then incubated with 1% BSA in PBS/Tween for 30 min to block nonspecific binding of antibodies. The anti-human vimentin monoclonal antibody (as the marker for DSCs) and HLA-G (Applied Biosystems) and cytokeratin-7 antibodies (as markers for trophoblast cells) were then added. The anti-human CD82 monoclonal antibody was used to detect whether DSCs, trophoblast cells, and BeWo cells express CD82 protein. The cells were incubated with a primary antibody or an isotypic control 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 hematoxylin. The experiments were repeated five times.

### CD82 Silence in DSCs

For siRNA transfection, the primary cultures of DSCs were seeded in 96-well plates. When cells had reached confluency, medium was changed to OPTIMEM (Invitrogen). The siRNA oligonucleotides targeting CD82 (set of three oligonucleotides; Stealth Select RNAi; Invitrogen) and Lipofectamine 2000 (Invitrogen) were mixed in OPTIMEM and then added to the cells at room temperature, with nontargeting siRNA oligonucleotides as negative control. After 6-h incubation, the cells were incubated in DMEM for a further 48 or 72 h in 5% $CO_2$ at 37°C, and the gene knockdown was confirmed by RT-PCR (48 h, six-well plates) and in-cell Western (72 h, 96-well plates).

### CD82 Transfection into BeWo Cells

An expression vector for the human *CD82* gene (nucleotides 181 to 985 in GenBank accession no. NM_002231.2), pcDNA3.1(+)-CD82, was kindly provided by K. Milde-Langosch (University Clinic Hamburg-Eppendorf). The plasmid transient transfection was generated according to the transfection guidelines by Lipofectamine 2000 (Invitrogen). In brief, $1 \times 10^5$BeWo cells were plated in 96-well plates without antibiotics. When the cells were 90%–95% confluent, 1 μl Lipofectamine 2000, 50 μl OPTIMEM, and 0.4 μg plasmid pcDNA3.1(+) vector (Invitrogen) or pcDNA3.1(+)-CD82 were mixed and incubated for 20 min at room temperature and then added to each well. After 6 h of incubation, these cells were incubated in DMEM for a further 48 or 72 h in 5% $CO_2$ at 37°C, until the gene overexpression was confirmed by RT-PCR (48 h, six-well plates) and in-cell Western (72 h, 96-well plates).

### RT-PCR

The total RNA was extracted from human decidua and villi, DSCs, trophoblast cells, BeWo cells, siRNA-transfected DSCs, or plasmid-transfected BeWo cells with Tri reagent (Molecular Research Center). The cDNA was generated with oligo(dT)18 primers using Revert Aid First Strand cDNA Synthesis Kit (Fermentas Life Science). The 50-μl PCR amplification of the single-strand cDNA was performed by 28 precycles at 95°C for 5 min, then denaturation at 94°C for 45 sec, annealing at 59°C for 45 sec, and elongation at 72°C for 45 sec using 2.5 U Taq polymerase (Fermentas Life Science). The primer sequences are indicated in Table 1. The amplified DNA was fractionated by 2% agarose gel (Oxiod) electrophoresis, and ethidium bromide-stained bands were photographed. The ratio of the intensity of the target gene to GAPDH was taken as the transcriptional level of interest. The experiments were repeated three times.

### Quantitative Real-Time PCR

Total RNA was extracted from transfected cells or human decidual tissues. Triplicate samples containing cDNA were prepared as mentioned in the previous section, *RT-PCR*. Taqman universal PCR master mix (Applied Biosystems), specific primers, and fluorescent dye-labeled Taqman MGB probes for target gene and GAPDH were mixed and analyzed on an ABI7000 thermal cycler (Applied Biosystems). The primer sequences are indicated in Table 1 and were synthesized by TaKaRa Biotechnology Co., LTD. The cycling conditions consisted of a denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 sec, a 60-sec annealing step at 62°C, and finally a holding temperature of 15°C. To determine the amount of gene product present in the sample, cycle time (Ct) was determined. The average Ct value was calculated from triplicate wells for each sample with each primer set. Most duplicate samples varied by <0.5 Ct. The relative gene expression was determined by calculating ΔCt values (ΔCt) by subtraction of the Ct value for GAPDH

TABLE 1: Primer sequences of CD82, invasiveness-related proteins, and GAPDH.

| Gene name | Size (bp) | Primer sequences | |
|---|---|---|---|
| *CD82* | 326 | Sense: | 5′-CTGGGGCTGTACTTTGCTTTC-3′ |
| | | Antisense: | 5′-CAGAAGCCCTTCCTCACAGAA-3′ |
| *MMP2* | 135 | Sense: | 5′-ATGACATCAAGGGCATTCAGGAG-3′ |
| | | Antisense: | 5′-TCTGAGCGATGCCATCAAATACA-3′ |
| *MMP9* | 94 | Sense: | 5′-ACGCACGACGTCTTCCAGTA-3′ |
| | | Antisense: | 5′-CCACCTGGTTCAACTCACTCC-3′ |
| *TIMP1* | 194 | Sense: | 5′-CCTTATACCAGCGTTATGAGATCAA-3′ |
| | | Antisense: | 5′-AGTGATGTGCAAGAGTCCATCC-3′ |
| *TIMP2* | 135 | Sense: | 5′-GGAGCACTGTGTTTATGCTGGAA-3′ |
| | | Antisense: | 5′-GACCGAGCGATTGCTCAAGA-3′ |
| *integrinβ1* | 105 | Sense: | 5′-TGTGTCAGACCTGCCTTGGTG-3′ |
| | | Antisense: | 5′-AGGAACATTCCTGTGTGCATGTG-3′ |
| *titin* | 106 | Sense: | 5′-GAAAGTGGCACAGGGTTCCATAA-3′ |
| | | Antisense: | 5′-AGCTCTCAGCCATTCCTGATTTG-3′ |
| *GAPDH* | 235 | Sense: | 5′-GGGGAGCCAAAAGGGTCATCATCT-3′ |
| | | Antisense: | 5′-GAGGGGCCATCCACAGTCTTCT-3′ |

primers from the Ct value for target gene primers. The relative fold expression of each gene was determined compared with control in the experiment. The experiments were carried out in triplicate.

## *In-Cell Western*

According to the description by Egorina et al. [30], we used a newly set up assay called in-cell Western to determine the in-cell protein level of CD82, MMP2, MMP9, TIMP1, TIMP2, titin, and integrinβ1. The procedure was as follows: siRNA-transfected DSCs and plasmid-transfected BeWo cells in 96-well plates were incubated with or without vehicle, U0126 (30 μmol/L; Cell Signaling Technology), or anti-integrinβ1 neutralizing antibody (R&D Systems) for another 24 h, and then cells were immediately fixed with 4% (vol/vol) formaldehyde in PBS for 20 min at room temperature. After washing with 0.1% (vol/vol) Triton, these cells were blocked by addition of 150 μl of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences) for 90

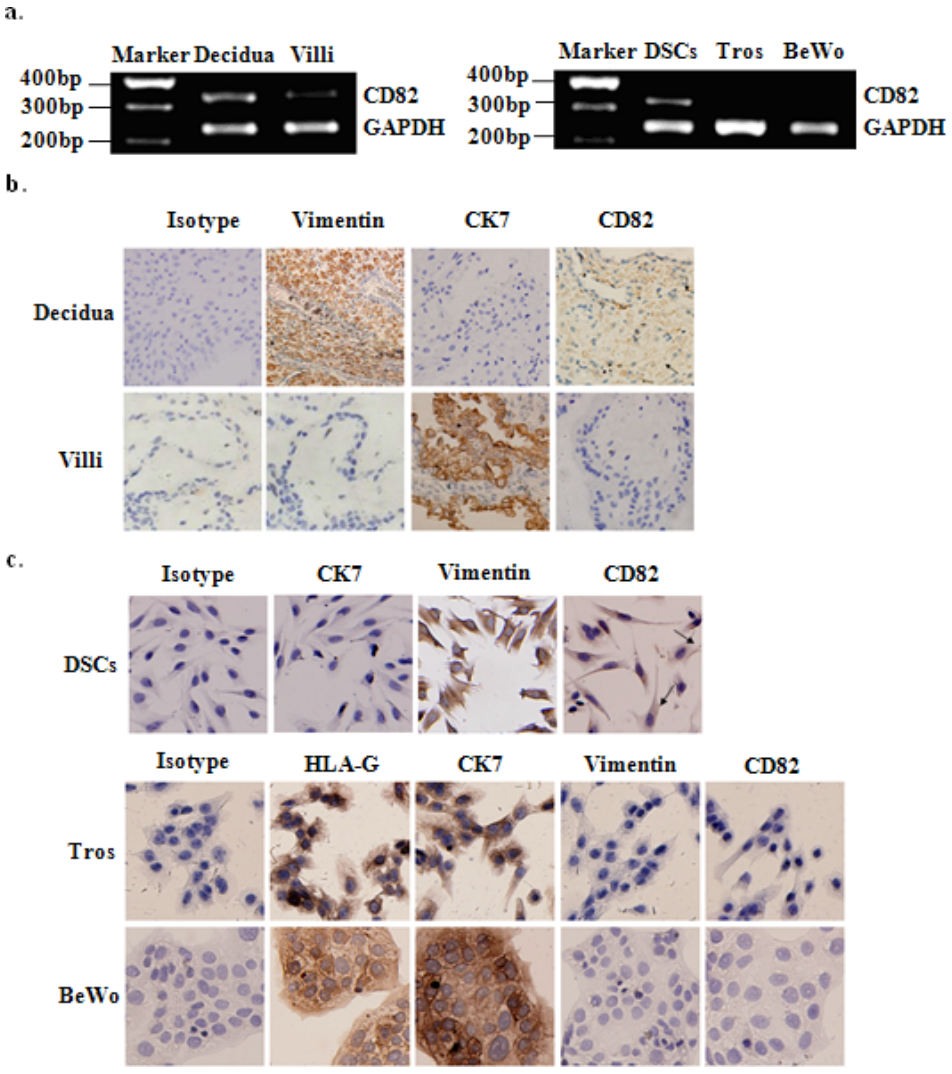


FIG. 1. CD82 is only expressed in DSCs and not in trophoblast cells of human first-trimester pregnancy. **a**) The transcription of CD82 at the maternal-fetal interface. **b**, **c**) The paraffin-embedded tissues, primary cultured DSCs, trophoblasts, and BeWo cells were analyzed by immunohistochemistry and immunocytochemistry, respectively. CD82 expression was localized to the plasma membrane (arrows) in the decidua. Decidual cells were also strongly positive for CD82, and the HLA-G-stained trophoblast cells were negative for CD82. Original magnification ×200. Results were highly reproducible in three independent experiments. Tros, trophoblast cells.

min at room temperature and then incubated with mouse anti-human CD82, or with mouse anti-human MMP2, MMP9, TIMP1, TIMP2 (R&D Systems), titin (Chemicon), or integrinβ1 (R&D Systems) primary antibody with actin (Santa Cruz Biotechnology) as control. After overnight treatment at 4°C, the wells were incubated with a corresponding second 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.). This procedure was carried out in the dark. Images of the target gene were obtained using the Odyssey Infrared Imaging System (LI-COR Biosciences Gmbh). The protein expression level was calculated as the ratio of the intensity of the target gene to that of actin. The experiments were carried out in triplicate and repeated three times.

Thereafter, we detected the level of phospho-MAPK3/1 in the CD82-silenced DSCs and the CD82-transfected BeWo cells by in-cell Western; cells were treated with anti-integrinβ1 neutralizing antibody for 24 h or with U0126 for 30, 60, and 90 min, respectively. The primary antibodies used here were mouse anti-human phospho-MAPK3/1 and rabbit anti-human total MAPK3/1 (Santa Cruz Biotechnology). The level of phospho-MAPK3/1 was shown by the ratio of the intensity of the phospho-MAPK3/1 to that of total MAPK3/1. The phospho-AKT and phospho-p38 (Santa Cruz Biotechnology) in cells without U0126 or anti-integrinβ1 neutralizing antibody were also evaluated. The experiments were carried out in triplicate and repeated three times.

### *Immunofluorescence (IF)*

The CD82-transfected BeWo cells and CD82-silenced DSCs were plated on coverslips in 24-well plates (BD Biosciences) for 96 h. Monolayers were fixed with 4% (vol/vol) paraformaldehyde for 10 min at room temperature, followed by 10-min incubation with 0.2% (vol/vol) Triton X-100. After washing with PBS, nonspecific binding was blocked with 10% FBS in PBS. Anti-CD82 rabbit polyclonal antibody (1:50; SC-5540; Santa Cruz Biotechnology) and anti-TIMP1 and anti-integrinβ1 mouse monoclonal antibodies diluted in PBS were added for 1 h, avoiding light, and then the slides were mounted in 0.1% (wt/vol) 4,6-diamidino-2-phenylindole (DAPI; 1:50; Invitrogen) for nuclear counterstaining for 5 min at room temperature and observed in an Olympus BX51 fluorescence microscope (Olympus). The secondary antibodies were Texas Red and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and anti-mouse IgG (1:250; Rockland). The experiments were carried out in triplicate and repeated three times.

### *Matrigel Invasion Assay*

The invasion of trophoblast cells across matrigel was evaluated objectively in an invasion chamber based on our previous procedure [31]. Briefly, the cell inserts (8-mm pore size, 6.5-mm diameter; Corning) coated with 15–25 μl matrigel were placed in a 24-well plate. The CD82-silenced DSCs were plated in the lower or upper chamber, and the primary trophoblast cells or CD82-transfected BeWo cells ($2 \times 10^5$) were plated in the upper chamber, which formed a coculture unit including direct and indirect. For direct coculture unit, the primary trophoblast cells were plated in the upper chamber until adhering to the matrigel. The media was removed, and the silenced DSCs were also plated in the upper chamber and cocultured. For indirect coculture unit, the primary trophoblast cells were plated in the upper chamber until adhering to the matrigel. The media was removed, and the silenced DSCs were also plated in the lower chamber and cocultured. In treated groups, when the silenced DSCs or CD82-transfected BeWo cells were adherent, mitogen-activated protein/extracelluar signal-regulated kinase (MEK)1/2 inhibitor U0126 (30 μmol/L) or anti-integrinβ1 neutralizing antibody (1 μg/$10^6$ cells) was added. The lower chamber was filled with 800 ml medium. The cells were then incubated at 37°C for 48 h. The inserts were removed and washed in PBS, and the noninvading 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 10 min at room temperature and stained with hematoxylin. The result was observed under an Olympus BX51+DP70 fluorescence microscope (Olympus). The cells migrated to the lower surfaces were counted in five predetermined fields at a magnification of 200×. Each experiment was carried out in triplicate and repeated three times.

### *Western Blot Analysis*

Total protein extracted from human decidual tissues from the early pregnancy termination (n = 6) and unexplained miscarriage (n = 6) were prepared using RIPA buffer. Then 60 μg protein was loaded onto a 10% polyacrylamide-SDS gel. The resolved proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) and incubated with a 1:500 dilution of mouse anti-human CD82 monoclonal antibody and a 1:1000 dilution of mouse anti-human β-actin monoclonal antibody (Santa Cruz Biotechnology) in PBS containing 0.05% (vol/vol) Tween-20 and 5% (vol/vol) fetal calf serum, respectively. After an extensive washing, the bound primary antibodies were detected by using a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc.), respectively, with a chemiluminescent detection system. The experiments were repeated three times.

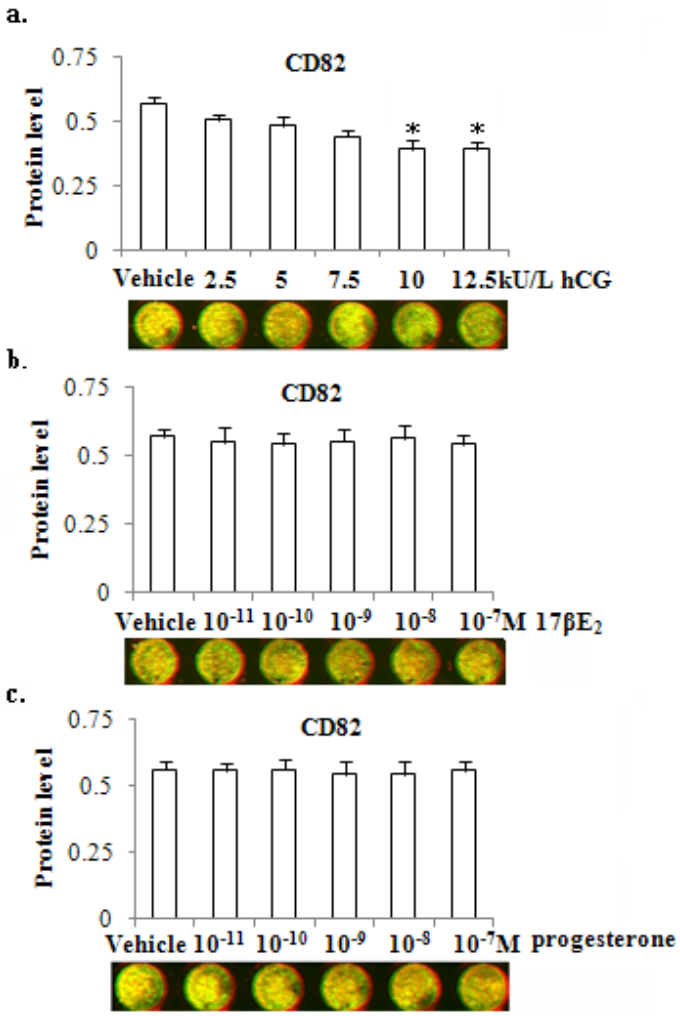


FIG. 2. hCG down-regulates the expression of CD82 in DSCs. **a**, **b**, **c**) The primary DSCs were treated with hCG, estrogen, or progesterone for 72 h. In-cell Western indicates that hCG down-regulates the expression of CD82 in DSCs, but the expression is not influenced by estrogen and progesterone. CD82, red; actin, green. Results were highly reproducible in three independent experiments. Error bars depict the SEM. $^*P < 0.05$, compared to the control. Original magnification ×200.

### *Statistics*

All values are presented as the mean ± SD. One-way ANOVA was used to detect the difference of invasion, CD82, and other molecule transcriptions and translations in human DSCs and BeWo cells. Differences were considered as statistically significant at $P < 0.05$.

## RESULTS

### *CD82 Is Only Expressed in DSCs but Not in Trophoblasts of Human First-Trimester Pregnancy*

The decidua and villi in the early pregnancy termination transcribed *CD82* mRNA, as shown by RT-PCR (Fig. 1a, left), and CD82 was only transcribed in primary DSCs, but not in

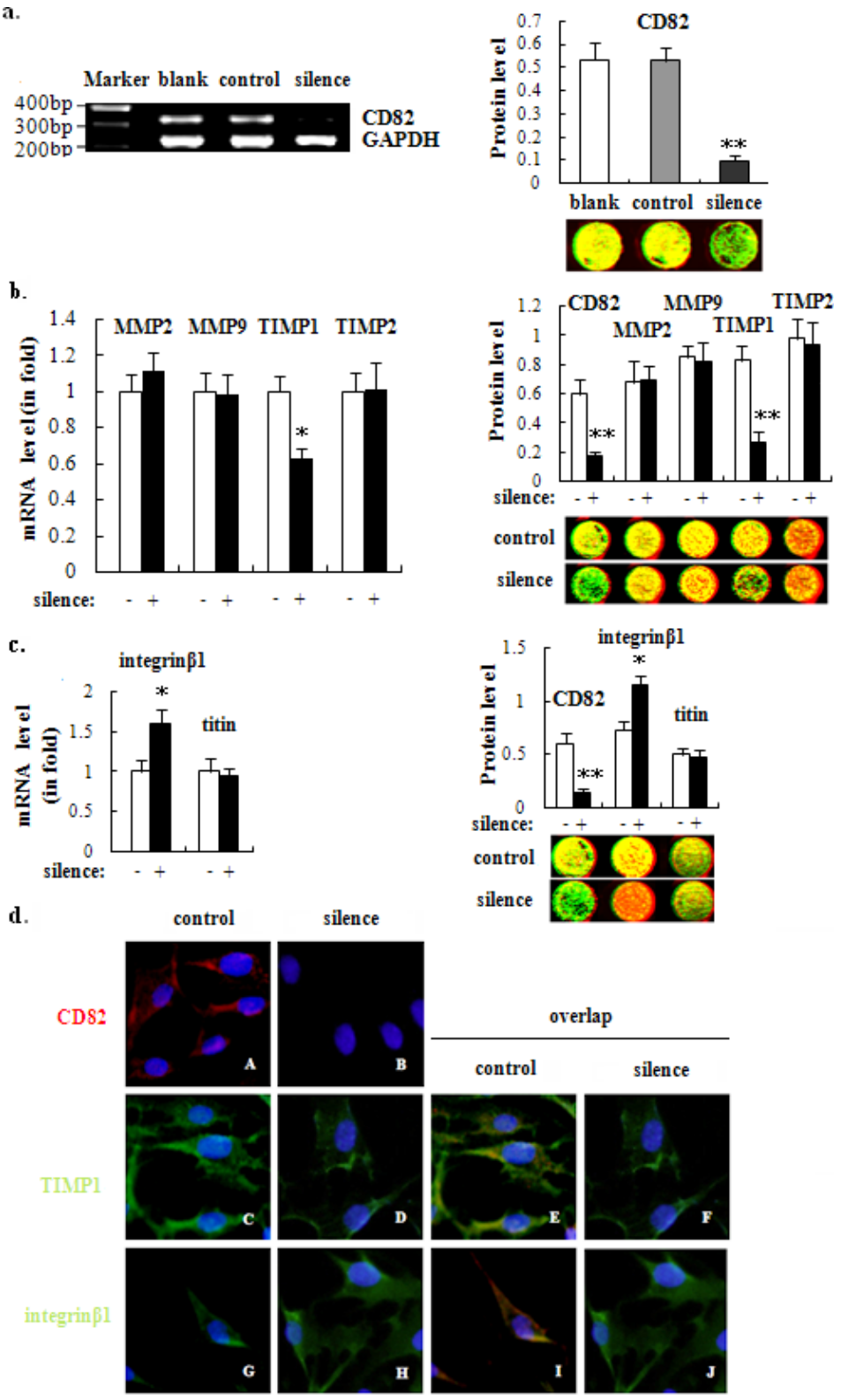


FIG. 3. CD82 up-regulates TIMP1 and down-regulates integrinβ1 in primary human DSCs. **a**) The primary DSCs were silenced for CD82 (si-CD82) for 72 or 96 h, and then the efficiency was demonstrated by RT-PCR (72 h) and in-cell Western (96 h). **b**, **c**) The effect of CD82 on transcription and expression of MMPs, TIMPs, titin, and integrinβ1 was detected by real-time RT-PCR and in-cell Western. **d**) Dual immunofluorescence was applied to detect the effect of CD82 on regulation of TIMP1 and integrinβ1 in the primary DSCs. CD82 antibodies followed by Texas Red-conjugated secondary antibody, red; TIMP1 and integrinβ1 antibodies followed by FITC-conjugated secondary antibody, green; nuclei counterstained with DAPI, blue; blank, no transfection; control, the nontargeting siRNA oligonucleotides; silence, CD82 was knocked down. Results were highly reproducible in three independent experiments. Error bars depict the SEM. $*P < 0.05$, $**P < 0.01$, compared to the si-negative control. Original magnification ×200.

primary trophoblast or BeWo cells (Fig. 1a, right). Immunohistochemistry was performed on paraffin-embedded decidua and villi with CD82 monoclonal antibody, with antibodies against vimentin, cytokeratin 7 (CK7), and HLA-G as control. The deciduas, as well as primary DSCs, were stained positive for CD82 on the plasma membrane (single arrow), but the villi, primary trophoblasts, and BeWo cells were negative for CD82 (Fig. 1, b and c).

### *hCG Down-Regulates the Expression of CD82 in DSCs*

To investigate the effects of the pregnancy-associated hormones (estrogen, progesterone, and hCG) on the expression of CD82 in DSCs, we determined the protein level of CD82 in DSCs by in-cell Western after treatment with different concentrations of 17β-estradiol ($10^{-11}$–$10^{-7}$mol/L), progesterone ($10^{-11}$–$10^{-7}$ mol/L), and hCG (2.5 kU/L–12.5 kU/L) for 72

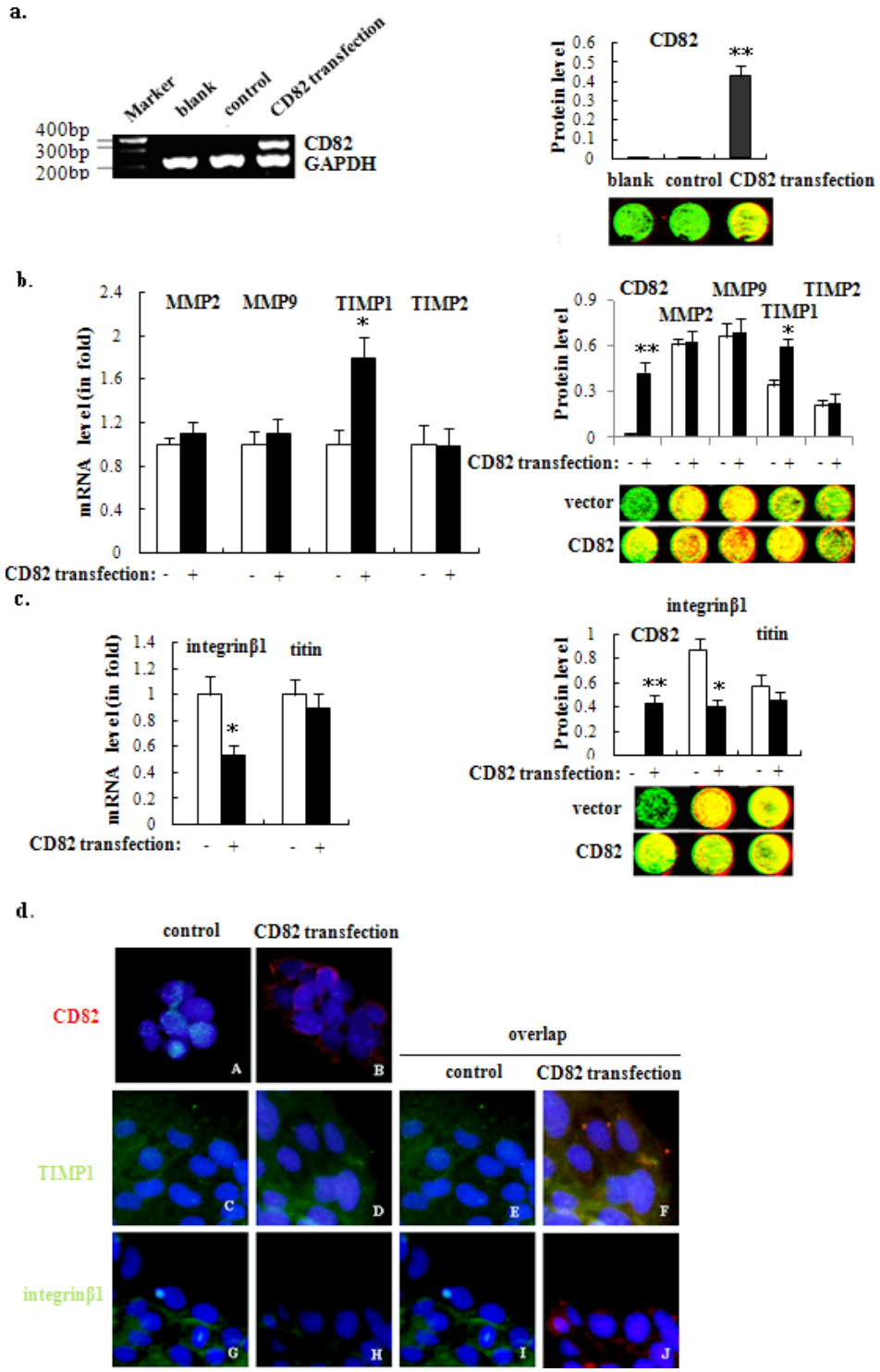


FIG. 4. The CD82 tranfection up-regulates TIMP1 and down-regulates integrinβ1 in BeWo cells. **a**) BeWo cells were transfected, respectively, with plasmid pCDNA3.1(+) vector or pCDNA3.1(+)-CD82 for 72 or 96 h, then overexpression was demonstrated by RT-PCR (72 h) and in-cell Western (96 h). **b**, **c**) The effect of CD82 on MMPs, TIMPs, titin, and integrinβ1. **d**) Dual immunofluorescence (96 h) was also applied to detect the regulation of CD82 by TIMP1 and integrinβ1 in BeWo cells. CD82 antibodies followed by Texas Red-conjugated secondary antibody, red; TIMP1 and integrinβ1 antibodies followed by FITC-conjugated secondary antibody, green; nuclei were counterstained with DAPI, blue; blank, no transfection; control, the transfected pCDNA3.1(+) vector; CD82 transfection, the transfected pCDNA3.1(+)-CD82. Results were highly reproducible in three independent experiments. Error bars depict the SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control. Original magnification ×200.

h. Human CG attenuated the expression of CD82, and the maximal inhibition occurred at a concentration of 10 kU/L (Fig. 2a; $P < 0.05$), but estrogen or progesterone did not modulate the expression (Fig. 2, b and c). These results suggest that the syncytiotrophoblast cells secreted hCG that probably participated in regulating the invasion in situ of extravillous trophoblast cells by down-regulating the expression of CD82 in DSCs.

### *CD82 Up-Regulates TIMP1 and Down-Regulates Integrinβ1 in Primary Human DSCs*

RT-PCR and in-cell Western were used to identify the CD82 expression in primary human DSCs. The *CD82* mRNA (Fig. 3a, left) and protein levels (Fig. 3a, right) of DSCs were significantly decreased after the CD82 silence ($P < 0.01$).

The interaction between DSCs and trophoblast cells induces expression of invasion-relevant genes by activating the intracellular signaling pathway. MMP2 and MMP9 are two important protein enzymes that degrade the ECM and that are involved in human first-trimester trophoblast invasion. The silencing of CD82 in DSCs significantly decreased the mRNA (Fig. 3b, left) and protein (Fig. 3b, right) levels of TIMP1 compared with the si-negative control ($P < 0.01$ and $P < 0.05$, respectively). However, the mRNA and protein levels of

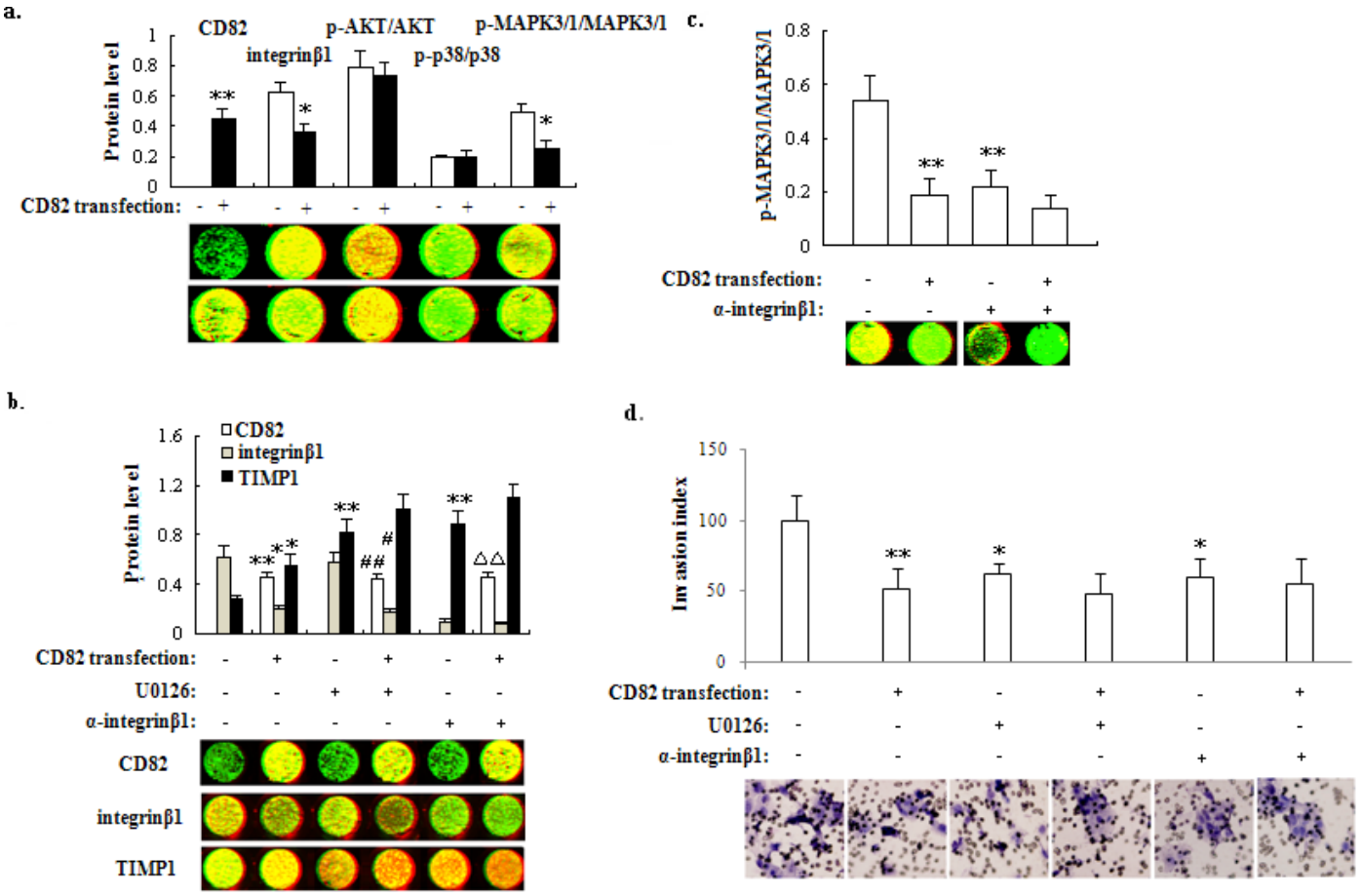


FIG. 5. CD82 transfection inhibits invasiveness of BeWo cells partly via the integrinβ1/MAPK/MAPK3/1 signaling pathway. **a**) In-cell Western indicates that the ratio of P-MAPK3/1 to T-MAPK3/1 in BeWo cells is decreased after 96 h of transfection with CD82. Here, CD82, integrinβ1, P-AKT1/2, P-P38, and P-MAPK3/1 are shown in red, and actin, T-AKT1/2, T-P38, and T-MAPK3/1 are shown in green. **b**) U0126 (30 μmol/L) or anti-integrinβ1 neutralizing antibody also increases the TIMP1 expression in BeWo cells after 24 h, but U0126 can not influence the expression of integrinβ1. TIMP1, red; actin, green. **c**) CD82 transfection inhibits the activated MAPK3/1, and anti-integrinβ1 neutralizing antibody appears to have the same effect on the activated MAPK3/1. **d**) CD82, U0126, or anti-integrinβ1 neutralizing antibody inhibits invasiveness of BeWo cells. Control, the transfected pCDNA3.1(+) vector; CD82 transfection, the transfected pCDNA3.1(+)-CD82. Results were highly reproducible in three independent experiments. Error bars depict the SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control; $\#P < 0.05$, $\#\#P < 0.01$, compared to the control and U0126 treatment. $\Delta\Delta P < 0.01$ compared to the control and anti-integrinβ1 neutralizing antibody treatment. Original magnification ×200.

MMP2, MMP9, and TIMP2 showed no statistical difference ($P > 0.05$) between the two groups.

We also determined the transcription and protein levels of integrinβ1 and titin that are associated with trophoblast invasion by RT-PCR and in-cell Western, respectively. Knockdown of CD82 in DSCs significantly increased the transcription and protein levels of integrinβ1 (Fig. 3c; $P < 0.05$), but did not influence the expression of titin (Fig. 3c; $P > 0.05$) compared with the si-negative control.

Subsequently, immunofluorescence was used to further evaluate the effect of CD82 on the expression of TIMP1 and integrinβ1 in DSCs. Figure 3d shows that the green fluorescence staining of TIMP1 was decreased and integrinβ1 was increased after silencing of CD82 in DSCs. Therefore, CD82 up-regulated TIMP1 and down-regulated integrinβ1 in primary human DSCs, thereby possibly controlling the invasion of trophoblast cells.

### *Overexpression of CD82 Up-Regulates TIMP1 and Down-Regulates Integrinβ1 in Trophoblastic Cell Line BeWo*

We also identified the CD82 expression in BeWo cells by RT-PCR and in-cell Western and showed that BeWo cells highly expressed CD82 mRNA (Fig. 4a, left) and protein (Fig. 4a, right; $P < 0.01$) after transfection by pcDNA3.1(+)-CD82, compared with transfection by the pcDNA3.1(+) vector.

In contrast, the mRNA (Fig. 4b, left) and protein (Fig. 4b, right) levels of TIMP1 were increased in BeWo cells transfected by pcDNA3.1(+)-CD82, compared with the pcDNA3.1(+) vector. However, the mRNA and protein levels of MMP2, MMP9, and TIMP2 showed no statistical difference ($P > 0.05$) between the two groups.

Likewise, we detected the transcription and protein levels of integrinβ1 and titin in BeWo cells by RT-PCR and in-cell Western. The overexpression of CD82 in BeWo cells significantly decreased the transcription and protein levels of integrinβ1 (Fig. 4c; $P < 0.05$), but didn't influence the expression of titin (Fig. 4c; $P > 0.05$) compared with the pcDNA3.1(+) vector.

Then, immunofluorescence was used to further evaluate the effect of CD82 on the expression of TIMP1 and integrinβ1 in BeWo cells. Figure 4d shows that the green fluorescence staining of TIMP1 was increased and that of integrinβ1 was decreased in BeWo transfected by pcDNA3.1(+)-CD82 when compared with the pcDNA3.1(+) vector, which suggests that overexpression of CD82 in BeWo cells inhibited the invasion,

probably through up-regulating TIMP1 and down-regulating integrinβ1.

### *Overexpression of CD82 Inhibits Invasiveness of BeWo Cells Partly via Integrinβ1/MAPK/MAPK3/1 Signaling Pathway*

Integrinβ1, PIK3, and MAPK signaling pathways are involved in modulation of migration and penetration of human cancer cells and trophoblast cells. Therefore, we determined whether CD82 inhibits the invasion of human first-trimester trophoblast cells via integrinβ1, PIK3, or MAPK signaling pathways. First, we detected the expression of the critical molecules of these signaling pathways and found that CD82 overexpression in BeWo cells significantly decreased the expression of integrinβ1 ($P < 0.05$) and the proportion of phospho-MAPK3/1 to total MAPK3/1 ($P < 0.05$), but did not influence the proportions of phospho-AKT to total AKT and of phospho-p38 to total p38 when compared with the pcDNA3.1(+) vector (Fig. 5a), which suggests that the integrinβ1 and MAPK/MAPK3/1 signaling pathways were involved in the CD82-mediated suppression of human trophoblast cell invasiveness.

We next determined whether the CD82-controlled expression of integrinβ1 and TIMP1 in BeWo cells was through the integrinβ1 and MAPK/MAPK3/1 signaling pathways. As shown in Figure 5b, the TIMP1 expression of BeWo cells was also increased by U0126 or anti-integrinβ1 neutralizing antibody, but the expression of integrinβ1 was not effected by U0126.

To determine the relationship of integrinβ1 to MAPK/MAPK3/1 signaling pathways in the CD82 actions, we detected the phospho-MAPK3/1 level by in-cell Western in BeWo cells transfected by the pcDNA3.1(+) vector or pcDNA3.1(+)-CD82. These cells were then treated with anti-integrinβ1 neutralizing antibody for 24 h. As shown, the proportion of phospho-MAPK3/1 to total MAPK3/1 in the CD82-expressed BeWo cells was significantly lower than that of the BeWo cells without CD82 expression ($P < 0.01$), and the proportion of phospho-MAPK3/1 to total MAPK3/1 in BeWo cells decreased with anti-integrinβ1 neutralizing antibody ($P < 0.01$); moreover, anti-integrinβ1 neutralizing antibody impaired the inhibitory effect of CD82 on phosphorylation of MAPK3/1 (Fig. 5c). Therefore, we propose that the integrinβ1/MAPK/MAPK3/1 signaling pathway was involved in the functional modulation of CD82 on human first-trimester trophoblast invasion.

The matrigel-based transwell assay demonstrated the effect of CD82 on the invasion of human first-trimester trophoblast cells and the invasiveness of BeWo cells transfected by CD82 after treatment with U0126 or anti-integrinβ1 neutralizing antibody (Fig. 5d); the number of cells that migrated to the lower surface were counted. The invasive index of the CD82-expressed BeWo cells was significantly lower than that of the control ($P < 0.01$), and U0126 or anti-integrinβ1 neutralizing antibody also decreased the invasion of BeWo cells, which demonstrates that the overexpressed CD82 in BeWo cells up-regulated the expression of TIMP1, and inhibited their invasion probably through the integrinβ1/MAPK/MAPK3/1 signaling pathway.

### *The DSC-Expressed CD82 Inhibits the Invasiveness of Trophoblast Cells in Coculture*

To demonstrate the effects of CD82 in DSCs on the invasion of human first-trimester trophoblast cells, we used the matrigel-based transwell assay to evaluate the invasiveness of primary trophoblast cells in indirect or direct coculture with DSCs transfected by si-CD82 with si-negative control. The number of cells that migrated to the lower surface was counted after 48 h of incubation. As shown in Figure 6, the invasive index of human first-trimester trophoblast cells was significantly higher in coculture with DSCs in CD82 silence than in that of the si-negative control ($P < 0.01$), and there was no difference between the direct and indirect coculture ($P > 0.05$), which suggests that the DSC-expressed CD82 inhibited the invasion of human first-trimester trophoblast cells by way of soluble molecules.

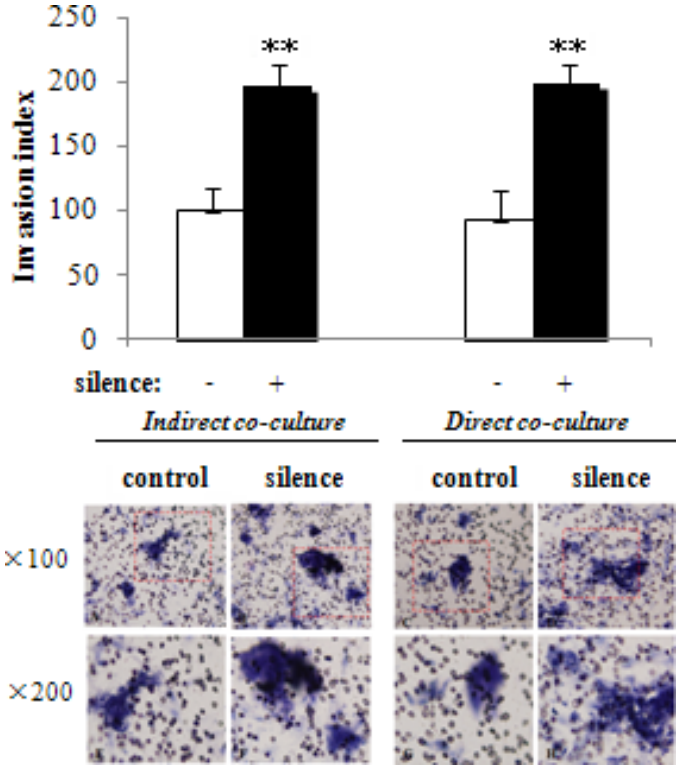


FIG. 6. The DSCs-expressed CD82 inhibits the invasiveness of human primary trophoblast cells in coculture. For direct coculture unit, the primary trophoblast cells were plated in the upper chamber until adhering to the matrigel. The media was removed, and then the CD82-silenced DSCs were added in the upper chamber and cocultured for 48 h. For the indirect coculture unit, the primary trophoblast cells were plated in the upper chamber until adhering to the matrigel. The media was removed, and then the CD82-silenced DSCs were also plated in the lower chamber and cocultured for 48 h. After direct or indirect coculture with the CD82-silenced DSCs, the invasiveness of the primary trophoblast cells is clearly enhanced. Control, the nontargeting siRNA oligonucleotides; silence, CD82 was knocked down. These pictures are representative of three individual experiments. Error bars depict the SEM. $^{**}P < 0.01$, compared to the control. Upper panels, original magnification ×100; lower panels are enlarged insets from upper panels, original magnification ×200.

### *The DSC-Expressed CD82 Inhibits the Invasiveness of Trophoblast Cells in Coculture Partly via the Integrinβ1/MAPK/MAPK3/1 Signaling Pathway*

In contrast to the results of the CD82-overexpressed BeWo cells, the expression of integrinβ1 ($P < 0.05$) and the proportion of phospho-MAPK3/1 to total MAPK3/1 ($P < 0.05$) in DSCs were obviously higher after CD82 silence than that of the si-negative control (Fig. 7a), and the proportions of

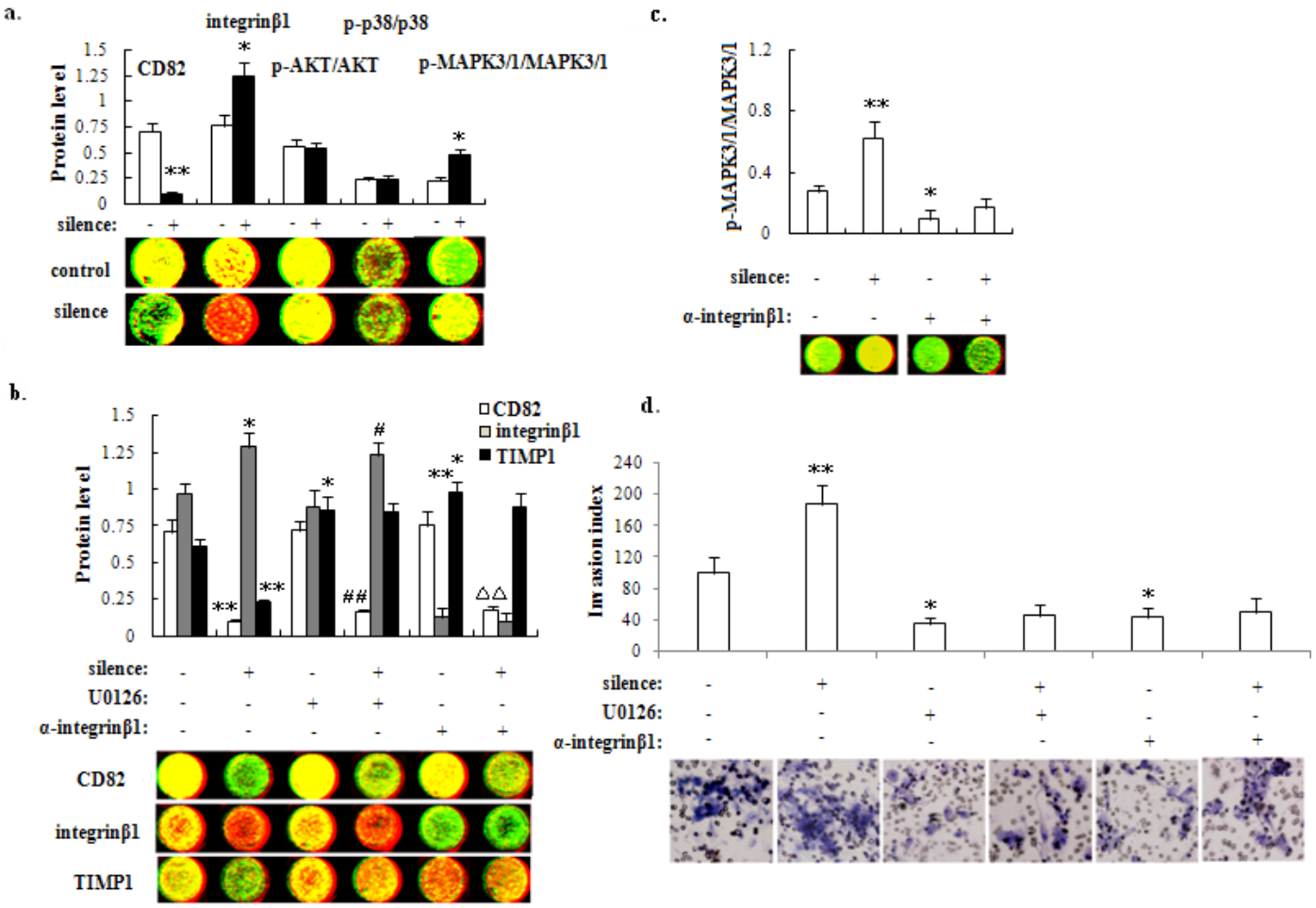


FIG. 7. The DSCs-expressed CD82 inhibits the invasiveness of trophoblast cells in coculture via the integrinβ1/MAPK/MAPK3/1 signaling pathway. **a**) In-cell Western indicates that the ratio of P-MAPK3/1 to T-MAPK3/1 is enhanced after 96 h of CD82 silence in the primary DSCs. Here CD82, integrinβ1, P-AKT1/2, P-P38, and P-MAPK3/1 are in red; actin, T-AKT1/2, T-P38, and T-MAPK3/1 are in green. **b**) CD82 silence up-regulates integrinβ1 expression and down-regulates TIMP1 expression in the primary DSCs, and anti-integrinβ1 neutralizing antibody abolishes the effects of the CD82 silence, but U0126 only restores the TIMP1 expression. **c**) CD82 silence leads to MAPK3/1 activation in the primary DSCs, and anti-integrinβ1 neutralizing antibody results in its inactivation. **d**) CD82 silence in the primary DSCs improves invasiveness of primary trophoblast cells in the coculture, and anti-integrinβ1 neutralizing antibody or U0126 can abolish the improvement. Control, the nontargeting siRNA oligonucleotides; silence, CD82 was knocked down. Results were highly reproducible in three independent experiments. Error bars depict the SEM. $*P < 0.05$, $**P < 0.01$, compared to the control; $\#P < 0.05$, $\#\#P < 0.01$, compared to the control and U0126 treatment; $\Delta\Delta P < 0.01$, compared to the control and anti-integrinβ1 neutralizing antibody treatment. Original magnification ×200.

phospho-AKT to total AKT and phospho-p38 to total p38 were also not changed between the two groups.

As shown in Figure 7b, the increase of TIMP1 induced by CD82 in DSCs was reversed by U0126 or anti-integrinβ1 neutralizing antibody, and the expression of integrinβ1 in DSCs was not effected by U0126.

As shown, the proportion of phospho-MAPK3/1 to total MAPK3/1 in the CD82-silenced DSCs was significantly higher than that of the si-negative control ($P < 0.01$). The proportion of phospho-MAPK3/1 to total MAPK3/1 in the DSCs was decreased after treatment with anti-integrinβ1 neutralizing antibody ($P < 0.01$), and anti-integrinβ1 neutralizing antibody impaired the inhibitory effect of CD82 on phosphorylation of MAPK3/1 in DSCs (Fig. 7c). Therefore, it is concluded that the integrinβ1/MAPK/MAPK3/1 signaling pathway was involved in functional modulation of CD82 in DSCs on the first-trimester human trophoblast invasion.

Our results also show that the invasive index of the human primary trophoblast cells was significantly higher in coculture with the CD82-silenced DSCs than in that of the control without silence ($P < 0.01$; Fig. 7d). The decreased invasiveness induced by CD82 might also be also abolished by U0126 or anti-integrinβ1 neutralizing antibody, which is associated with the protein levels of TIMP1 and integrinβ1 in DSCs (Fig. 7b).

It can be concluded that the CD82 in DSCs regulated the expression of TIMP1, participated in intercellular communication with human trophoblast cells, and then controlled trophoblast invasion by deactivating the integrinβ1/MAPK/MAPK3/1 signaling pathway.

### *The Expression of CD82 Increases in Decidua from Miscarriage*

To further demonstrate the association of CD82 expression at the human maternal-fetal interface with pregnancy outcome, we collected the decidua from the normal early pregnancy termination and the unexplained miscarriage and detected the expression of CD82 by real-time PCR, immunohistochemistry, and traditional Western blot. As shown, the mRNA level of *CD82* in the decidua from the unexplained miscarriage was 97-fold higher than that of the normal early pregnancy ($P < 0.01$;

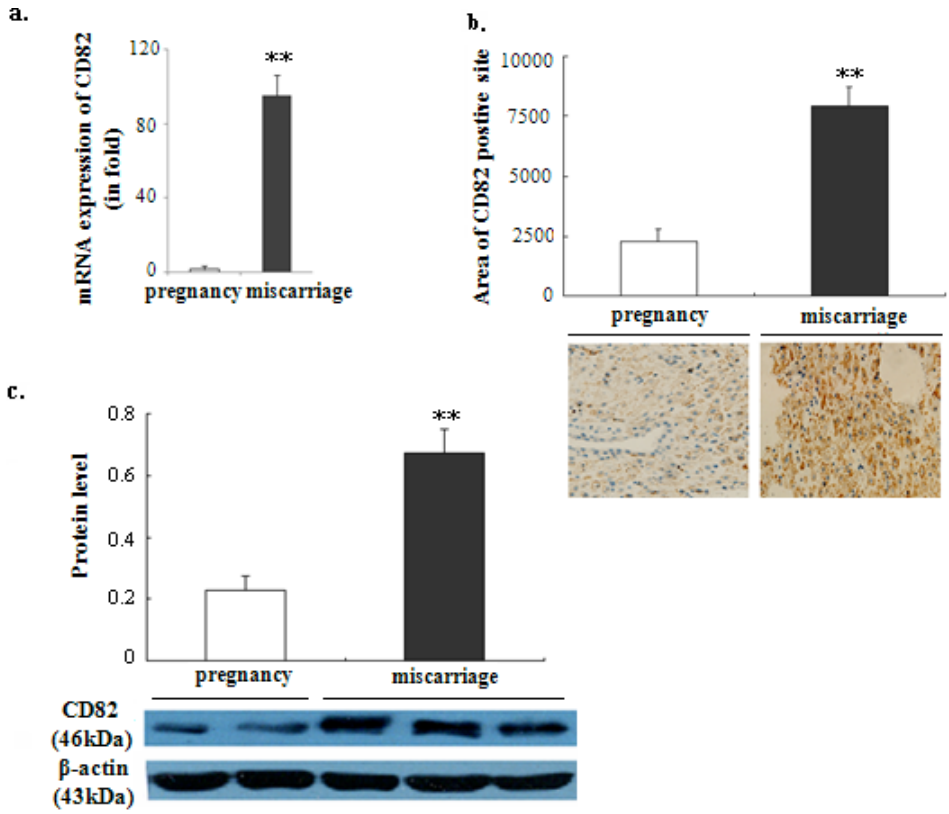


FIG. 8. The transcription and protein translation of CD82 increase significantly in the decidua of the unexplained miscarriage. CD82 transcription and translation in human decidua was analyzed by quantitative real-time PCR (**a**), immunohistochemistry (**b**), or Western blot (**c**). The relative fold expression of the CD82 in decidua is compared between the miscarriage and normal early pregnancy. These pictures are representatives of six individual experiments. Error bars depict the standard error of the mean. Pregnancy, the decidua from normal early pregnancy; miscarriage, the decidua from the unexplained miscarriage. $^{**}P < 0.01$, compared to pregnancy. Original magnification (**b**) ×200.

Fig. 8a). Consistent with transcription level, the decidua from the unexplained miscarriage had a much higher CD82 protein expression than that of the normal early pregnancy termination, based on immunohistochemistry and Western blot ($P < 0.01$; Fig. 8, b and c), which suggests that the CD82 overexpression in decidua restricted the appropriate invasion of trophoblasts, leading to early pregnancy wastage.

## DISCUSSION

Successful pregnancy depends on the ability of trophoblast cells to invade the uterine decidual stroma and to gain access to the maternal circulation, which is a mechanism similar to that of tumor cells. However, as opposed to malignant invasion, the trophoblast invasion is strictly limited in normal pregnancy. These events are regulated by the cross-talking of paracrine and autocrine factors between the trophoblast cells and DSCs at the maternal-fetal interface [32]. DSCs secrete a lot of cytokines and express proteins, such as TIMP1, that control the invasiveness of the trophoblast cells. As a wide-spectrum tumor metastasis suppressor gene, CD82 is expressed in the primary DSCs but not in the primary trophoblast cells, so CD82 might be the media of cross-talking between DSCs and trophoblast cells.

Therefore, in the present study, we have investigated whether the DSCs-expressed CD82 regulates the invasion of trophoblast cells. As shown in Figure 3 and Figure 5, we have demonstrated that human DSCs from the first-trimester pregnancy express CD82 that inhibits the invasion of trophoblast cells through up-regulating the transcription and translation of TIMP1. DSCs and trophoblast cells produce TIMP1, which controls MMP secretion of DSCs and trophoblast cells [12]. MMPs are partly responsible for placentation and spiral artery remodeling [8]. MMPs are involved in pregnancy complications, including not only spontaneous abortion but also preeclampsia, fetal growth restriction, and so on, that result from an insufficient invasion of trophoblasts.

Our observations show that CD82 deactivates MAPK3/1 and down-regulates the expression of integrinβ1, and anti-integrinβ1 neutralizing antibody reinforces the deactivation of MAPK3/1 and the increase in expression of TIMP1 induced by CD82, which suggests that CD82 stimulates the expression of TIMP1 via the integrinβ1/MAPK/MAPK3/1 signaling pathway as well as other signal pathways involved in the invasion of trophoblast cells. The mechanism of CD82-mediated tumor metastasis suppression is not fully understood, but a series of the CD82-associated molecules, such as EGFR, integrinβ1, and E-cadherin are involved [22, 23].

Trophoblast invasion involves protelysis and remodeling of the uterine decidua. In addition to the MMPs, the integrin repertoire of the endometrium and decidua may play an important role in successful implantation. According to a timed expression correlating with embryo attachment, the αvβ3 and α4β1 integrins are considered markers of uterine receptivity [33]. The αvβ3 integrin has been shown to be highly expressed at the time of embryo attachment, and aberrant expression of αvβ3 is associated with infertility [34]. The miscarriage has been found to have a lower expression of α4β3 and α5β1 integrins in the endometrium during the implantation window than that of unexplained infertility [35]. Moreover, the trophectoderm also express several integrins, α3, α5, β1, β3, β4, and β5, that are implicated in blastocyst attachment to the endometrial surface [36, 37]. In female mice lacking a functional integrinβ1 gene, embryos develop normally to the blastocyst stage but fail to implant properly and die. In our study, CD82 in DSCs down-regulates the expression of integrinβ1, which suggests a mechanism of CD82 in DSCs that controls the invasiveness of trophoblast cells.

Pregnancy is characterized by high levels of estrogen, progesterone, and hCG, which play important roles in the

implantation process. Human CG can stimulate trophoblast migration through IGF2 [38] and MMP9 [39]. Progesterone may decrease invasion and gelatinase expression in trophoblast cells of the first trimester, but increase the invasion and MMP2 expression in trophoblast cells of the late pregnancy [40]. We have demonstrated that hCG attenuates CD82 expression in DSCs, but estrogen or progesterone has no effect on the CD82 expression. So we conclude that hCG secreted by syncytiotrophoblastic cells might increase invasiveness of the extravillous trophoblast cells through down-regulating the expression of CD82 in DSCs. As is well known, several studies have reported the expression of hCG in a variety of cancers, including trophoblastic and testicular germ cell tumors, bladder cancer, colorectal cancer, ovarian cancer, lung cancer, pancreatic cancer, cervical cancer, prostate cancer, and breast cancer [41–44]. It is further reported that such cancers have poor prognosis and adverse survival rate. The role of hCG or its subunits with respect to the biology of the tumor cells is not fully clear. In view of our results, we presume that abnormal levels of hCG secreted by the cancer cells might promote their invasion and metastasis by down-regulating the expression of CD82.

Interestingly, we have found that the transcription and translation of CD82 in decidual tissues from unexplained miscarriage are significantly higher than that of the normal early pregnancy, which suggests that the CD82 overexpression at the maternal-fetal interface would lead to the early pregnancy wastage.

In summary, our study has demonstrated for the first time that CD82 expressed in DSCs participates in intercellular communication with trophoblast cells, which is different from our previous understanding that CD82 in tumor cells mainly inhibits the motility and invasion of itself. Further research may focus on the functional molecules and proteins that regulate the expression of CD82, such as the anti-hCG vaccine, which will help to potentially control both pathological trophoblastic disease and tumor.

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