Chemokine CCL24 promotes the growth and invasiveness of trophoblasts through ERK1/2 and PI3K signaling pathways in human early pregnancy

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Abstract

Chemokine CCL24, acting through receptor CCR3, is a potent chemoattractant for eosinophil in allergic diseases and parasitic infections. We recently reported that CCL24 and CCR3 are co-expressed by trophoblasts in human early pregnant uterus. Here we prove with evidence that steroid hormones estradiol (E), progesterone (P), and human chorionic gonadotropin (hCG), as well as decidual stromal cells (DSCs) could regulate the expression of CCL24 and CCR3 of trophoblasts. We further investigate how trophoblast-derived CCL24 mediates the function of trophoblasts *in vitro*, and conclude that CCL24/CCR3 promotes the proliferation, viability and invasiveness of trophoblasts. In addition, analysis of the downstream signaling pathways of CCL24/CCR3 show that extracellular signal-regulated kinases (ERK1/2) and phosphoinositide 3-kinase (PI3K) pathways may contribute to the proliferation, viability and invasiveness of trophoblasts by activating intracellular molecules Ki67 and matrix metalloproteinase 9 (MMP9). However, we did not observe any inhibitory effect on trophoblasts when blocking c-Jun N-terminal kinase (JNK) or p38 pathways. In conclusion, our data suggests that trophoblast-derived CCL24 at the maternal-fetal interface promotes trophoblasts cell growth and invasiveness by ERK1/2 and PI3K pathways. Meanwhile, pregnancy-related hormones (P and hCG), as well as DSCs could up-regulate CCL24/CCR3 expression in trophoblasts, which may indirectly influence the biological functions of trophoblasts. Thus, our results provide a possible explanation for the growth and invasion of trophoblasts in human embryo implantation.

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Introduction

Embryo implantation is a complex process which requires not only the growth and differentiation of villi, but also trophoblasts invade into maternal uterine arteries so as to establish sufficient blood supply for the conceptus (Timeva *et al.* 2014). When fetal-derived villi contact with maternal decidua, a subpopulation of trophoblasts located at the tips of anchoring villi undergo proliferation and differentiation, and develop into invasive extravillous trophoblasts (EVTs). Finally, EVT infiltrate into decidual spiral arteries to remodel the vessel walls (Chen *et al.* 2012). The entire process is precisely regulated by modulators at the maternal-fetal interface; for example, decidual immune cells (DICs), DSCs, female steroid hormones and cytokines (Paiva *et al.* 2011, Oreshkova *et al.* 2012). However, failure to achieve this process may result in pregnant complications, such as abortion, preeclampsia, fetal growth restriction (FGR), gestational trophoblastic diseases, and so on (Kwak-Kim *et al.* 2010, Kinoshita *et al.* 2012).

Chemokines, whose principle role is orchestrating immune response in the body, are a large family of chemotactic cytokines (Zlotnik & Yoshie 2012). CCL24 is a member of CCL chemical family. Because of its similar effect with eotaxin-1 (CCL26) and eotaxin-3 (CCL28) on attracting eosinophils, it is also called eotaxin-2 (Zhebrun *et al.* 2014). CCR3 is the only receptor of CCL24 (Zlotnik & Yoshie 2012). However, other CCL chemokines such as CCL5, CCL7, CCL8, CCL12, CCL13, CCL15, CCL26 and CCL28 share the receptor with CCL24, even if they have different affinities (Vinader & Afarinkia 2012). CCR3 is mainly presented on eosinophils, and is also found on basophils, monocytes, Th2 lymphocytes, epithelial cells as well as airway smooth muscles (Zhebrun *et al.* 2014). Thus, CCL24 is considered a contributor to kinds of atopic disorders, parasitic infections and numerous systemic diseases (Owczarek *et al.* 2010, Kinoshita *et al.* 2012).

The maternal-fetal interface is a specific chemokine-rich environment and several chemokines have been found there (He *et al.* 2012, Wallace *et al.* 2013). Our previous work provided evidence that trophoblasts are an important source of CCL24 in human early pregnant uterus (Li *et al.* 2013). At the same time, CCR3 is detected on human villi and trophoblast cell line HTR8/SVneo (Chau *et al.* 2013, Li *et al.* 2013). However, whether trophoblasts have a self-regulation through CCL24/CCR3 axis and what the downstream mechanisms are still largely unknown. The aim of the present study was to investigate the effect of CCL24/CCR3 on the biological behaviors of trophoblasts, and to find out possible signaling pathways in this process. Meanwhile, we identified local factors at the maternal-fetal interface that may influence the expression of CCL24/CCR3 by trophoblasts

Materials and methods

Tissue collection, isolation and culture of trophoblasts and DSCs

The study was approved by the Ethical Committee of the Obstetrics and Gynecology Hospital, Fudan University. All tissue samples were obtained with the consent of patients.

Villi and deciduas were collected from first-trimester pregnant women (age 20–35 years, gestational age 6–9 weeks) who chose termination for no medical reasons. Before their curettage operations, their blood tests, including steroid hormones, and ultrasound examination of the fetuses were normal. In addition, those who were overweight, had the habit of smoking or drinking, had or acute or chronic diseases were excluded. After the curettage operations, villi ($n=10$, age: 27 ± 6.77 years, gestational age: 7 ± 1.41) were soon collected and stored in 10% buffered formalin at 4 °C for wax embedding. For primary culture, sterile villi ($n=242$, age: 26 ± 2.01 years, gestational age: 8 ± 0.49) or deciduas ($n=26$, age: 24 ± 3.75 years, gestational age: 7 ± 1.53) samples were immediately put into ice-cold high D-glucose DMEM (HG-DMEM; Gibco Grand Island, NY, NY, USA), with 1% penicillin and streptomycin in the medium. Tissues were transported to the laboratory within 30 min, and then trophoblasts and DSCs were isolated according to our previous procedures (Li *et al.* 2010). These methods supplied about 85% cytokeratin-positive purified trophoblasts, and about 98% vimentin-positive and cytokeratin-negative DSCs. Cells were cultured with HG-DMEM plus 10% BSA (Hyclone, Logan, UT, USA), and incubated at 37 °C in a 5% CO₂ incubator.

Immunohistochemistry for detecting the expression of CCR3 on villi

Paraffin sections (5 µm) of villi were dehydrated in graded ethanol. After blocking the endogenous peroxidase

activity by 3% H₂O₂ for 15 min, samples were incubated with goat anti-human CCR3 antibody (15 µg/ml, R&D Systems, Minneapolis, MN, USA), or goat IgG isotype in a humid chamber overnight at 4 °C. All sections were washed three times with PBS, and then overlaid with peroxidase-conjugated anti-goat IgG (Golden Bridge International, Inc, Beijing, China). The color reaction was developed with 3,3-diaminobenzidine (DAB), and then counterstained with hematoxylin. Results were observed by Olympus BX51+DP70 microscope (Olympus, Tokyo, Japan).

Flow Cytometry for detecting CCR3 on trophoblasts

Flow cytometry was applied to evaluate the purity of primary cultured trophoblasts and the expression of CCR3 on trophoblasts. Cytokeratin 7 (CK7) is a protein that specifically expressed in epithelial tissues, and is used as a maker of trophoblast in villi (Li *et al.* 2010). Freshly isolated trophoblasts were gently washed with PBS, and then mixed with mouse anti-human CK7-FITC MAB (BD, Franklin Lakes, NJ, USA) and CCR3-APC MAB (Biolegend, San Diego, CA, USA). Isotypic control antibodies were used. After incubation in darkness for 30 min at room temperature, cells were analyzed immediately by a flow cytometer (FACS Calibur, BD). All samples were analyzed by flow cytometry three times and the experiment was repeated eight times.

In order to evaluate whether DSCs influence the expression of CCR3 on trophoblasts, we co-cultured trophoblasts and DSCs (1×10^5 cells/well) (the ratios of DSC:trophoblasts were 1:3, 1:2, 1:1 or 2:1 respectively) in 12-well plates for 48 h, then CCR3 expression on trophoblasts in co-culture unit was analyzed by flow cytometry.

In addition, we treated trophoblasts (2×10^5 cells/well) with 17β-E₂ (E, 10^{-8} M), progesterone (P, 10^{-8} M), or human chorionic gonadotropin (hCG, 5 kU/l) for 48 h. Then, the supernatant was collected for detecting the level of CCL24, and cells were harvested for CK7 and CCR3 staining by flow cytometry.

ELISA for detecting the level of CCL24

ELISA was taken to evaluate the level of CCL24 that secreted by trophoblasts after treatment with E, P or hCG for 48 h. Cell culture supernatant was collected as previously mentioned, and centrifuged to remove cellular debris, and then stored at −80 °C. The ELISA for CCL24 (R&D Systems) was accomplished following the manufacture's instruction. The sensitivity of this product is 2.5 pg/ml.

BrdU cell proliferation assay and MTT assay

Trophoblasts were plated at the density of 1×10^4 cells/well in 96-well flat-bottom plates. Before

treatment, trophoblasts were starved for 12 h, with HG-DMEM that included 1% BSA as culture medium. Then, cells were stimulated by recombinant human CCL24 (rhCCL24, 0.1, 1, 10 or 100 ng/ml), anti-CCL24 neutralizing antibody (a-CCL24, 0.08, 0.4, 2 or 10 µg/ml), anti-CCR3 neutralizing antibody (a-CCR3, 0.32, 1.6, 8 or 40 µg/ml) or vehicle for 48 h. All of those reagents were purchased from R&D Systems. Then, BrdU cell proliferation assay (Millipore, Billerica, MA, USA) was used to evaluate the proliferation ability of trophoblasts according to the manufacturer's instruction. Each experiment was performed in triplicate, and repeated six times.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric assay for assessing cell viability. It is based on the conversion of MTT into formazan crystals, which has a purple color (van Meerloo *et al.* 2011). We treated trophoblasts with rhCCL24, a-CCL24 or a-CCR3 (all concentrations were the same with BrdU assay) for 48 h, then washed the plates with sterile PBS, and added 180 µl of conditioned medium plus 20 µl of 5 mg/ml MTT solution per well. From then on, plates were conserved or handled in dark. After 4 h of cell culture in the incubator, we gently removed culture fluid, added 150 µl of DMSO to dissolve purple crystals, stirred the plates in a gyratory shaker for 5 min, and finally read the plates at 490 nm within 1 h. Each experiment was performed in triplicate, and repeated six times.

Matrigel invasion assay

The transwell plates (24-well, pore size 8 µm) for invasion assay were purchased from Becton Dickinson Discovery Lab-ware. First of all, the inner bottoms of upper chambers were coated with 15 µl/well matrigel solution (the work concentration was 1.5mg/ml; BD), then we placed the plates in humid 37 °C incubator for matrigel solidification. Four hours later, HG-DMEM conditioned medium (600 µl/well) with rhCCL24 (0, 0.1, 1, 10 or 100 ng/ml) was added into the lower chambers of the plates. At the same time, trophoblasts (2×10^5 cells, 200 µl medium) were seeded directly into the upper chambers. The plates were incubated for 48 h at 37 °C in a 5% CO₂ incubator, so cells could migrate through the matrigel membrane. After incubation, cells in the upper chambers were removed using cotton-tipped swabs, while other cells which had invaded through the matrigel membrane and reached the outer surface of upper chambers were carefully handled. We fixed cells with 10% formalin for 10 min and then stained them with hematoxylin for 15 min. After rinsing the upper chambers twice with distilled water, results were observed under the Olympus BX51+DP70 microscope (Olympus, Tokyo, Japan). We counted the number of cells that had passed through the membrane and clung to the bottom side of the upper chambers.

Each experiment was performed in triplicate and repeated four times.

Treatment with MAPK/ERK1/2, PI3K, JNK or p38 pathway inhibitor

Trophoblasts were treated with signaling inhibitors for MAPK/ERK1/2 (U0126, 10 µM), PI3K (LY294002, 10 µM), JNK (SP600125, 10 µM) or p38 (SB203580, 10 µM) with or without rhCCL24. All inhibitors were purchased from Cell Signal Technology (Danvers, MA, USA). First, trophoblasts were seeded into plates and treated with rhCCL24. We plated cells 1×10^4 /well into 96-well flat-bottom plates for viability and proliferation assays, and added rhCCL24 to the medium at the concentration of 1 ng/ml. For invasion, ELISA or flow cytometry assay, we seeded 5×10^5 cells/well in 24-well flat-bottom plates and stimulated cells by 10 ng/ml rhCCL24. Forty-eight hours later, the viability, proliferation and invasiveness of trophoblasts were detected as procedures previously described.

At the same time, cell culture supernatant was collected for analyzing the concentration of matrix metalloproteinase-9 (MMP9) and metalloproteinase inhibitor-1 (TIMP1). In the present study, we tested the level of MMP9 and TIMP-1 to investigate roles of CCL24 in trophoblast invasion regulation. Human MMP9 and TIMP-1 ELISA Kit were purchased from Shanghai Excell Biology, Inc (Shanghai, China). The experiments were performed by following the manufacture's instruction. The sensitivity of the two products was 30 pg/ml.

In addition, the intracellular Ki67 and Bcl-2 of trophoblasts were detected by flow cytometry. We treated trophoblasts with ERK and PI3K inhibitors as previously mentioned, and then analyzed the effect of inhibitors on Ki67 and Bcl-2 expression. Trophoblasts were washed with cold PBS, treated with FOXP3 Fix/Perm Buffer Set (Biolegend) by following instruction, stained with Ki67-PE MAB or Bcl-2-FITC MAB (BD) in dark for 30 min, and finally analyzed cells by flow cytometry.

Statistical analysis

All values are expressed as mean $\pm$ s.d. Statistical analysis was performed using *t*-test or one-way ANOVA with Statistical Package for the Social Sciences Software version 11.5. Differences were considered as statistically significant if $P < 0.05$.

Results

Human first-trimester trophoblasts express CCR3 and DSCs promote it expression

To determine the expression and localization of CCR3 at trophoblasts, we performed immunohistochemistry for paraffin-embedded villi. From Fig. 1A, an abundant

Figure 1 Human first-trimester trophoblasts express CCR3 and DSCs promote its expression. (A) Immunohistochemical location of CCR3 in villi. The CCR3 protein was observed at the trophoblast layer as well as the cell column. Original magnification: $\times 400$. (B) Flow cytometry results showed the expression of CCR3 on freshly isolated trophoblasts. We used CK7 as a marker of trophoblasts. The purity of primary cultured trophoblasts was more than 85%, and CCR3 was detected on the surface of trophoblasts. (C and D) DSCs promote the expression of CCR3 on trophoblasts. After trophoblasts were directly co-cultured with DSCs (1×10^5 cells/well) (the ratios of DSCs: trophoblasts were 1:3, 1:2, 1:1 and 2:1) for 48 h, the level of CCR3 on trophoblasts increased significantly. The CK7-negative population represented DSCs, and trophoblasts were CK7-positive. DSCs, decidual stromal cells; Tros, trophoblasts. The blue arrows indicate the cytotrophoblasts, the red arrows indicate the syncytiotrophoblasts, and the black star indicates the trophoblastic cell column. $**P < 0.01$ and $***P < 0.001$; NS, no statistical difference.

expression of CCR3 was observed at the trophoblast layer as well as the cell column which is formed by trophoblasts. Besides, CCR3 protein was detected at both the cytomembrane and cytoplasm of trophoblasts. Further, we primary cultured trophoblasts and investigated the level of CCR3 on trophoblasts. CK7, a marker of epithelial cells, was chosen to identify trophoblasts, because trophoblasts are fetal originated epithelial cells. The purity of freshly isolated trophoblasts reached 85% as we tested it by flow cytometer. Meanwhile, the positive rate of CCR3 on primary cultured CK7⁺ trophoblasts was $(8.79 \pm 1.85)\%$ (Fig. 1B).

At the maternal-fetal interface, the complicated cross-talk between fetal derived trophoblasts and maternal derived DSCs has been illustrated before. Trophoblasts were recognized to directly contact with DSCs (Li *et al.* 2010, 2011). Thus, we speculated that DSCs might influence the biological function of trophoblasts by regulating the expression of CCR3 on trophoblasts. We then co-cultured trophoblasts with DSCs in different proportion (the ratios of DSC:trophoblasts were 1:3, 1:2, 1:1 or 2:1), and detected the level of CCR3 on CK7⁺ trophoblasts. From Fig. 1C and D, even when the ratio of DSCs and trophoblasts were 1:3, DSCs could significantly promote CCR3 expression. Along with the increase of DSCs proportion in the co-culture system, CCR3 level in trophoblasts was gradually increased.

It suggests that DSCs up-regulate CCR3 expression in trophoblasts in a cell number-dependent manner.

E, P and hCG regulate CCL24/CCR3 expression of trophoblasts

During human early pregnancy, sex steroid hormones E, P and hCG are at high concentrations in peripheral blood and uterus. Moreover, these hormones are the most vital factors to control trophoblast growth and invasion during embryo implantation and placentation (Szekeres-Bartho *et al.* 2009, Chen *et al.* 2011, Park & Yang 2011). To study whether E, P and hCG modulate trophoblasts through regulating the expression of CCL24 and CCR3, we treated freshly isolated trophoblasts with these hormones *in vitro*. As shown in Fig. 2A and B, hCG (5 kU/l) significantly increased the level of CCL24 ($P < 0.05$) as well as CCR3 ($P < 0.001$) that expressed by trophoblasts when compared to control group. We also observed a promoting effect of P (10^{-8} M) on CCR3 ($P < 0.01$), but we did not find any notable increase of CCL24. In addition, our data showed that E (10^{-8} M) was able to reduce the secretion of CCL24 by trophoblasts ($P < 0.01$), while there was no effect on CCR3 expression. These results suggest that steroid hormones E, P and hCG regulate the expression of CCL24 and CCR3 of trophoblasts, but in a complex way.

Figure 2 E, P and hCG regulate CCL24/CCR3 expression of trophoblasts. The expression of CCL24 (A) and CCR3 (B) by trophoblasts after they were treated with E, P and hCG. Freshly isolated trophoblasts were treated with E (10^{-8} M), P (10^{-8} M), and hCG (5 kU/l) for 48 h, then the supernatant was tested by ELISA and the cells were harvested for flow cytometry. Ctrl, control; E, 17 β -estradiol; P, progesterone; hCG, human chorionic gonadotropin; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control group.

CCL24 promotes the proliferation, viability and invasiveness of trophoblasts

Based on our finding that trophoblasts secrete chemokine CCL24 and also express receptor CCR3, we further investigated whether CCL24 could impact the biological behaviors of trophoblasts. We performed BrdU proliferation assay, MTT assay and matrigel invasion assay to evaluate the effect of CCL24 on the proliferation ability, viability and invasiveness of trophoblasts respectively. Our results showed that rhCCL24 significantly promoted the proliferation and viability of trophoblasts at the dose of 1 ng/ml (Fig. 3A and B, $P < 0.01$), which is approximately equal to the concentration we detected from the supernatant of trophoblasts (Li *et al.* 2013). Meanwhile, rhCCL24 stimulated the invasiveness of trophoblasts at the concentrations from 1 to 100 ng/ml, but the maximum effect was observed at 10 ng/ml ($P < 0.001$, Fig. 3C and D). In contrast, either a-CCL24 or a-CCR3 was able to eliminate the stimulatory effect of rhCCL24 on trophoblasts. Overall, these data suggest that CCL24 is an

important promoter for the growth and invasiveness of trophoblasts in human early pregnancy.

CCL24 enhances the proliferation and viability of trophoblasts through MAPK/ERK1/2 and PI3K signaling pathways

To further study the potential signaling pathways downstream of CCL24/CCR3 axis, we treated trophoblasts with signaling inhibitors for MAPK/ERK1/2, PI3K, JNK or p38 pathways, with or without rhCCL24. Then we repeated BrdU proliferation assay and MTT assay to evaluate the proliferation and viability of trophoblasts, and test the expression of proliferation-associated and apoptosis-associated molecules in cells. As shown, we observed that rhCCL24 increased the proliferation of trophoblasts in the control groups (Fig. 4A, $P < 0.01$), which confirmed our previous results (Fig. 3A). In addition, inhibitors for ERK1/2 and PI3K significantly inhibited the proliferation of trophoblasts, when compared with the control group that have not been stimulated by rhCCL24 (Fig. 4A, $P < 0.01$). Meanwhile, in the presence of rhCCL24, the two inhibitors also decreased the proliferation of trophoblasts (Fig. 4A, $P < 0.001$). However, the same inhibitory effect was not observed in JNK or p38 inhibitor treatment groups, even though rhCCL24 increased the proliferation ability of trophoblasts in both groups (Fig. 4A).

Since we have figured out that MAPK/ERK1/2 and PI3K pathways are downstream signalings of CCL24/CCR3 in trophoblasts, the inhibitors for ERK1/2 and PI3K were again added to analyze their role in regulating the viability of trophoblasts. From the results, treatment with rhCCL24 undoubtedly increased the viability of trophoblasts in the control groups (Fig. 4B, $P < 0.05$). The inhibitor for PI3K, but not for ERK1/2, decreased the viability of cells (Fig. 4B, $P < 0.001$). Moreover, the two inhibitors could decrease the effect of rhCCL24 when compared with the control group, but a greater role was observed in the PI3K inhibitor group (Fig. 4B).

We further analyzed which molecules are mediated by the MAPK/ERK1/2 pathway and PI3K pathway in trophoblast proliferation. Protein Ki67 is a cellular marker for proliferation, while Bcl-2 family contains anti-apoptotic proteins (including Bcl-2, Bcl-xL, and Bcl-w) as well as pro-apoptotic proteins (including Bax, Bad, Bak and Bok) (Cory & Adams 2002). They are associated with or may be necessary for cell growth and survival (Ali *et al.* 2012). Therefore, we measured the level of intracellular Bcl-2 and Ki67 proteins after treatment trophoblasts with the two inhibitors. Interestingly, we found that about 80% of trophoblasts were Ki67 positive (Fig. 4C and D), but the level of Bcl-2 in trophoblasts was hardly detected (Fig. 4E and F). In addition, rhCCL24 could increase the expression of Ki67 (Fig. 4C and D, $P < 0.001$), but no significant difference was observed for Bcl-2 protein (Fig. 4E and F). After treatment with inhibitors for ERK and PI3K, the level of intracellular Ki67 of trophoblasts

Figure 3 CCL24 promotes the proliferation, viability and invasiveness of trophoblasts. Trophoblasts were treated with recombinant human CCL24 (rhCCL24; 0.1, 1, 10 or 100 ng/ml), anti-CCL24 neutralizing antibody (a-CCL24; 0.08, 0.4, 2 or 10 µg/ml), or anti-CCR3 neutralizing antibody (a-CCR3; 0.32, 1.6, 8 or 40 µg/ml) for 48 h. (A) BrdU cell proliferation assay was performed to examine the proliferation of trophoblasts. We set the OD value of control group as 1, and calculated the proliferation index of treatment groups. (B) MTT assay was used to detect the viability of trophoblasts. The result was shown as viability index. (C and D) Matrigel invasion assay was performed to examine the invasiveness of trophoblasts. (C) Representative pictures show the trophoblasts which invaded through the matrigel coated transwells; original magnification: $\times 400$. (D) The relative numbers of trophoblasts which have invaded through transwells. We set the total number of control group as 1, and calculated the invasion index. Ctrl, control; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control group.

decreased significantly (Fig. 4C and D, $P < 0.05$). Meanwhile, the two inhibitors also prohibited the promoting effect of rhCCL24 (Fig. 4C and D, $P < 0.05$). However, we did not see any apparent regulation to Bcl-2 by the two inhibitors (Fig. 4E and F).

Due to the extremely low expression of Bcl-2 in trophoblasts, we then tested two other Bcl-2 family members: anti-apoptotic protein Bcl-xL and pro-apoptotic protein Bax. The expressions of Bcl-xL and Bax in trophoblasts were much higher than Bcl-2, with the positive rates about 40 and 20% respectively (Supplementary Figure 1, see section on supplementary data given at the end of this article). When we treated trophoblasts with the inhibitor for ERK1/2 pathway, we observed up-regulating expressions of Bax and Bcl-xL. However, we did not observe any regulation by rhCCL24 to Bcl-xL and Bax (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Collectively, CCL24 could up-regulate the proliferation and viability of trophoblasts through MAPK/ERK1/2 and PI3K signaling pathways. Among this process, Ki67, but not Bcl-2, may be an important participator.

CCL24 promotes MMP9 production and enhances the invasiveness of trophoblasts through PI3K signaling pathway

Given that CCL24/CCR3 regulate the proliferation and viability of trophoblasts through MAPK/ERK1/2 and PI3K pathways, we investigated whether they play roles in regulating the invasiveness of trophoblasts. MMP family is involved in the breakdown of extracellular matrix, yet the TIMP family are natural inhibitors of the MMP members (Yamamoto *et al.* 2015). Both of them are important participators in the process of cell invasion. Similarly, we treated trophoblasts with the inhibitors for ERK1/2 and PI3K, with or without the presence of rhCCL24, and then repeated matrigel invasion assay. RhCCL24 promoted the invasiveness of trophoblasts (Fig. 5A, $P < 0.01$), which was consistent with our previous results. The inhibitor for PI3K was able to inhibit trophoblast invasion (Fig. 5A, $P < 0.001$) even when compared in the rhCCL24 treatment groups (Fig. 5A, $P < 0.001$). However, there was no notable regulation performed by PI3K inhibitor.

Figure 4 CCL24 enhances the proliferation and viability of trophoblasts through MAPK/ERK1/2 and PI3K signaling pathways. (A) CCL24 promotes the proliferation of trophoblasts through MAPK/ERK1/2 and PI3K pathways. The inhibitors for MAPK/ERK1/2 (10 μ M), PI3K (10 μ M), JNK (10 μ M) or p38 (10 μ M) pathways, with or without rhCCL24 (1 ng/ml), were added to trophoblasts for 48 h. We repeated the BrdU cell proliferation assay and calculated the proliferation index. (B) The PI3K inhibitor reduces the viability of trophoblasts. Trophoblasts were treated with inhibitors for MAPK/ERK1/2 or PI3K for 48 h, and MTT assay was performed to evaluate the viability of cells. After the same treatment, the intracellular level of Ki67 (C and D) and Bcl-2 (E and F) in trophoblasts were tested by flow cytometry. ERKi, inhibitor for MAPK/ERK1/2 (U0126); PI3Ki, inhibitor for MAPK/ERK1/2 (LY294002). Data were shown as mean $\pm$ s.d. NS, no significant difference. * P <0.05, ** P <0.01 and *** P <0.001 compared to the control group; # P <0.05 and ## P <0.01 compared in the same inhibitor treatment groups, but with or without rhCCL24; Δ P <0.05, $\Delta\Delta$ P <0.01 and $\Delta\Delta\Delta$ P <0.001 compared to the rhCCL24 control group.

Figure 5 CCL24 promotes MMP9 production and enhances the invasiveness of trophoblasts through PI3K signaling pathway. (A) The PI3K inhibitor, but not MAPK/ERK1/2 inhibitor, reduces the invasiveness of trophoblasts. Trophoblasts were treated with the inhibitors for MAPK/ERK1/2 (10 μ M) and PI3K (10 μ M), with or without rhCCL24 (10 ng/ml). Matrigel invasion assay was repeated. The result was presented by invasion index. Cell culture supernatant from inhibitors treatment trophoblasts were collected, and tested for the concentration of MMP9 and TIMP1 by ELISA. (B) and (C) show the concentration of MMP9 and TIMP1 in the supernatant respectively. Data are shown as mean $\pm$ s.d. NS, no significant difference. * P <0.05, ** P <0.01 and *** P <0.001 compared to control group; # P <0.05 compared in the same inhibitor treatment groups, but with or without rhCCL24; $\Delta\Delta$ P <0.01 and $\Delta\Delta\Delta$ P <0.001 compared to the rhCCL24 control group.

TIMP-1 and MMP9 are well-known for their role in mediating the breakdown of extracellular matrix, and they are important regulators for cell invasion. Thus, we collected the supernatant of inhibitors-treated trophoblasts, and measured the concentration of TIMP-1 and MMP9 by ELISA. Our results showed that trophoblasts

secreted high level of TIMP-1 and MMP9, the concentrations reached at (13530.29 ± 807.50) pg/ml and (1011.46 ± 82.77) pg/ml respectively (Fig. 5C and B). RhCCL24 significantly increased the level of soluble MMP9 in the supernatant (Fig. 5B, P <0.05). Meanwhile, inhibitors for both ERK1/2 and PI3K were able to reduce the secretion of MMP9 by trophoblasts (Fig. 5B, P <0.001), and they also decreased the stimulatory effect of rhCCL24. However, rhCCL24 and two inhibitors did not influence TIMP-1 level (Fig. 5C, P >0.05).

Discussion

During the first trimester of human pregnancy, successful embryo implantation and placentation are essential requirements for the establishment of normal gestation. Trophoblasts, as the main component of chorionic villi, play a leading role in the process. Therefore, the behaviors of trophoblasts, such as the proliferation, viability and invasion are vitally important (Chen *et al.* 2012). Even though a lot of researches have been done to study the biological changes of trophoblasts in embryo implantation, the mechanism has not been fully illustrated yet. In the present work, we demonstrated that trophoblast-derived CCL24 could regulate the proliferation ability, viability and invasiveness of trophoblasts through MAPK/ERK1/2 and PI3K signaling pathways. At the same time, the local factors of maternal-fetal interface like steroid hormone E, P, and hCG as well as DSCs influence the expression of CCL24/CCR3 by trophoblasts, which may finally regulate the biological behaviors of trophoblasts and embryo implantation.

Chemokines are a group of chemotactic cytokines, which usually act to induce the migration and activation of leukocytes in immune responses (Vinader & Afarinkia 2012). However, the human early pregnant uterus is also a chemokine abundant environment. Trophoblasts, DSCs, DICs and decidual epithelial cells are the sources of various chemokines (Li *et al.* 2011, Erlebacher 2013). Among these, trophoblasts are recognized as the most important origination of chemokines; for instance, chemokine CXCL6, CXCL8, CXCL12, CCL14, and CCL4 (Jovanovic *et al.* 2010, Li *et al.* 2011, Zhang *et al.* 2013). We had reported that trophoblasts secrete chemokine CCL24 (Li *et al.* 2013). In the present study, we further verified the existence of receptor CCR3 on the surface of trophoblasts, which is in agreement with one previous report that demonstrated the expression of CCR3 on trophoblast cell line HTR8/SVneo (Chau *et al.* 2013). These observations gave a clue that trophoblasts probably exert self-regulation in the local uterus, while the CCL24/CCR3 axis maybe involved in this process.

In the process of embryo implantation, once trophoblasts attach and penetrate into decidua, they emerge in an entirely specific environment. Fetal-derived trophoblasts form an extensive crosstalk with maternal-derived cells such as DSCs and DICs, and they are also exposed

to high concentration of steroid hormones E, P and hCG (Szekeres-Bartho *et al.* 2009, Chen *et al.* 2011, Park & Yang 2011). DSCs do not secrete CCL24, but they induce trophoblasts to secrete a higher level of CCL24 (Li *et al.* 2013). In this study, we found that DSCs also up-regulated CCR3 expression on trophoblasts. Thus, DSCs may facilitate the function of CCL24/CCR3 axis in trophoblasts by increasing the expression of both chemokine and receptor. However, the molecular mechanism needs to be further explored. Trophoblast can also influence the maternal immune system during pregnancy by expressing soluble and cell surface molecules, such as HLA-G (Rizzo *et al.* 2007, Carosella *et al.* 2008, Oreshkova *et al.* 2012) and IDO (Munn *et al.* 1998). These molecules limit the proliferation and activation of T, APC (antigen presenting cell) and NK cells in decidua. CCL24 secreted by trophoblasts may also involved in immune regulation at the maternal-fetal interface by dialogue with CCR3⁺DIC, which needs to be further studied.

Steroid hormones E, P, hCG are the key regulating factors in pregnancy (Cha *et al.* 2012). Both E and hCG are synthesized by trophoblasts, and hCG can maintain the production of P by the corpus luteum (Chen *et al.* 2012). P and hCG are classically known for their stimulatory roles in trophoblasts invasion and functional differentiation (Halasz & Szekeres-Bartho 2013). However, the role of E in regulating trophoblasts remains unclear. The receptor of estrogen ER α was found in differentiating cytotrophoblast cells of term placenta (Bukovsky *et al.* 2003). Nevertheless, it was proposed that E might not have significant impact on trophoblasts based on the fact that E deficiency usually did not result in fetal abnormalities (Chen *et al.* 2012). Chen *et al.* (2011) found that hCG stimulated the migration of cells but did not affect cell number, high dose of P inhibited the migration of trophoblast cell line HTR8/SVneo as well as the number of cells, but E had no effect. However, our results showed that hCG and P increased the expression of CCL24 or CCR3 by trophoblasts, while E had an opposite function.

One cause of the difference may be that cell line HTR8/SVneo cannot display the real features of normal trophoblasts. It is also possible that E, P and hCG influence trophoblasts through other ways. All of this evidence indicates that steroid hormones could directly regulate the expression of CCL24 and CCR3 by trophoblasts. On the other hand, the receptors of E, P, and hCG exist in most uterine tissue compartments. They regulate the function of endometrial stromal cells (ESCs) and DSCs soon after decidualization occurs (Cha *et al.* 2012, Chen *et al.* 2012). In pregnant uterus, trophoblasts, DSCs, and steroid hormones form a very complicated dialogue interface. We have reported that these steroid hormones could promote the growth of DSCs through up-regulating CCR3 expression on DSCs (Li *et al.* 2013). DSC is also an important stimulator for CCL24 and CCR3

expression in trophoblasts. Therefore, steroid hormones might also up-regulate CCL24/CCR3 expression of trophoblasts by strengthening dialogue between DSC and trophoblast.

Chemokines regulate the main events that occurred at the maternal-fetal interface, such as the endometrium decidualization, vessel remodeling and immune tolerance (Kuang *et al.* 2009, Godbole *et al.* 2011). Moreover, chemokines were also proven to modulate the biological functions of trophoblasts, and finally regulate the process of embryo implantation. CXCL12 (stromal cell-derived factor-1, SDF-1) has an anti-apoptotic effect on human trophoblasts (Jaleel *et al.* 2004), and CXCL16 is able to induce the proliferation and invasion of first-trimester human trophoblasts in an autocrine manner (Huang *et al.* 2006). Similarly, the present work showed that CCL24 promotes the proliferation ability, viability and invasiveness of trophoblasts, which implied a beneficial role of CCL24/CCR3 in embryo implantation.

In eosinophils, the function of CCL24/CCR3 axis can be exerted by activating MAPK/ERK1/2, PI3K, and p38 signaling pathways (Shamri *et al.* 2013). Meanwhile, these signaling are universally associated with regulating cell's growth, survival and invasiveness. For instance, CCL2 enhances the survival and invasiveness of endometrial stromal cells by activating MAPK/ERK1/2 and PI3K pathways (Li *et al.* 2012); IL-25 promotes the proliferation of DSCs by activating Akt and JNK pathways (Wang *et al.* 2014); JNK and p38 pathways were also proved to inhibit the growth and induce the apoptosis of human colorectal carcinoma cells (Zhang *et al.* 2014). Therefore, in the present study, the role of MAPK/ERK1/2, PI3K, JNK and p38 signaling pathways were analyzed when trophoblasts were treated with CCL24. Our data suggest that when the proliferation, viability and invasiveness of trophoblasts are induced by CCL24/CCR3 axis, the MAPK/ERK1/2 and PI3K pathways, but not JNK and p38 pathways, are activated, which is consistent with the results of most previous work.

A relevant question was which molecules are involved under the MAPK/ERK1/2 and PI3K pathways when CCL24 promotes the proliferation, viability and invasiveness of trophoblasts. In cell cycle, Ki67 is present during all active phases but absent in resting phase, so the expression of Ki67 protein in human cells is strictly related to cell proliferation (Ali *et al.* 2012). Bcl-2 inhibits apoptosis of cells but, paradoxically, it also suppresses proliferation (Zinkel *et al.* 2006). Our results in the current study revealed a high expression of Ki67 in trophoblasts, and MAPK/ERK1/2 and PI3K pathways could increase its expression. However, no significant regulation of Bcl-2 was observed. Thus, CCL24 regulates the proliferation of trophoblasts might be Ki67-mediated, but not Bcl-2. In addition, we investigated the possible molecules under the MAPK/ERK1/2 and PI3K pathways that might induce the invasion of

Figure 6 A schematic model of CCL24 promotes the proliferation, viability and invasiveness of trophoblasts. Trophoblasts co-express chemokine CCL24 and receptor CCR3 at the maternal-fetal interface. Trophoblasts-derived CCL24 promotes the expression of Ki67 and MMP9 via MAPK/ERK1/2 or PI3K signaling pathways, and consequently induces the proliferation, viability and invasiveness of trophoblasts. The specific environment in human early pregnant uterus: direct contact with DSCs as well as high concentration of P and hCG regulate the expression of CCL24/CCR3 on trophoblasts, and might amplify trophoblast's self-regulation. Thus, trophoblasts might mediate their own biological functions through CCL24/CCR3 axis, and finally participate in controlling embryo implantation and placentation. DSCs, decidual stromal cells; Tros, trophoblasts; E, 17 β -estradiol; P, progesterone; hCG, human chorionic gonadotropin.

trophoblasts. MMP9 is an enzyme that could degrade the extracellular matrix in the process of cell migration and invasion, while TIMP1 is a natural inhibitor of it (Yamamoto *et al.* 2015). MMP9 was previously found as a downstream molecule of chemokines, and acted to increase the invasiveness of trophoblasts (Chen *et al.* 2011, Zhang *et al.* 2013). Consistent with these reports, the present work showed that MMP9 is regulated by PI3K signaling pathway when CCL24 promotes the invasion of trophoblasts. However, even though we found the MAPK/ERK1/2 pathway could influence MMP9, we did not observe its effect on regulating the invasiveness of trophoblasts. Therefore, other factors may inhibit the function of MAPK/ERK1/2 signal. Although trophoblasts are rich in TIMP1, we did not find out any pathways that mediate its expression in this study. Actually, the extent of trophoblast invasion is accurately regulated in normal pregnancy. Uncontrolled invasion is related to choriocarcinoma, while invasiveness deficiency could result in abortion, preeclampsia, FGR and so on (Kwak-Kim *et al.* 2010, Kinoshita *et al.* 2012). In our current study, CCL24/CCR3 signal regulates MMP9 level, but does not influence TIMP1 level of trophoblasts. However, other factors at the maternal-fetal interface, such as CD82 (Li *et al.* 2010), can control trophoblasts invasion

through up-regulating TIMP1 expression. The balance of MMP9/TIMP1 constructed by maternal-fetal micro-environment plays an important role in maintaining a status of moderate invasion. However, the mechanism also needs to be further researched.

Collectively, as shown in Fig. 6, the present study for the first time demonstrates chemokine CCL24 as an important mediator in the self-regulating process of trophoblasts. Trophoblasts-derived CCL24 promotes the expression of Ki67 and MMP9 via MAPK/ERK1/2 and or PI3K signaling pathways, and consequently induces the proliferation, viability and invasiveness of trophoblasts. The specific environment in human early pregnant uterus: direct contact with DSCs as well as high concentration of P and hCG regulate the expression of CCL24/CCR3 on trophoblasts, and might amplify trophoblast's self-regulation. These findings contribute to our understanding about the complex process of embryo implantation, and may provide a clue for searching effective treatment to pregnancy complications.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-15-0119>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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