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CXCL14 Inhibits Trophoblast Outgrowth Via a Paracrine/Autocrine Manner During Early Pregnancy in Mice

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CXCL14, a member of chemokine family, was previously known to participate in many pathophysiological events, such as leukocytes recruitment and tumor suppression. However, it remained largely unknown whether CXCL14 is a physiological player during early pregnancy. In this regard, our recent global gene microarray analysis has observed an implantation-specific expression profile of CXCL14 mRNA during early pregnancy in mice, showing its higher levels at implantation sites compared to inter-implantation sites, implicating a potential role of CXCL14 in the periimplantation events. In the present investigation, using Northern blot, in situ hybridization and immunostaining, we further demonstrated that uterine CXCL14 expression was specifically induced at embryo implantation site and expanded with subsequent decidualization process in a spatiotemporal manner. The implanting embryo also showed a highlighted expression of CXCL14 in the blastocyst trophectoderm and its derived ectoplacental cones (EPCs) during postimplantation development. In vitro functional study revealed that CXCL14 could significantly inhibit both primary and secondary trophoblast attachment and outgrowth, correlated with a stage-dependant downregulation of MMP-2 and/or MMP-9 activity. Moreover, it was found that biotinylated CXCL14 could specifically bind to trophoblast cells in vitro and in vivo, suggesting trophoblast cell, perhaps expressing the unidentified CXCL14 receptor, is a bioactive target of CXCL14. Collectively, our findings provide evidences supporting the contention that CXCL14 is an important paracrine/autocrine modulator regulating trophoblast outgrowth at the maternal–fetal interface during the process of pregnancy establishment. This study is clinically related since CXCL14 is also highly expressed in human receptive endometrium and trophoblasts.

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The initiation of implantation requires synchronized development of the embryo to the blastocyst stage, and the well-differentiated endometrium into a receptive state. As soon as the blastocyst attachment reaction happens, the primary trophoblast begin to invade through the epithelial layer of uterine wall, followed by decidualization of uterine stroma and further sophisticated interactions between invading trophoblast and decidualizing cells towards successful placentation (Wang and Dey, 2006). This well-controlled invading processes between trophoblast and uterine cells are subtly balanced by groups of positive and negative regulators locally produced at the maternal–fetal interface. Among these numerous regulators, the chemokine family, which is previously well known by their role in leukocyte chemotaxis, has been recently found to play important roles during early pregnancy, such as governing the migration of trophoblast (Salamonsen et al., 2007).

Recent global gene expression studies in human menstrual cycle have shown that among numerous chemokine members, the *CXCL14*, also known as *BRAK* (breast and kidney expressed chemokine), has a surge up-regulation in the mid-secretory phase of endometrium, which coincides the predicted time of embryo implantation (Talbi et al., 2006). Similarly, our own microarray data (Mouse 430 2.0, Affymetrix, Santa Clara, CA) on day 5 of mouse pregnancy also revealed that the *CXCL14* was significantly up-regulated in the implantation sites compared to the inter-implantation sites. The similar expressional finding in both human and mice species, to our knowledge, have suggested an important role of CXCL14 during the window of embryo implantation, and therefore become the interest of current investigation.

CXCL14 belongs to a large family of the chemokines, which were a group of structurally related molecules that regulate activation and trafficking of leukocytes via members of the seven-transmembrane G protein-coupled receptors (Fernandez and Lolis, 2002). CXCL14 was first found in human normal breast and kidney tissues, and further found to express

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in many other normal tissues, and also in some kinds of malignant tissues (Hromas et al., 1999; Frederick et al., 2000; Augsten et al., 2009; Pelicano et al., 2009). As a unique member of chemokine family, CXCL14 is currently suggested as a chemoattractant for dendritic cells (DC), activated monocytes, neutrophils, and natural killer cells (NK) under different pathophysiological conditions (Kurth et al., 2001; Shellenberger et al., 2004; Shurin et al., 2005; Starnes et al., 2006; Salogni et al., 2009). It has been also reported that CXCL14 was a potent mediators of neoangiogenesis and tumor growth, invasion, metastasis (Schwarze et al., 2005; Ozawa et al., 2006; Augsten et al., 2009), which shares many similarities with the process of trophoblast invasion during early pregnancy (Fitzgerald et al., 2008). To date, the specific receptor of CXCL14 has not yet been found. Recently, targeted deletion of *CXCL14* in mice has resulted in compromised fertility (Meuter et al., 2007 and personal communication with authors). As the homozygous pairs were reported to produce reduced litter size and some knockout females did not produce newborns. However, the underling mechanisms of this phenomenon are still unexplored.

The observed reproductive phenotype in CXCL14 null mice coupled with its potential role in cell migration has encouraged us to hypothesize that CXCL14 might play important roles in trophoblast function during early pregnancy, since a lot of pregnant failures were associated with abnormal trophoblast invasion at periimplantation stage (Wilcox et al., 1999; Song et al., 2002). In this investigation, we showed a site-strict and cell-specific expression of *CXCL14* within the implantation sites at both maternal and embryonic compartment. Further functional study revealed CXCL14 as an inhibitory factor against trophoblast outgrowth through downregulating MMP-2 and 9. Also, binding assays of CXCL14 were performed at the maternal–fetal interface to predict the potential site of unidentified CXCL14 receptor.

Materials and Methods

Animals and treatments

Kunming white strain mice (Experimental Animal Center, Institute of Genetics and Development, Chinese Academy of Sciences) were maintained in the animal facility of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. The Guidelines for the Care and Use of Animals in Research were followed. Mice were allowed free access to water and food with a constant photoperiod (12L:12D). Adult female mice (25–30 g, 7–8 weeks old) in estrus were mated with fertile males of the same strain at room temperature (25°C). The following morning of finding a vaginal plug was designated as day 1 of pregnancy.

DNA microarray analyses

Implantation (IS) and inter-implantation (IIS) sites were monitored by an intravenous injection of a blue dye and divided by sharp dissection at 0800–0900 h a.m. on day 5 (n = 10 mice). Uterine tissues were flash frozen and stored at −80°C. Total RNA was extracted with Trizol reagent (Invitrogen, Eugene, OR). cDNA and Biotinylated cRNA were prepared according to Affymetrix protocols. Samples were hybridized overnight to high-density Mouse Genome 430 2.0 array (Affymetrix), at the Shanghai Hujing Biotech Co., Ltd (Shanghai, China). Then the chips were scanned using a Scanner3000, and the data were extracted using the Affymetrix GeneChip Operating Software version 1.2 (Affymetrix). Each sample was hybridized on two chips for reducing the false positive rate.

Northern blot analyses

Total uterine RNAs were extracted with Trizol reagent (Invitrogen), and were quantified by absorbance at 260 nm as well as by ethidium bromide staining after electrophoresis through agarose gels. Samples of total RNA (20 μg) were then blotted overnight to Hybond-N membrane by electrical transfer. The cDNA probe (bases 442–1058 of Mus musculus CXCL14: GenBank Accession No. NM_019568) was labeled with ^{32}P and hybridization was carried out overnight at 60°C. The blot was subjected to autoradiography at −70°C using BioMax MS film (Amersham, UK). To determine the relative amounts of RNA transferred to the membrane, blots were stripped and hybridized with a ^{32}P-labeled 18S RNA probe.

RNA extraction and RT-PCR

Total RNA was extracted using Trizol Reagents and reverse transcribed in 25 μl of reaction mixture containing 30 U avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The PCR was conducted in a total volume of 50 μl for 30 cycles of denaturation at 94°C of 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 45 sec, with a final extension step of 10 min at 72°C. The primers used in this study include CXCL14 (5-GGG TCC AAG TGT AAG TGT TC-3; 5-GTA GTG CTG TGA ACG GTC TC-3) and 18S (5-AAT CAG GGT TCG ATT CCG GA-3; 5-CCA AGA TCC AAC TAC GAG CT-3). The amplified products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide. Images of the RT-PCR agarose gels were acquired with a High Performance CCD camera and quantification of the bands was performed by ChemiDocXRS (BioRAD, Hercules, CA). Band intensity of the genes determined was compared with the band intensity of 18S as an internal control, and the relative level was acquired.

Indirect immunofluorescence

Preimplantation embryos at different developmental stages were flushed from the oviduct or uterus on days 2–4 of pregnancy. All embryos were then fixed in 4% paraformaldehyde (30 min, room temperature). After fixation, embryos were washed three times in PBS and permeabilized in PBS containing 0.2% TritonX-100 (12 min, room temperature). After rinsing three times in PBS, embryos were incubated in 5% BSA for 30 min at room temperature to block nonspecific binding of the antibodies. Then, embryos were incubated with anti-CXCL14mAb (R&D Systems, Minneapolis, MN) at 4°C overnight followed by secondary antibody (fluorescein isothiocyanate-labeled anti-rat IgG) for 1 h at 37°C. Nuclei were stained with 5 μg/ml of propidium iodide (Sigma, St. Louis, MO) for 10 min. Embryos were viewed under a laser-scanning confocal microscope (Leica, Heidelberg, Germany). Rat preimmune IgG was used as a negative control.

In situ hybridization

In situ sense and antisense probe templates were prepared by amplifying a 351bp fragment of CXCL14 cDNA (GenBank accession no: NM_019568, nucleotides 334–684), using sense and antisense primers modified with either T7 or SP6 sequences. The resulting templates were then transcribed with the digoxigenin-labeling kit (Roche Molecular Biomedicals, Mannheim, Germany) according to the manufacturer's protocol. After deparaffinization and proteinase K treatment, the tissue sections were prehybridized and then incubated in the hybridization buffer containing 1 μg/ml of digoxigenin-labeled sense or antisense BRAK cRNA probe overnight. After serial washing and RNase treatment, samples were blocked and incubated with alkaline phosphatase-conjugated antidigoxigenin antibody (Roche Molecular Biochemicals; 1:2,000 dilution) in the blocking buffer at room temperature for 1 h. After intense washing, signal was visualized through the use of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega) until the color developed to the desired extent.

Blastocyst attachment and outgrowth assays in vitro

Blastocyst attachment and outgrowth assays were performed as described previously (Qin et al., 2005). In brief, blastocysts were obtained by flushing the uterine horns with Ham's F-12 medium on day 4 morning of pregnancy and transferred in 96-well plates precoated with fibronectin (10 μg/ml; Sigma), containing Ham's F-12 (supplemented with 0.4% BSA) plus 0, 1, 10, 50, 100, 200, 1,000 ng/ml of recombinant mouse CXCL14 (rmCXCL14) protein (R&D Systems). It was determined that the recombinant CXCL14 at the concentration of 100 ng/ml has an optimal effect for blastocyst attachment and outgrowth at 48 h after initiation of culture.

The blastocysts were then divided into four treatment groups: the medium only (control group, n = 40, four wells), the medium plus 100 ng/ml of rmCXCL14 (rmCXCL14 group, n = 40, four wells), the medium plus 30 μg/ml of rat anti-mouse CXCL14 IgG (anti-CXCL14 group, n = 40, four wells), and medium plus 30 μg/ml rat IgG (IgG group, n = 40, four wells). When primary giant trophoblast cells were visible around the attachment site of the attached blastocysts, we designated the blastocysts as outgrowth. Blastocyst attachment was examined at 48 h of culture. Blastocyst attachment was only examined once, because gentle pipetting was required to determine whether the embryo would detach from the bottom of plates. The ratios of blastocysts with attachment and outgrowth relative to the total number of embryos were calculated.

Outgrowth assays of EPCs

Ectoplacental cones (EPCs) from the mice on day 8 of pregnancy were dissected out under sterile conditions and transferred to 4-well (30 EPCs per well) plates precoated with Matrigel (8 mg/ml; BD). The EPCs were cultured in Ham's F-12 (supplemented with 0.4% BSA). Recombinant mouse CXCL14 (100 ng/ml), rat anti-mouse CXCL14 IgG (30 μg/ml), or normal rat IgG was supplemented to freshly isolated EPCs, respectively. Outgrowth assays in vitro were performed according to the methods established by our group (Dai et al., 2003; Liu et al., 2008). When secondary trophoblast giant cells (sTGCs) were visible around the attachment site of the attached blastocysts, we designated the blastocysts as outgrowth. The percentage of EPCs outgrowth was evaluated with outgrown EPCs to the number of total EPCs. The outgrowth area, which is occupied by sTGC, was recorded by inverted microscopy and measured with NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Detection of CXCL14 binding sites

Recombinant CXCL14 protein was biotinylated by EZ-link biotinylation reagent (Pierce). Proteins were incubated with 1 ml of 0.3 mg/ml EZ-link biotinylation reagent in PBS on ice for 2 h. After incubation, samples were dialyzed to remove the free biotin and measured their protein concentrations. Trophoblast giant cells from EPCs and frozen tissues sectioned were fixed in 4% paraformaldehyde (30 min, room temperature). Sample were blocked with Block Ace at room temperature for 1 h and then incubated with biotinylated protein (100 μg/ml) at room temperature for 1 h. After incubation, the samples were incubated with avidin-FITC (1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at room temperature for 1 h. Nuclei were counterstained with 5 μg/ml of propidium iodide (Sigma) for 10 min. Samples were viewed under a fluorescence inverted microscope (Nikon, Tokyo, Japan). Nonlabeled CXCL14 and biotin were used as negative controls.

Gelatin zymography

The conditioned medium of blastocysts and EPCs culture was collected at 24 and 48 h. Protein content of conditioned media was measured according to the method of Bradford, and 250 ng protein mixed with 4× sample buffer (8% SDS (w/v), 0.04% Bromophenol Blue (w/v), 40% glycerol (v/v), and 0.25M Tris) and then subjected to electrophoresis in a 10% polyacrylamine gel containing 0.5 mg/ml gelatin (Sigma). The gel was washed in 2.5% Triton X-100 and 50 mM Tris/HCl, at pH 7.5 for 1 h to remove the SDS and incubated for 16–18 h in calcium assay buffer (50 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.5) at 37°C. After staining with 0.2% Coomassie Brilliant Blue R250 in 50% methanol, and 10% acetic acid, the gel was destained with 10% acetic acid. The lytic bands were quantified by computer-aided densitometry. Each experiment was repeated three times.

Statistical analysis

All experimental treatments were carried out in triplicate and each experiment was repeated at least three times. Values were presented as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by LSD's post hoc test, and a value of $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS 11.5.

Results

CXCL14 expression is higher at implantation site with the onset of embryo implantation and ongoing decidualization

Our whole-genome microarray study comparing mRNAs from mouse implantation sites and inter-implantation sites on day 5 of pregnancy has shown that the expression intensity of CXCL14 mRNA in implantation sites was 4.1-fold higher compared with inter-implantation sites (Fig. 1A). This result was further confirmed by Northern blot analysis as showed in Figure 1B. The changing profiles of *CXCL14* mRNA during periimplantation period were next performed by RT-PCR as showed in Figure 1C,D. From D1 to D4 of pregnancy, the *CXCL14* expression was generally low or undetectable, the slightly increased expression observed on D1 and D4 may reflect an estrogen regulation of this gene (proestrous estrogen on D1 and preimplantation estrogen surge from corpus luteum on D4), as demonstrated in Supplementary Figure 1. In contrast with D1–D4, the expression level of *CXCL14* dramatically increased with the initiation of implantation from D5 onward, but this increased expression were only restricted within the implantation sites, while the level of inter-implantation sites remained low or undetectable (Fig. 1C,D).

CXCL14 spatiotemporally localized on both mouse uterus and embryo in a cell-specific manner during periimplantation

The dynamic changing level of CXCL14 has next led us checking the site-specific localization of this gene during mouse periimplantation. By using in situ hybridization, here we found that from D1 to D4 of pregnancy, *CXCL14* were located in the epithelial (both luminal and glandular) and subepithelial region with low or undetectable level in accordance with RT-PCR results (Fig. 2A–C). While on day 5, the expression of CXCL14 significantly increased at the primary decidualization zone (PDZ) surrounding the site of embryo implantation. This expression pattern in the decidua, however, ceased to grow with the expanding of decidualization, and began to decrease from D6 onward, then disappeared from the PDZ on D7 and D8 while remained in outer region of PDZ, which is termed as secondary decidualization zone (SDZ; Fig. 2D–G). During D7 and D8, an increasing level of *CXCL14* was also observed at the mesometrial part of decidualizing stroma as shown in Figure 2F,G, a site considered for future placenta formation.

Notablely, through D5–D8 of pregnancy (Fig. 2D–G), the growing embryo also showed an evidenced expression of CXCL14, with particularly strong signal in the region of EPCs on D7 and D8 (Fig. 2F,G), which is a major source of trophoblast

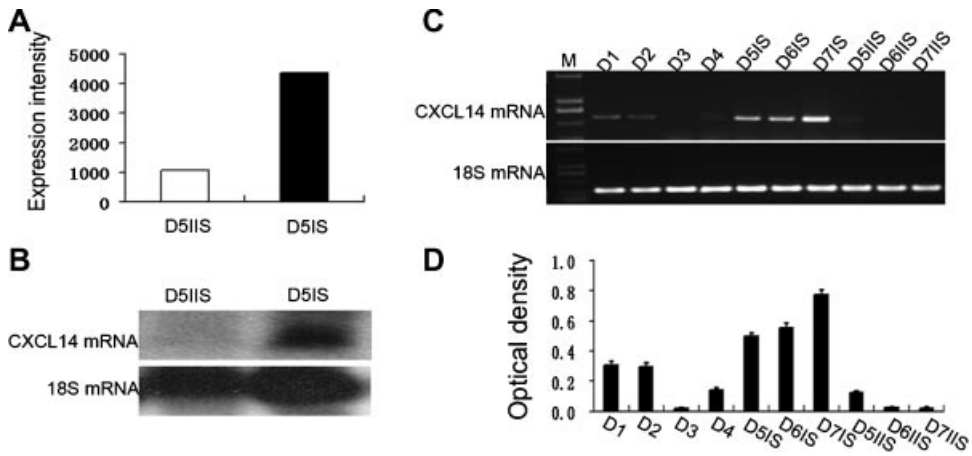

Fig. 1. Levels of *CXCL14* mRNA spatiotemporally expressed in periimplantation mouse uteri. A: Relative expression intensity of *CXCL14* mRNA was analyzed by DNA microarray comparing implantation site (IS) and inter-implantation site (IIS) of day 5 pregnancy mouse uterus (n = 10). Values for expression intensity were derived from integration of hybridization signals. B: Northern blot confirmation of DNA microarray results. Autoradiography of a membrane probed sequentially with a ^{32}P-labeled cDNA probe for *CXCL14* and 18S rRNA. C: RT-PCR analysis of *CXCL14* mRNA in mouse uteri (n = 3 per day) from days 1 to 7 of pregnancy. D: Optical indensity of *CXCL14* mRNA (*CXCL14*/18S mRNA) during periimplantation uteri. The results (mean ± SEM, n = 3) were calculated from values of *CXCL14* mRNA relative to that of 18S mRNA (internal control).

cells within the implantation sites. This finding has inspired us further examine whether CXCL14 was also expressed in preimplantation embryos. And indeed, our immunofluorescence staining showed that the CXCL14 were expressed in the D4 blastocyst with strong signal in the trophectoderm (Fig. 3B), whereas the morula showed very weak or undetectable signals (Fig. 3A), suggesting a potential role of CXCL14 in trophoblast function.

CXCL14 inhibits trophoblast outgrowth in vitro

Given the abundant expression of CXCL14 on trophectoderm and trophoblast of periimplantation embryo, and several reports showing CXCL14 as a tumor migration regulator (Schwarze et al., 2005; Ozawa et al., 2006; Pelicano et al., 2009), here we want to address the question as to whether CXCL14 participated in regulating trophoblast invasion during embryo implantation, a process similar to tumor invasion in many aspects. Here an in vitro culture system was utilized to assess the ability of blastocyst attachment and outgrowth in the presence of rmCXCL14 or CXCL14 antibody. Blastocyst attachment status was checked at 48 h after initiation of in vitro culture. As shown in Figure 4A, compared with the control groups, the attachment rate of blastocysts onto the fibronectin substrate were significantly inhibited by rmCXCL14 while promoted by CXCL14 antibody after 48 h of culture ($P < 0.01$). There's no significant differences observed between control and rat IgG group. The extent of blastocyst outgrowth was

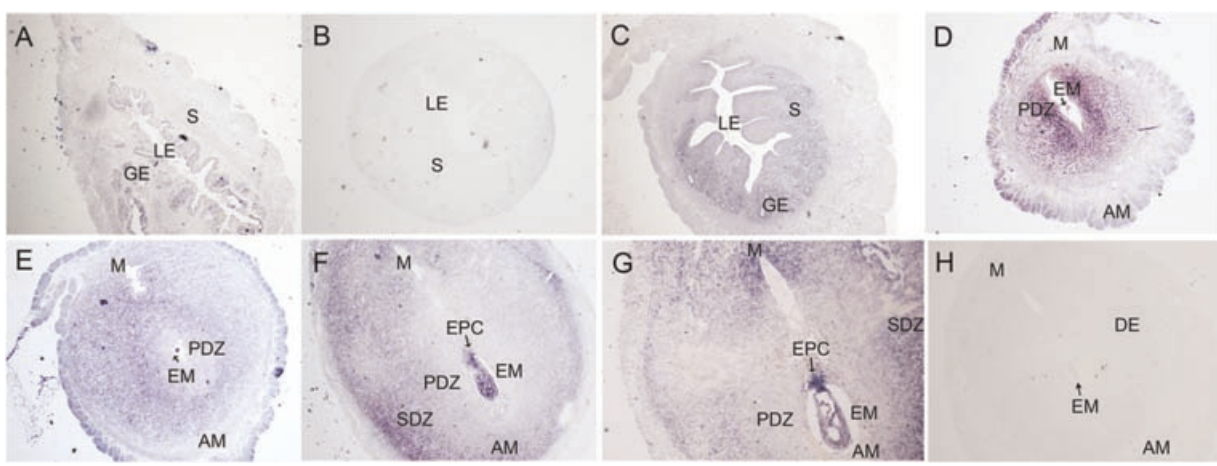

Fig. 2. Localization of *CXCL14* mRNA in mouse uteri during periimplantation. In situ hybridization shows localization of *CXCL14* mRNA as blue-purple precipitates. Uterine cross-sections from days 1(A), 3(B), 4(C), 5(D), 6(E), 7(F), and 8(G) of pregnancy were subjected to in situ hybridization using a digoxigenin-labeled CXCL14 antisense cRNA probes. H: Negative control: day 6 of pregnancy, sense probes of CXCL14 were used. Similar results were obtained in 2–3 mice for each checkpoint. GE, glandular epithelium; LE, luminal epithelium; S, stroma; EM, embryo; AM, antimesometrial pole; M, mesometrial pole; PDZ, primary decidual zone; SDZ, secondary decidual zone; DE, decidua. Original magnification, 40×.

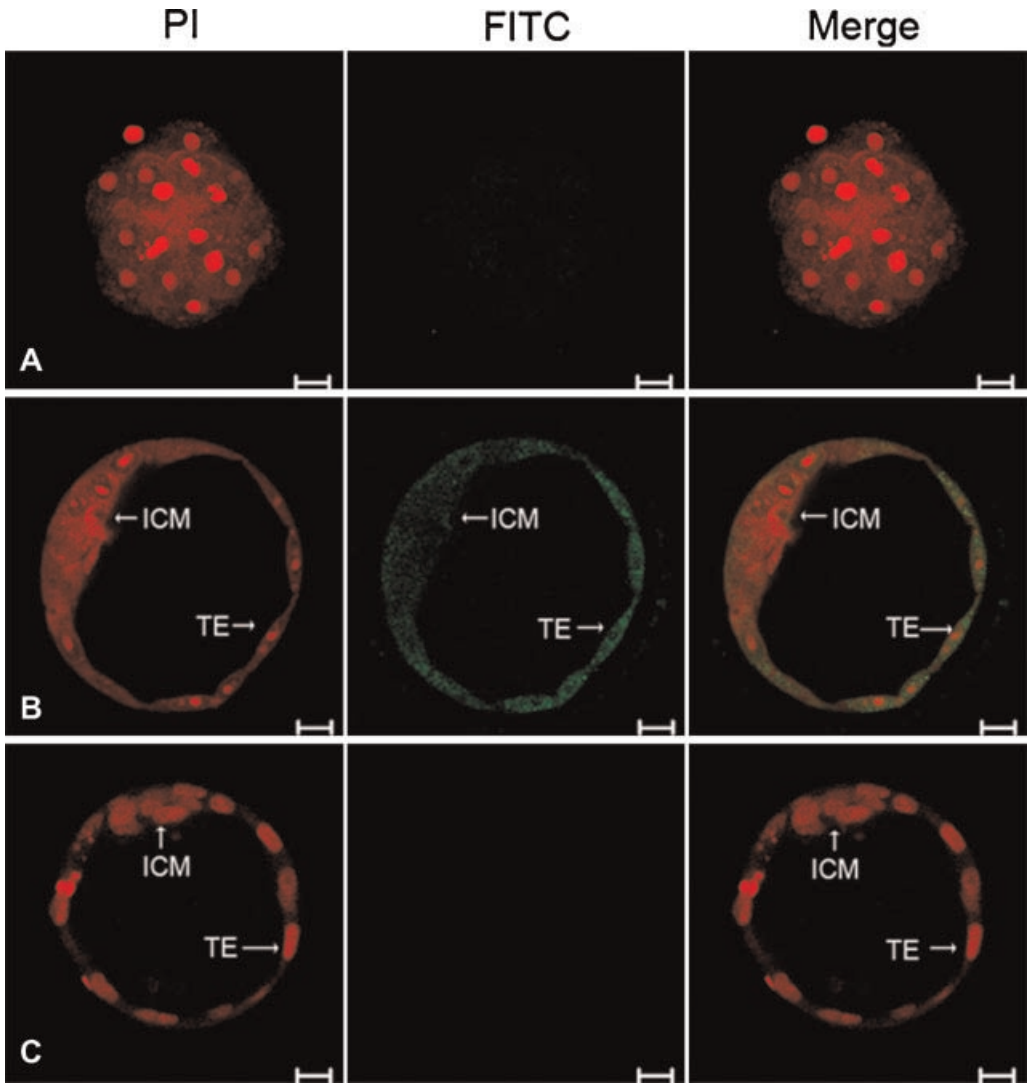

Fig. 3. Immunofluorescence detection of CXCL14 in mouse preimplantation embryos. Green signal represents CXCL14 staining with FITC-conjugated secondary antibody and red signal indicates nuclear staining with PI. A: Morula. B: Blastocyst. Note the highlighted expression of CXCL14 protein at the trophectoderm of blastocyst. ICM, inner cell mass; TE, trophectoderm. C: Negative control: primary antibodies replaced by rat IgG. Scale bars, 50 μm.

checked at three time points (24, 36, and 48 h). As shown in Figure 4B, after 36 and 48 h in vitro culture, an inhibitory effect on blastocyst outgrowth was evidently observed in rmCXCL14-treated groups, while a promontory effect was observed at 48 h when cultured with CXCL14 antibody ($P < 0.01$). We have further performed Ki67 staining of blastocysts after cultured with control medium and medium plus CXCL14, as to examine whether CXCL14 affect the proliferation ability of the blastocyst. As shown in Supplementary Figure 3. CXCL14 (100 and 200 ng/ml) treatment group does not show obvious differences in regards to ki67 staining, also, the blastocysts seems morphologically normal compared with control group. These results suggested that the reduced outgrowth performance after CXCL14 treatment is not due to reduced proliferation.

To further study the role of CXCL14 during trophoblast cell outgrowth and migration, we isolated EPCs from day 8 mouse pregnant uteri, a structure predominantly composed of invasive sTGCs derived from blastocyst trophectoderm (Liu et al., 2008), EPCs were cultured in the presence of either rmCXCL14, CXCL14 antibody, or normal rat IgG. The results showed that the trophoblastic outgrowth capacity were significantly reduced in the CXCL14-treated group, as demonstrated by the outgrowth rate and outgrowth area of EPCs, while the CXCL14 antibody shows an opposite effect as shown in Figure 5A–C. These results demonstrated that CXCL14 has an inhibitory role on both primary and secondary trophoblast invasion.

CXCL14 inhibits trophoblast outgrowth via MMP-2 and 9 downregulation in vitro

To shed additional light on the mechanisms by which CXCL14 regulates trophoblast outgrowth, we next examined whether CXCL14 could regulate MMP-2 and 9 production of EPCs, which have been considered as two key factors regulating trophoblast invasion (Staun-Ram and Shalev, 2005). After 24 and 48 h of in vitro culture, EPCs-conditioned medium was collected and analyzed for collagenase activity by gelatin zymography. At 24 h checkpoint, no significant differences were

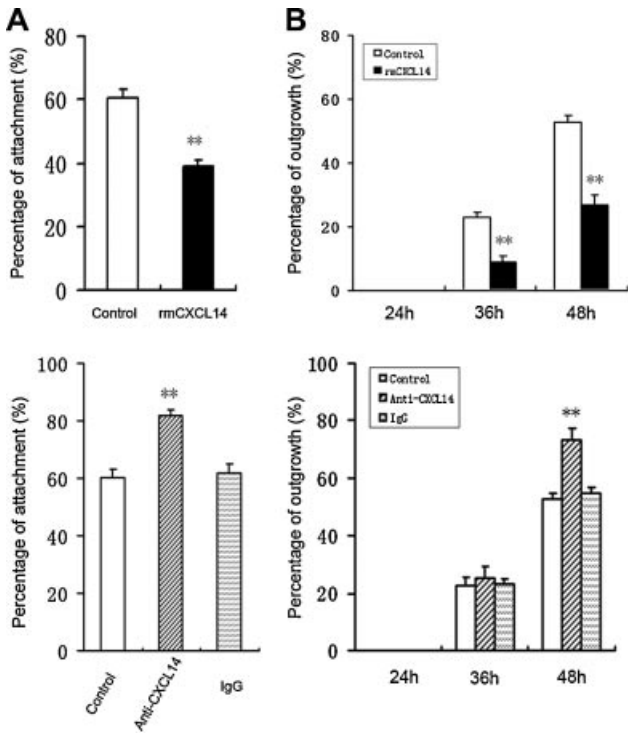

Fig. 4. CXCL14-inhibited attachment and outgrowth of blastocyst in vitro. Blastocyst attachment and outgrowth assays were performed in 96-well plates precoated with 10 μg/ml of FN. A: Rate of blastocyst attachment. Recombinant mouse CXCL14 (rmCXCL14) added exogenously to the culture media-inhibited blastocyst attachment at 48 h culture (top), whereas blastocyst attachment was promoted by the treatment with anti-mouse CXCL14 antibody (anti-CXCL14) (bottom). B: Rate of blastocyst outgrowth. Rate of blastocyst outgrowth was reduced with the use of rmCXCL14 at 36 and 48 h after initiation of culture (top), whereas outgrowth increased by the treatment with anti-CXCL14 antibody (bottom). Blastocysts incubated in normal cultural media were served as controls. Results were shown as mean ± SEM (n = 4). $^{**}P < 0.01$ compared with the control group.

observed between three groups (data not shown). While after 48 h culture, MMP-2 and 9 levels were significantly suppressed in CXCL14-treated group and promoted in CXCL14 antibody-treated group ($P < 0.05$ and $P < 0.01$; Fig. 6A–C). These results have demonstrated a direct or indirect effect of CXCL14 on regulating trophoblast MMPs production during the process of trophoblast outgrowth.

CXCL14 could specifically bind to trophoblast cells both in vitro and in vivo

Since CXCL14's-specific receptor(s) has not been identified to date, binding assays were performed to test whether the trophoblast cell is the direct target of secreted CXCL14 and to explore the binding site of biotinylated CXCL14. As shown in Figure 7A–C, the biotinylated CXCL14 uniformly stained the trophoblast giant cells grown out from EPCs in culture, while biotin and no labeled CXCL14 group did not show positive signals. Furthermore, in vivo binding assay of biotinylated CXCL14 was performed on frozen tissues section of day 8 pregnant mouse uteri. As demonstrated in Figure 7D, the biotinylated CXCL14 could specifically bind to the EPC region of D8 implantation site, while the surrounding decidual cells shows no or very weak binding signal.

DISCUSSION

The current study has established the spatialtemporal expression pattern of CXCL14 during mouse periimplantation. The cell-specific expression of CXCL14 at implantation sites on both uterine and embryonic compartment has led us into two lines of findings. At one side, our in vitro functional study demonstrated that recombinant CXCL14-inhibited trophoblast cell attachment and outgrowth while the CXCL14 antibody had an opposite effects. This inhibitory effect of CXCL14 was further found to associate with downregulation of trophoblast MMP-2 and 9 activity. On the other side, by using biotinylated recombinant CXCL14 binding experiment, we find that at the maternal–fetal interface, the major binding site of secreted CXCL14 was trophoblast cells derived from blastocyst, but not the stroma cells from the uterus, indicating that the trophoblast cells were the bioactive target of CXCL14 in the context of early pregnancy, and would express the unidentified CXCL14 receptor(s).

In early pregnant uterus, the process of implantation requires a highly coordinated and complex dialogue between the embryo and maternal tissue, numerous locally produced modulators and signaling events are involved during this reciprocal interaction. Recent evidences have shown that, several members of the chemokine family could regulate trophoblast migration in a way similar to that of leukocyte recruitment (Hannan and Salamonsen, 2007), which exerts their function via binding to the cognate receptors within the context of maternal–fetal interface. To our best knowledge, most of the currently studied chemokines with a regulatory role during early pregnancy shows a promontory effect on trophoblast migration. However, our results showed that CXCL14 played an evidenced inhibitory role on trophoblast attachment and outgrowth, this unique feature of CXCL14 on trophoblast migration were also in accordance with several reports that CXCL14 have a tumor suppressive function (Schwarze et al., 2005; Ozawa et al., 2006). Furthermore, our parallel study in human pregnancy also showed an inhibitory effect of CXCL14 when culturing human trophoblast in vitro (unpublished data in our lab). These data has suggested a unique role of CXCL14 in balancing the invasive ability of trophoblast against other promoting chemokines. Since the receptor of CXCL14 has not been found to date by testing several cognate receptors of the chemokine family (CXCR1, 2, 3, 4) (Cao et al., 2000; Sleeman et al., 2000), it is possible that CXCL14 might has an unique receptor beyond the currently known receptors of chemokine family (Hannan and Salamonsen, 2007; Hess et al., 2007). In this regard, using in vitro binding assay to predict the potential site of CXCL14 receptor would be sound and ensures further identification of its true face. Here our results showed that the biotinylated CXCL14 uniformly stained sTGCs of EPCs in vitro and in vivo, demonstrating the functional binding sites of its potential receptor(s). These results has also strengthen our belief that CXCL14 secreted form both the trophoblast and surrounding uterine tissue could directly act on trophoblast cell in a paracrine/autocrine manner. However, it should also be mentioned that there are also studies demonstrating that CXCL14 promote cell migration in other specific systems (Augsten et al., 2009; Pelicano et al., 2009) Implicating that CXCL14 might have potentially diverse roles under different pathophysiological conditions.

During early pregnancy, trophoblast invasion are closely correlative with the expression of MMPs (Staun-Ram and Shalev, 2005; Cohen and Bischof, 2007; Ferretti et al., 2007), which are capable of degrading extra cellular matrix. According to the existing literature, at the stage of early pregnancy, MMP-2 and 9 were the major participants during embryo implantation, which due to their ability to degrade Collagen IV, the main component of the basement membrane, thus, enabling the

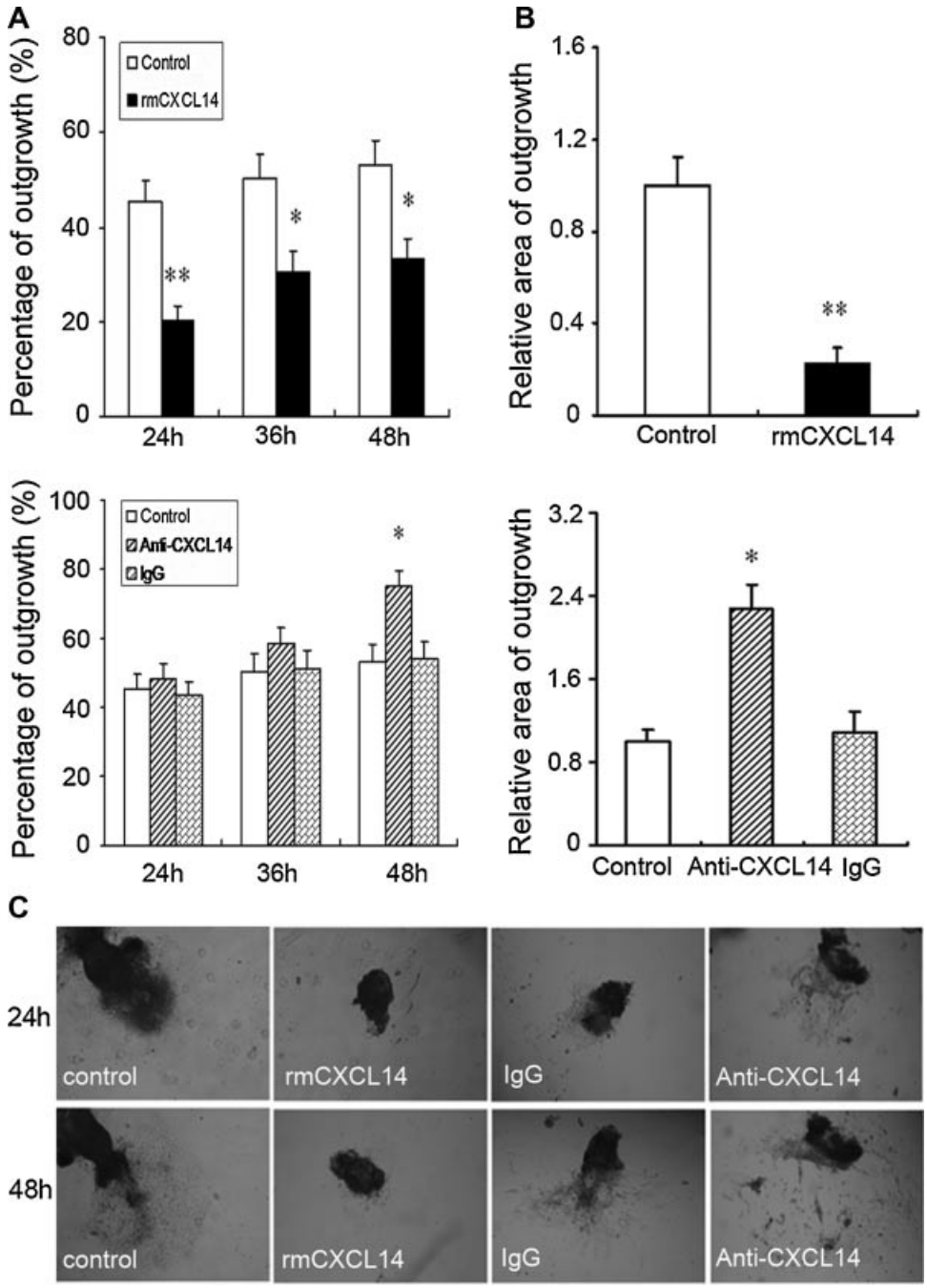

Fig. 5. Effect of CXCL14 on EPCs outgrowth in vitro. Outgrowth assays of EPCs were performed in 4-well plates precoated with Matrigel (8 mg/ml). A: Outgrowth percentage of EPCs. rmCXCL14 (top) and anti-CXCL14 antibody (bottom) were added into the culture medium and cultured with EPCs for 24, 36, and 48 h, and outgrowth percentage of EPCs was recorded at each checkpoint. B: Outgrowth area of EPCs. Outgrowth area of EPCs was reduced with the treatment of rmCXCL14 at 48 h culture (top), whereas was promoted by the treatment with anti-CXCL14 (bottom). Data represented as fold change in the outgrowth area of EPCs (±SEM, n = 30) compared to controls. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group. C: Demonstrative photos showed morphologic observation of EPCs in the presence of rmCXCL14 or anti-CXCL14 antibody or rat IgG. Photos were taken under the light microscope after 24 and 48 h of culture. Original magnification, 40×.

invasion of the trophoblast cells through the decidua and into the maternal vasculature (Staun-Ram and Shalev, 2005). Since CXCL14 could inhibit migration of both blastocyst (rich in primary trophoblast giant cells) and EPCs outgrowth (rich in sTGCs), which were previously reported to have different predominance in MMP-2 and 9 content (Hulboy et al., 1997). We tested the effect of CXCL14 on MMP-2, and 9 production in both targets. Our data indicated that CXCL14 could downregulate MMP-2 in the blastocyst (data not shown) while downregulate both MMP2, and 9 production in the EPCs. These results are in correspondance with previously reported relationship between chemokine and MMPs secretion in other systems (Cross and Woodroofe, 1999; Van and Libert, 2007). The relative abundance of MMP-2, and 9 in blastocyst and EPCs

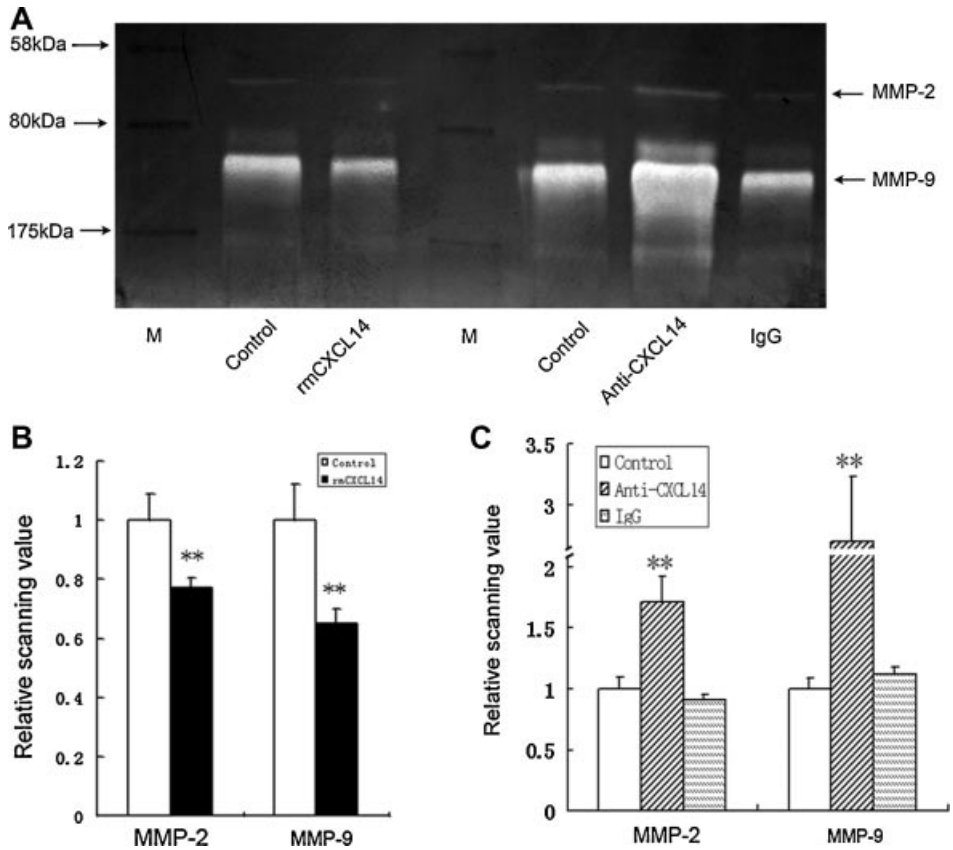

Fig. 6. CXCL14-inhibited MMP-2 and 9 production in cultured EPCs. A: Representative gelatin zymography showed the relative secretion of MMP-2 (72-kDa) and MMP-9 (92-kDa) in EPCs culture medium with treatment of rmCXCL14, anti-CXCL14 antibody and rat IgG at 48 h culture. B,C: Relative densitometric analysis showed that the MMP-2 and 9 production were inhibited in rmCXCL14-treated group (B), while promoted in anti-CXCL14-treated group (C). Results represented as fold change in the production of collagenase (±SEM, n = 3) compared to controls. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

also support previous findings that MMP-2 is the main gelatinase in primary trophoblast giant cells while MMP-9 is the main gelatinase in sTGCs (Staun-Ram and Shalev, 2005). The underlining mechanisms of how CXCL14 achieve this effect still kept unknown, mainly due to the lack of knowledge on its receptor and subsequent signaling pathway. Future identification of CXCL14 receptor may further facilitate understanding of these detailed processes.

In addition to the evidenced role of CXCL14 on trophoblast migration, the highly uterine expression of *CXCL14* at embryo implantation on D5 might also suggest that *CXCL14* could contribute to the establishment of PDZ at this time. While from D7 to D8, when the SDZ were establishing in the outer region of PDZ, the *CXCL14* again showed a timely increased expression at these parts of decidua (Fig. 2). Interestingly, this expression pattern of *CXCL14* is somewhat similar with another immunoregulator gene *PTX3*, and the *PTX3* null female mice do show a compromised implantation and decidualization (Tranguch et al., 2007). Similar expression pattern of *CXCL14* in the decidualizing stroma was also observed in the oil-induced deciduoma from D5 to D8 (Supplementary Fig. 2), suggesting its close relationship with uterine decidualization. Alternatively, the uterine expression of CXCL14 might also play a role in uNK cell recruitment thus regulate early pregnancy (Hanna et al., 2006; Carlino et al., 2008), because there have been reports about CXCL14's role on NK cell recruitment (Starnes et al., 2006). Our lab also found an overlapped distribution of CXCL14 and uNK cells in the uterus section from days 7 to 9 postimplantation (data not shown). However, a clear map of CXCL14's effects on uNK cell recruitment in the context of uterus were still vague, since a recent report has showed opposite results against its chemoattractant roles on many types of leukocytes based on knockout models (Meuter et al., 2007).

To date, the CXCL14 knockout mice have been generated by two independent labs. Although a comprehensive analysis of reproductive phenotype has not been performed, both labs has reported different degree of subfertility phenotype of CXCL14 null mice (Meuter et al., 2007; Nara et al., 2007). It should be noted that a reduced Mendelian frequency of $CXCL14^{-/-}$ mice was reported by both labs when intercrossing heterozygous mice. And one lab has reported that the littersize of a knockout × knockout breeding pair was generally small while some knockout females could not produce newborns. [personal communication with the authors], this phenotype is particularly interesting to us, because our current result has suggested that the deletion of CXCL14 might result in enhanced abnormal trophoblast invasion and cause overdestruction of newly formed decidual cells, which further hamper the process of placentation and subsequent pregnancy. However, detailed investigation in regards to when and how the deletion of CXCL14 cause an compromised fertility were further needed by using CXCL14 knockout mice.

In conclusion, the current study has for the first time showed a map of expressional profiles of CXCL14 during mouse

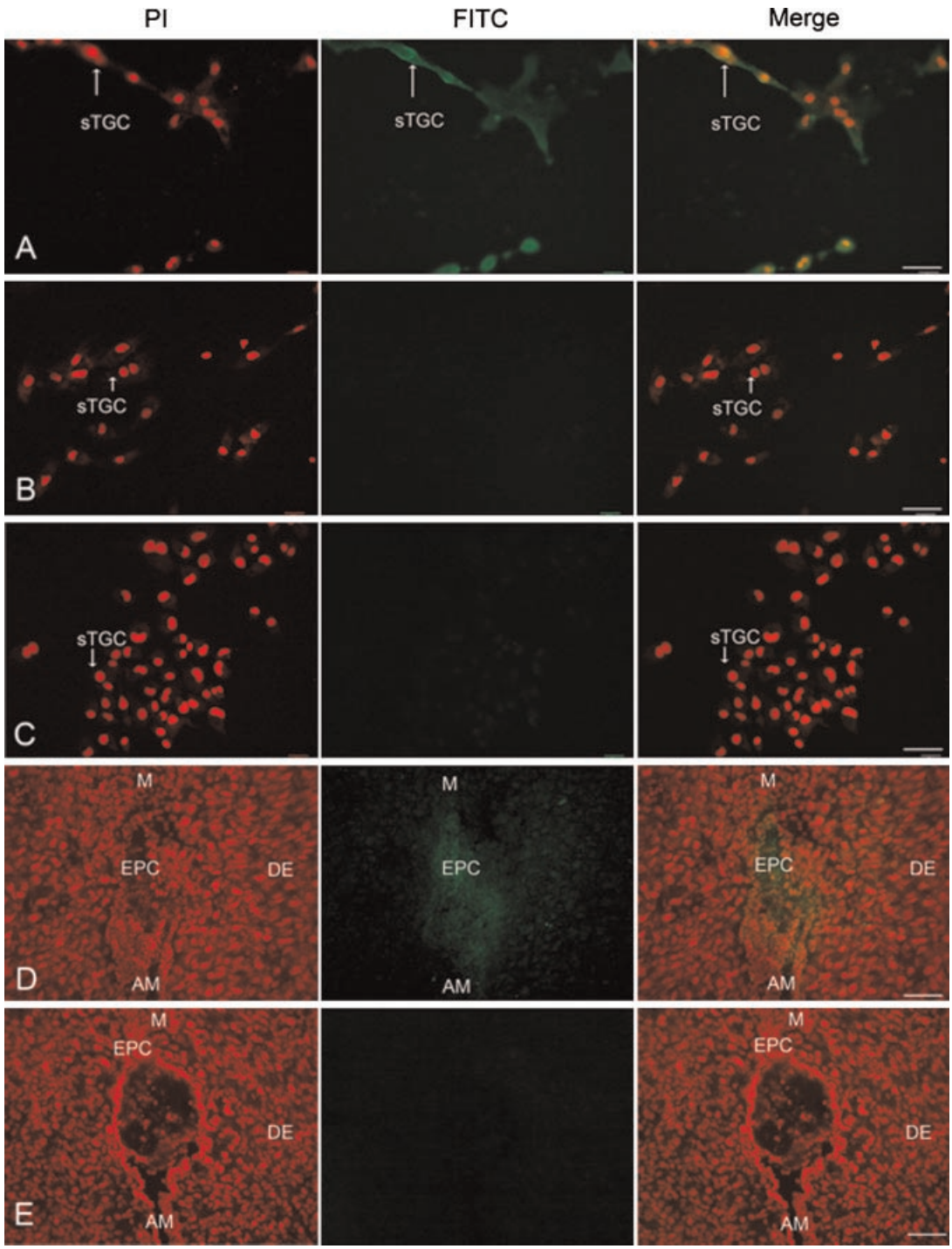

Fig. 7. Biotinylated CXCL14 specifically bound to mouse trophoblast of EPCs in vitro and in vivo. Recombinant CXCL14 proteins were biotinylated using the EZ-link biotinylation reagent. Biotinylated CXCL14 (A), no labeled CXCL14 (B), and biotin (C) were incubated with the trophoblast cells grown out from EPCs for 1 h and visualized with streptavidin-FITC and PI for nuclear counterstaining. Biotinylated CXCL14 (D) and biotin (E) was incubated with frozen tissue sections of day 8 implantation site. Note the EPCs region showed strong binding signal of biotinylated CXCL14 while the surrounding decidual cells showed weak or no signal. AM, anti-mesometrial pole; DE, decidua; M, mesometrial pole, Scale bar, 100 μM.

periimplantation. It is demonstrated that the CXCL14 plays an inhibitory role on trophoblast outgrowth during early pregnancy in a paracrine/autocrine manner. Our data has provided evidence that different member of chemokine family could play opposing effect within the context of maternal–fetal interface, ensuring an optimized environment for the well-controlled process of trophoblast invasion. This study also provided future possibilities to identify the currently unknown CXCL14 receptor(s) and warrants future clinical research on CXCL14, because CXCL14 is also specifically expressed in

human endometrium and trophoblast (Red-Horse et al., 2001, 2004; Talbi et al., 2006).

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