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*Original Article*

# L-22 enhances the invasiveness of endometrial stromal cells of adenomyosis in an autocrine manner

Qing Wang[1], Li Wang[2], Jun Shao[1], Yan Wang[1], Li-Ping Jin[1,3], Da-Jin Li[1], Ming-Qing Li[1,3]

*[1]Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, People's Republic of China; [2]Department of Pathology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, People's Republic of China; [3]Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, People's Republic of China*

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**Abstract:** It has reported that interleukin-22 (IL-22) promotes the invasion of tumor cells. IL-22 in the endometriotic milieu stimulates the proliferation of human endometrial stromal cells (ESCs). The present study aimed to elucidate whether and how IL-22 regulates the invasion of ESCs from adenomyosis. The expression of IL-22 and its receptors in normal endometrium, eutopic endometrium and ectopic lesion was analyzed by immunohistochemistry; the invasiveness of ESCs *in vitro* was verified by Matrigel invasion assay; and the effects of IL-22 on the correspondent functional molecules were investigated by ELISA and flow cytometry. Here we found that IL-22 and its receptors IL-22R1 and IL-10R2 in eutopic endometrium and ectopic lesion of adenomyosis were significantly higher than that of normal endometrium. Recombinant human IL-22 (rhIL-22) increased IL-22R1 and IL-10R2 levels on ESCs. Moreover, rhIL-22 promoted the invasiveness of ESCs, and inhibited the expression of metastasis suppressor gene CD82, stimulated the secretion of IL-8, RANTES, IL-6 and VEGF of ESCs. On the contrary, the neutralizing antibody for IL-22 reversed these effects. Our current study has demonstrated that IL-22 has a positive feedback on the expression of its receptors IL-22R1 and IL-10R2 on ESCs. This autocrine effect of IL-22 promotes the invasion of ESCs possibly through regulating invasion-related molecules, suggesting that the abnormal high expression of IL-22 may play an important role in ESCs invasion and finally contribute to the origin and development of adenomyosis.

**Keywords:** IL-22, endometrial stromal cells, invasion, adenomyosis

## Introduction

Adenomyosis is a common gynecological disorder. Unlike endometriosis, adenomyosis is a myometrial lesion characterized by the presence of ectopic endometrial glands and stroma located deep within the surrounding myometrium with adjacent myometrial hyperplasia and hypertrophy [1]. Abnormal stromal cell invasion has been proposed in the etiology of adenomyosis [1, 2], but the features in the microenvironment that regulate myometrial penetration by the overlying endometrium and the changes that trigger the development of uterine adenomyosis remain unclear.

A series of research has shown that chemokines produced in the ectopic endometrium in women with adenomyosis may contribute to the pathophysiology of adenomyosis, such as IL-6, IL-8, CCL2 (also known as monocyte chemoattractant protein-1) and RANTES (Regulated upon Activation, Normal T-cell Expressed and Presumably Secreted) [3-6]. In addition to the roles in inflammatory responses by the recruitment of leukocytes into the peritoneal cavity of patients with endometriosis, our previous works showed that these cytokines promotes the survivin and invasion of ESCs in the endometriotic milieu under the control of estrogen and metastasis suppressor molecules (such as CD82, NME1) [7-10].

Interleukin-22 (IL-22), is a member of the IL-10 family of cytokines, is mainly produced by hematopoietic cells involved in both innate and adaptive immunity [11, 12], such as $CD4^+$ Th17 cells, NK cells, and lymphoid tissue inducer-like

cells [13-15]. IL-22 targets cells through the IL-22R receptor, which is composed of IL-22R1 and IL-10R2 [16, 17]. The IL-22 receptor is found on cells of nonhematopoietic origin in the skin, kidney, liver, lung, and gut, allowing for IL-22-mediated regulation of local epithelial, endothelial, and stromal cells responses after infection or exposure to inflammatory stimuli [12, 18, 19]. Recently, a growing body of evidences indicated the IL-22 in tumor microenvironment stimulates the growth and invasion of cancer cells [20-22].

Recently, we have reported that IL-22 promotes the proliferation of ESCs through regulation of IL-8 and CCL2, and may further participate in progress of endometriosis [23]. However, there are still questions whether IL-22 regulates the invasion of ESCs through modulating the invasion-related molecules in eutopic endometrium of adenomyosis. Therefore, the present study is undertaken to identify the expression and role of IL-22 in the invasiveness of ESCs in eutopic endometrium from women with adenomyosis.

## Materials and methods

*Tissue collection, isolation and culture of ESC*

All tissue samples were obtained with informed consent in accordance with the requirements of the Research Ethics Committee in Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College. The normal endometrium tissues from fertile women (n = 10), and eutopic endometrial tissues and ectopic lesions from women (n = 10) with adenomyosis were obtained, undergoing hysterectomy for pain or other benign indications. All the samples were confirmed histologically according to established criteria [24].

The eutopic endometrial tissues (n = 16) from women with adenomyosis were collected under sterile conditions and transported to the laboratory on ice in DMEM (Dulbecco's modified Eagle's medium)/F-12 (Gibco, USA) with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA). The ESCs were isolated according to the previous methods [8, 9]. Immunocytochemistry showed >95% vimentin-positive and cytokeratin-negative ESCs.

*Immunohistochemistry*

The IL-22, IL-22R1 and IL-10R2 protein levels in the ectopic lesions (n = 10) and eutopic endometrial tissues from women with (n = 10) or without (n = 10) adenomyosis were dehydrated in graded ethanol and incubated with hydrogen peroxide in 1% bovine serum albumin in Tris-buffered saline (TBS) to block endogenous peroxidase. The samples were then incubated with mouse anti-human IL-22 monoclonal antibody (25 ug/ml, R&D system, Inc., MN, USA), mouse anti-human IL-22R1 (15 ug/ml, R&D system), mouse anti-human IL-10R2 (25 ug/ml, R&D system) or mouse IgG isotype antibody 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 antibody (SP-9002; Golden Bridge International, Inc., Beijing, China) and the reaction was developed with 3, 3'-diaminobenzidine (DAB), the sections counterstained with hematoxylin. The experiments were repeated five times.

*Treatment with recombinant human IL-22 protein and anti-human IL-22 neutralizing antibody*

ESCs ($1 \times 10^5$ cells/well) in 24-well plates were treated with recombinant human IL-22 (rhIL-22) protein (10 ng/ml, R&D Systems) or anti-human IL-22 neutralizing antibody (α-IL-22, 0.5 ug/ml, R&D Systems) for 48 h, with vehicle as control. Then the supernatant was collected and analyzed the cytokines concentration by ELISA. The invasiveness and the expression of IL-22R1, IL-10R2 and CD82 expression of ESCs were detected by Martigel invasion assay and flow cytometry, respectively.

*Enzyme-linked immunosorbent assay (ELISA)*

The culture supernatant was harvested, centrifuged to remove cellular debris, and stored at -80°C until being assayed by ELISA for analyzing the secretion levels of IL-8, RANTES, IL-6, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Shanghai ExCell Biology, Inc, Shanghai, China).

*Matrigel invasion assay*

The invasion of the ESCs (n = 6) across Matrigel was evaluated objectively in invasion chamber, based on our previous procedure [8]. Briefly, the cells inserts (8 um pore size, 6.5 mm diameter, Corning, USA) coated with 15-25 ul Matrigel were placed in a 24-well plate. The primary ESCs of $2 \times 10^4$ were plated in the upper chamber (the media contained 1% charcoal

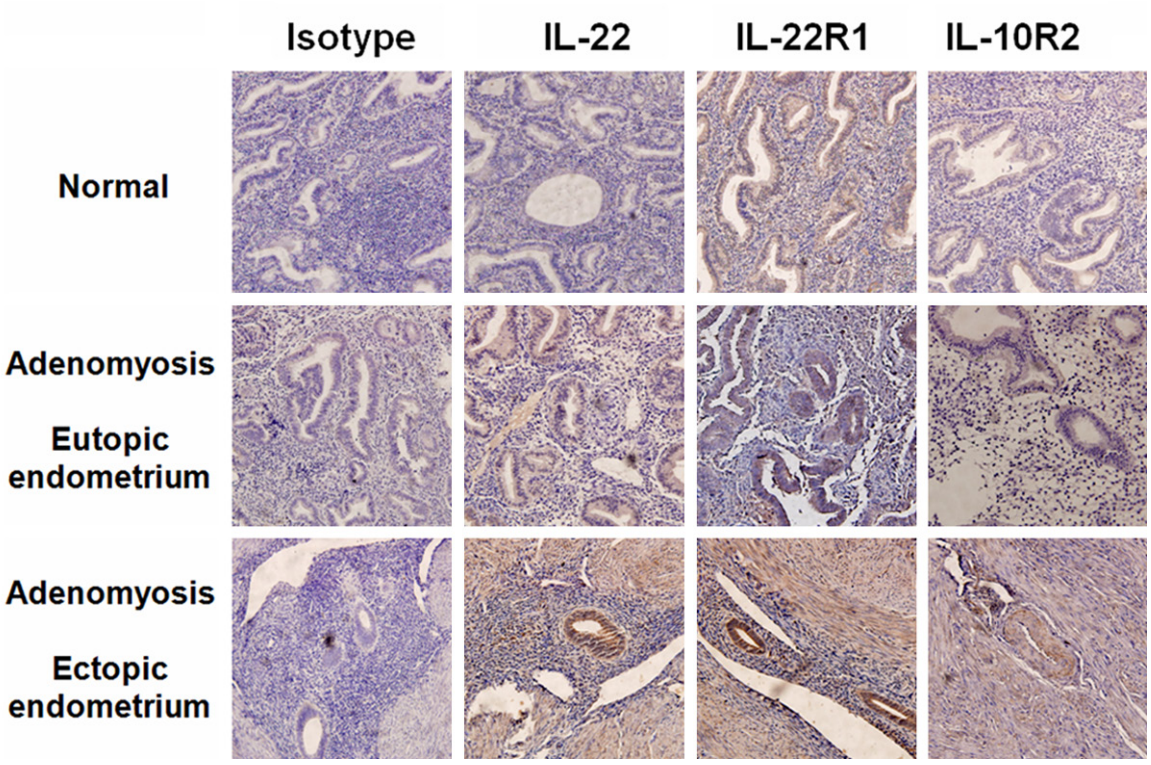



**Figure 1.** The expression of IL-22 and its receptors is increased in the eutopic endometrium and ectopic lesion from women with adenomyosis. The expression of IL-22 and it receptors (IL-22R1 and IL-10R2) in the endometrium from women of healthy control (n = 10), eutopic endometrium (n = 10) and ectopic lesion (n = 10) from women with adenomyosis was analyzed by immunohistochemistry. Original magnification: ×200.

stripped FCS). RhIL-22) protein (10 ng/ml, R&D Systems) or α-IL-22 (0.5 ug/ml, R&D Systems) was added, respectively. The lower chamber (the media was contained 5% charcoal stripped FCS) was filled with 800 ml medium. The cells were then incubated at 37°C for 48 h. The inserts were removed, washed in PBS and the non-invading cells together with the Matrigel were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in methanol for 10 min at room temperature and stained with hematoxylin. The result was observed under Olympus BX51+DP70 microscope (Olympus, Tokyo, Japan). The cells migrated to the lower surfaces were counted at a magnification of ×200. The invasion index of each group was calculated as the ratio of the cells number migrated to the lower surfaces to the vehicle control. Each experiment was carried out in triplicate, and repeated three times.

*Flow cytometry*

In addition, we analyzed the expression of IL-22R1, IL-10R2 and CD82 on ESCs (n = 6) by flow cytometry. ESCs were resuspended and washed with PBS, fixed to permeabilize the member and then incubated with mouse anti-human IL-22R1-Percp 5.5, IL-10R2-FITC and CD82-PE monoclonal antibody (Biolegend, USA) for another 30 min at room temperature. Meanwhile, the isotypic control was used. After incubation, the cells were washed and analyzed immediately by a FACS Calibur flow cytometer (Becton Dickinson, NJ, USA) by using Cellquest software (Becton Dickinson). Statistical analysis was conducted by using isotype matched controls. The experiments were repeated three times.

*Statistics*

All values are shown as the mean ± SD. *T*-test analysis of variance was used to detect the difference of IL-22R1, IL-10R2, CD82, IL-8, RANTES, IL-6, VEGF and FGF expression and the invasiveness in ESCs. Differences were considered as statistically significant at $P < 0.05$.

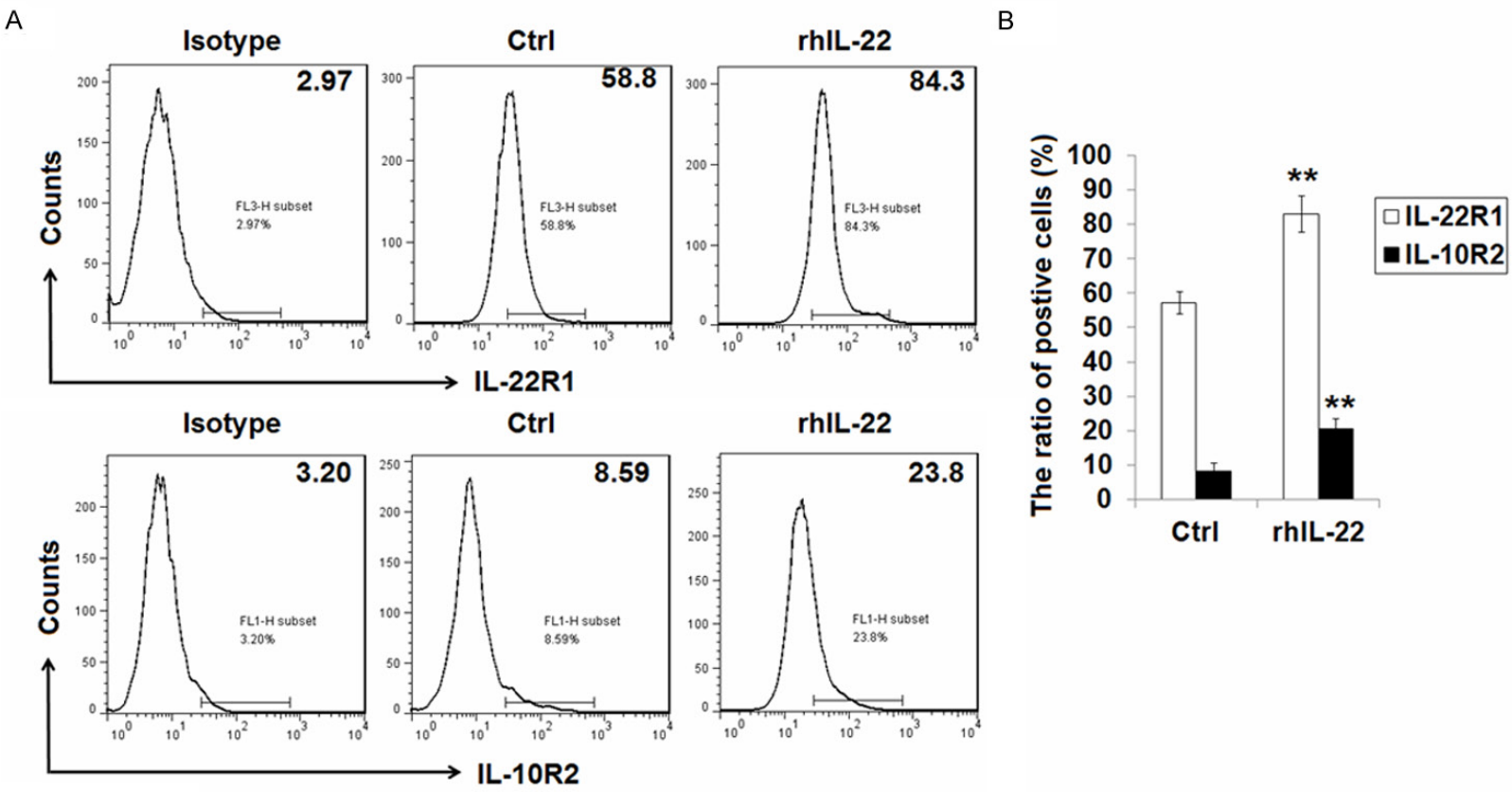



**Figure 2.** Recombinant human IL-22 up-regulates the expression of IL-22R1 and IL-10R2 on ESCs. A, B: ESCs (n = 6) of eutopic endometrium from women with adenomyosis were treated with recombinant human (rh) IL-22 (10 ng/ml) for 48 h. Then flow cytometry was used to analyze the expression of IL-22R1 and IL-10R2 on ESCs. These pictures are representatives of three individual experiments. Data are expressed as the mean ± SD. $^{**}P < 0.01$ compared to the vehicle control.

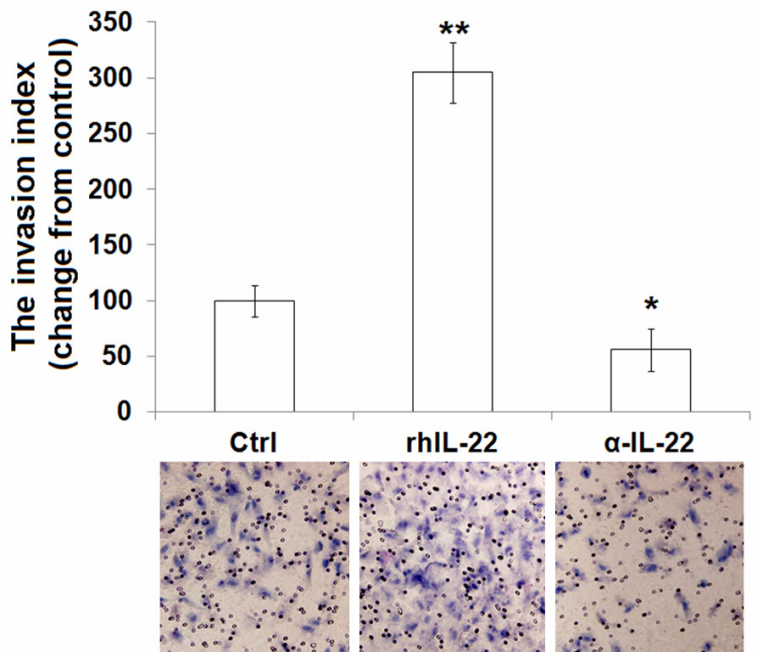



**Figure 3.** IL-22 enhances the invasiveness of ESCs. After treatment with rhIL-22 (10 ng/ml) or anti-human IL-22 neutralizing antibody (α-IL-22, 0.5 ug/ml) for 48 h, the matrigel invasion assay was performed to detect the invasiveness of ESCs (n = 6) of eutopic endometrium from women with adenomyosis. Original magnification: ×200. These pictures are representatives of three individual experiments. Data are expressed as the mean ± SD. $P < 0.05$ and $^{**}P < 0.01$ compared to the vehicle control.

## Results

*The expression of IL-22 and its receptors is increased in the eutopic endometrium and ectopic lesion from women with adenomyosis*

Firstly, we compared the expression of IL-22 and its receptors (IL-22R1 and IL-10R2) in normal endometrium, eutopic endometrium and ectopic lesion of adenomyosis by immunohistochemistry. As depicted in **Figure 1**, the expression of IL-22 in ectopic lesion was higher than that of eutopic endometrium from women with adenomyosis, both in the stromal cells and glandular epithelial cells. Moreover, the myometrial cells surrounding with ectopic lesion also high expressed IL-22 (**Figure 1**). However, we had not detected the positive staining of IL-22 in all healthy endometrium samples. Relative to the stromal cells, glandular epithelium preferentially expressed IL-22R1 and IL-10R2. Compare to healthy control, IL-22R1 and IL-10R2 staining was stronger in eutopic endometrium and ectopic lesion of adenomyosis. These results suggest that the abnormal levels of IL-22 and its receptor in adenomyotic stromal cells may participate in regulating the biological behaviors of ESCs, and further be involved in the origin and development of adenomyosis.

*Recombinant human IL-22 up-regulates the expression of IL-22R1 and IL-10R2 on ESCs*

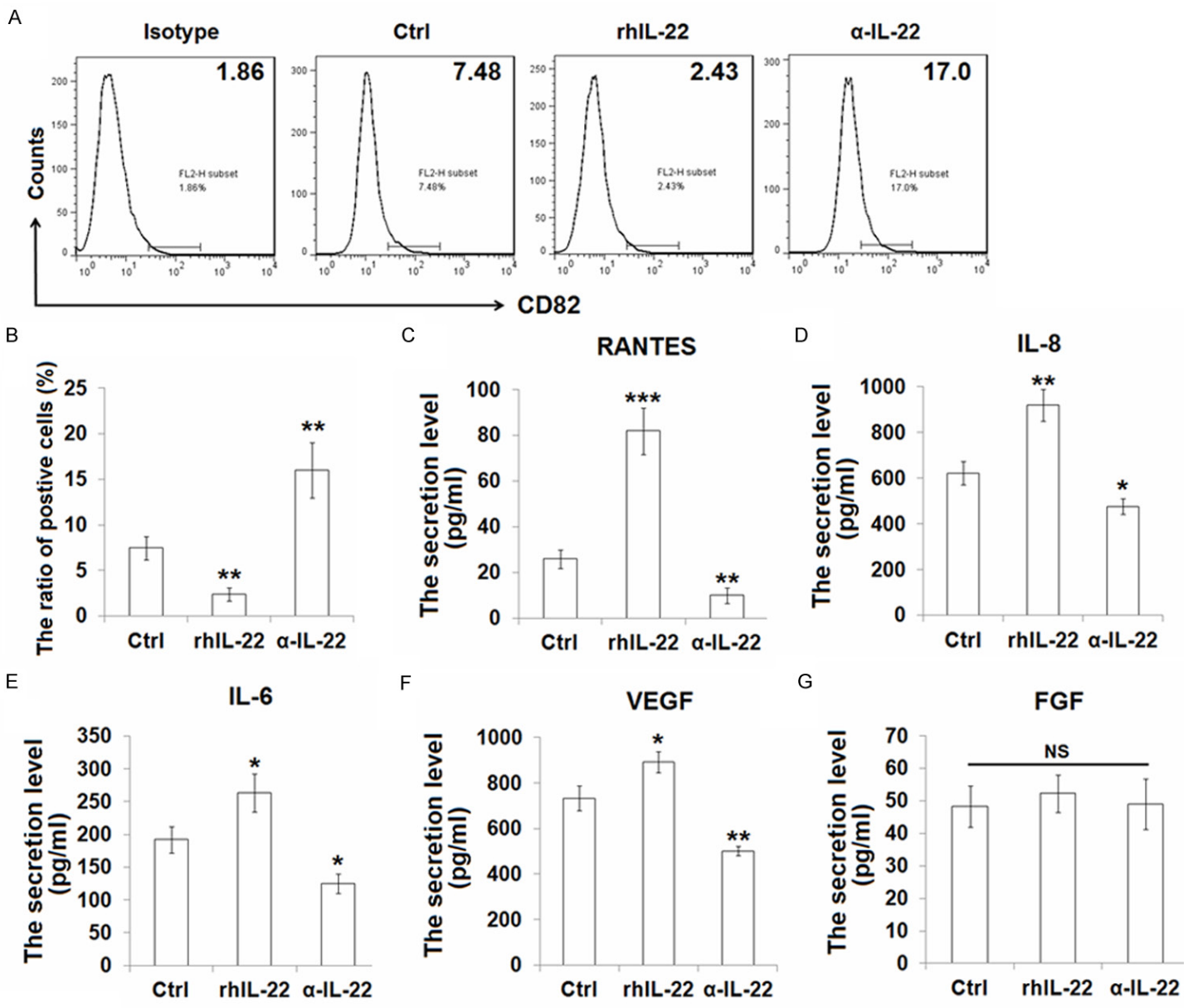
In order to measure the effect of IL-22 on its receptor, we treated ESCs of eutopic endometrium from women with adenomyosis with rhIL-22 (10 ng/ml) for 48 h. Then we found that rhIL-22 significantly up-regulated the expression of IL-22R1 ($P < 0.01$) (**Figure 2A**, **2B**) and IL-10R2 ($P < 0.01$) (**Figure 2A**, **2B**) on ESCs. These data indicate that IL-22 has an autocrine amplification effect on IL-22 signal through up-regulating the expression of IL-22R1 and IL-10R2.

*IL-22 enhances the invasiveness of ESCs*

We next investigated the effect of endogenous and exogenous IL-22 on the invasiveness of ESCs. Date presented in **Figure 3** showed that exogenous rhIL-22 (10 ng/ml) markedly increased the invasiveness of ESCs of eutopic endometrium from women with adenomyosis ($P < 0.01$) (**Figure 3**). On the contrary, blocking endogenous IL-22 with anti-human IL-22 neutralizing antibody (α-IL-22, 0.5 μg/ml) inhibited ESCs invasion ($P < 0.05$) (**Figure 3**). These findings suggest that both the exogenous IL-22 and IL-22 derived from ESCs enhance the invasiveness of ESCs. In addition, endometrial glandular epithelial cells and myometrial cells in uterus-secreted IL-22 may stimulate the invasiveness of ESCs invading to myometrium in a paracrine manner.

*IL-22 down-regulates CD82 expression and stimulates the secretion of IL-8, RANTES, IL-6 and VEGF of ESCs*

Taking into account the important role of cytokines and metastasis suppressor molecules in the regulation of ESC growth and invasion


A
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1.86
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FL2-H subset
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FL2-H subset
2.43%
FL2-H subset
17.0%
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IL-6
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FGF
NS
The secretion level (pg/ml)
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**Figure 4.** IL-22 down-regulates CD82 expression and stimulates the secretion of IL-8, RANTES, IL-6 and VEGF of ESCs. ESCs (n = 6) of eutopic endometrium from women with adenomyosis were incubated with rhIL-22 (10 ng/ml) or α-IL-22 (0.5 ug/ml) for 48 h, then the expression of CD82 on ESCs was analyzed by flow cytometry (A, B). In addition, the secretion levels of RANTES (C), IL-8 (D), IL-6 (E), VEGF (F), and FGF (G) in the supernatant of ESCs were detected by ELISA, respectively. Data are expressed as the mean ± SD. $P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the vehicle control. NS: no statistically difference.

[4-10], we further investigated the influence of IL-22 on metastasis suppressor molecule CD82 expression, and RANTES, IL-8, IL-6, VEGF and FGF levels of ESCs of eutopic endometrium from women with adenomyosis. As shown in **Figure 4**, the expression of CD82 on ESCs was obviously down-regulated after treatment with rhIL-22 ($P < 0.01$) (**Figure 4A**, **4B**). Instead, α-IL-22 had the opposite effect on CD82 expression of ESCs ($P < 0.01$) (**Figure 4A**, **4B**).

Subsequently, we found that stimulation with rhIL-22 resulted in a significant increase of RANTES, IL-8, IL-6 and VEGF of ESCs ($P < 0.05$, $P < 0.01$ or $P < 0.001$) (**Figure 4C-F**). Meanwhile, these cytokines levels were obviously decreased in the α-IL-22 treatment group ($P < 0.05$ or $P < 0.01$) (**Figure 4C-F**). However, not only rhIL-22 but also α-IL-22 had on significant influence on FGF secretion of ESCs ($P > 0.05$) (**Figure 3G**).

Thus, it could be concluded that IL-22 up-regulated the expression of the invasion-related molecules and repressed the expression of metastasis suppressor molecule CD82 in ESCs, stimulated the invasiveness of ESCs, and might be involved in the origin and development of adenomyosis.

## Discussion

Adenomyosis is influenced by estrogens, and its pathogenesis may be linked to local hyperestrogenism [25], which may be mediated through the action of aromatase on androgen precursors or by the activity of estrone sulfatase or dehydrogenases [26]. Wang *et al.* reported that estrogen receptor alpha (ERα) was positively correlated with the expression of IL-22 in peripheral blood [27]. Partly similar with the study by Qin *et al.* [28], in our study, we observed that IL-22 and its receptor IL-22R1 and IL-10R2 levels in eutopic endometrium and ectopic lesion of adenomyosis were significantly higher than that in normal endometrium. Thus, the high level of IL-22 in ectopic lesion of adenomyosis may be mediated by local hyperestrogenism. However, this postulation needs to be further investigated.

IL-22 has important functions in host defense at mucosal surfaces as well as in tissue repair [29]. Adenomyosis is also caused by trauma, known as tissue injury and repair [30]. The response to any implant is wound healing comprised of inflammation and tissue remodeling. The wound healing process involves extensive tissue remodeling through production of extracellular matrix (ECM) components, remodeling enzymes, cellular adhesion molecules, growth factors, cytokines and chemokine genes. It has reported that IL-22 production is influenced by cytokines such as IL-17, IFN-α, IFN-γ, or TNF-α. These cytokines also strongly enhance IL-22R and IL-10R2 expression, thereby increasing their responsiveness to IL-22 [31, 32]. Moreover, IFN-α differentiates monocytes into DCs that produce IL-23, a crucial driver for IL-22 production [33]. We found that rhIL-22 could up-regulate the expression of IL-22R1 and IL-10R2. It has reported that IL-22 also promotes the production of inflammatory mediators, such as IL-6, granulocyte colony-stimulating factor (G-CSF), IL-1β, and lipopolysaccharide (LPS)-binding protein (LBP) [31, 34]. These researches indicate that abnormal high level of IL-22 and its receptor may be one of the results of endometrium injury and inflammation. Furthermore, IL-22 can lead to the amplification of this positive feedback effect in an autocrine manner.

Recent researches have shown that IL-22 also regulates the biological behaviors of several cancer cells, such as proliferation, apoptosis, migration and invasion [20, 22, 35-37]. Our previous work has demonstrated that IL-22 stimulates the proliferation of ESCs from endometriosis [23]. Thus we postulated that IL-22 might not only participate in tissue remodeling of endometrium and myometrium, but also modulate the biological function of ESCs of adenomyosis, which are associated with the occurrence and continuation of adenomyosis. Then we found that both exogenous and endog-

enous IL-22 enhanced the invasiveness of ESCs of eutopic endometrium from women with adenomyosis *in vitro*.

Taking into account the key role of metastasis suppressor molecules in regulating the invasiveness of ESCs [7, 10, 38], we further analyzed the IL-22 on the expression of CD82 in ESCs, and found that rhIL-22 and α-IL-22 resulted in the decrease and increase of CD82 level on ESCs of eutopic endometrium from women with adenomyosis, respectively. This finding indicates that IL-22 in eutopic endometrium and ectopic lesion of adenomyosis may promote the invasion of ESCs through down-regulating CD82 expression.

Cytokines and chemokines are the important mediators for ESCs function [39, 40]. IL-22 is essential for the release of chemokines such as CXCL1, CXCL5, and CXCL9, as well as IL-6 and G-CSF from airway epithelial cells during pneumoniae infection [34]. Subsequently, we investigated the role of IL-22 in the regulation of IL-8, RANTES, IL-6, VEGF and FGF, and confirmed that IL-22 from ESCs led to the significant elevation of IL-8, RANTES, IL-6 and VEGF levels in the supernatant. These findings suggest that high level of IL-22 may promote the invasion of ESCs through stimulating the secretion of IL-8, RANTES, IL-6 and VEGF. As well known that angiogenesis also plays a key role in the origin and development of adenomyosis. As angiogenic factors, VEGF and FGF were involved in this process [41, 42]. Therefore, IL-22 may induce the angiogenesis in adenomyosis by stimulating the production of IL-8, IL-6, VEGF not FGF.

Therefore, based on other researches and our findings, it can concluded that IL-22 possibly induced by estrogen and endometrium inflammation, on the one hand, stimulates tissues inflammation and triggers tissues remodeling; on the other hand, participates in the formation of immune microenvironment in ectopic foci through stimulating IL-8, RANTES, IL-6 and VEGF production. These integral effects will promote the invasion and angiogenesis of ESCs and contribute to the origin and development of adenomyosis. Our data bring new insight into the effective mechanisms of IL-22 in pathogenesis of adenomyosis. Further research is warranted to elucidate the reason for abnormal high level of IL-22 in eutopic endometrium and ectopic lesion from women with adenomyosis, and explore the functions and significance of estrogen and IL-22 in the coordination between ESCs and other cells of endometriotic tissue and the progress of adenomyosis.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Ming-Qing Li, Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, No. 413, Zhaozhou Road, Shanghai 200011, China. Tel: 86-21-63457331; Fax: 86-21-63457331; E-mail: mqli1311@gmail.com

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