

Macrophage-derived Netrin-1 participates in endometriosis-associated pain

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Research

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Abstract

Background: Endometriosis is a common disease in reproductive-age women and usually causes pelvic pain. Endometriosis pain is considered as a kind of neuropathic pain and infiltrating nerve fiber in endometriotic lesions may play an important role. Netrin-1 is widely reported as an axon guidance cue that regulates axonal attraction or rejection in neural injury and regeneration. In this study, we aim to determine the role of Netrin-1 in endometriosis-related pain.

Methods: Peripheral blood, peritoneal fluid, and endometrial tissues were sampled from women with (n=37) and without (n=23) endometriosis. Serum Netrin-1 concentrations, endometrial expression levels of Netrin-1 and its receptors including DCC, A2BAR, UNC5B, UNC5C and DSCAM were assessed. The polarization phenotypes of the peritoneal macrophages were identified by detecting the marker expression of M1 (CD86+) /M2 (CD163+) macrophages via flow cytometry. Lipopolysaccharide (LPS) and interferon gamma (IFN- γ) stimulated human monocytic cell line (THP-1) and rat alveolar macrophage-derived cell line (NR8383) cells to induce M1 phenotype macrophages. The expression levels of M1 markers and Netrin-1 in THP-1/NR8383 cells were determined.

Results: The expression levels of Netrin-1 in serum and endometriotic lesions were significantly higher in women with endometriosis when compared with those in women without endometriosis ($P<.05$), and both were correlated with pain symptoms ($P<.05$). Netrin-1 was co-expressed with CD 68 (a macrophage marker) in endometriotic lesions, and was synthesized and secreted by THP-1 and NR8383 cells in process of M1 polarization. In women with endometriosis, peritoneal macrophages were polarized towards M1 phenotype. In addition, increased expression of DCC and A2BAR, and decreased expression of UNC5B, UNC5C and DSCAM in endometriotic lesions were found.

Conclusions: These results suggest that Netrin-1 production by macrophages in endometriotic lesions may play an important role in endometriosis pain.

Background

Endometriosis is a common gynecological disease among women of childbearing age, characterized by pain and infertility [1]. Pain symptoms in patients with endometriosis include dysmenorrhea, dyspareunia, dysuria, defecation pain and chronic pelvic pain, which have a significant impact on women's quality of life [2]. However, the exact mechanisms of endometriosis-associated pain remain unclear up to date despite extensive research efforts [3, 4].

In 2000, Anaf et al. firstly reported S100-labeled nerves infiltrating in endometriotic lesions, and the percentages of nerves were significantly higher in the lesions of women suffered from severe endometriosis pain [5]. In subsequent studies, elevated specific makers for sensory, sympathetic, and parasympathetic nerves [6–8] and growth factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurite growth factor 2 (NEGF2) [9–11] were identified in different types of endometriotic lesions, which were correlated with endometriosis pain. Obviously, endometriosis pain

may be considered as a kind of neuropathic pain. Moreover, in a rat model of endometriosis, auto-transplanted endometriotic lesions developed autonomic and sensory innervation [12]. Growth-associated protein 43 (GAP-43), a marker for neurite outgrowth and regeneration, was expressed in nerve fibers infiltrating in the endometriotic lesions of peritoneal and deep infiltrating endometriosis [13, 14]. However, it is still unclear how the nerve fibers in endometriotic lesions sprout abnormally.

Netrins is a member of the laminin superfamily and has a similar amino acid terminal sequence [15]. The name Netrin is derived from the Sanskrit *Netr*, meaning 'guide' as its role in axon guidance [16]. Until now, three secreted Netrins (Netrins 1, 3 and 4), and two glycosylphosphatidylinositol-anchored membrane proteins, Netrins G1 and G2, have been identified in mammals. Netrin Gs regulate axon guidance by forming synaptic interactions between neurons with the transmembrane Netrin G ligands NGL1 and 2. The secreted Netrins can bind to the receptors of Deleted in Colorectal Cancer (DCC), neogenin or Uncoordinated (UNC5) A–D, causing axonal attraction or rejection [17, 18]. Recent studies have shown that Netrins also participate in angiogenesis, cell proliferation, migration and tumorigenesis by binding to the receptors of DCC, neogenin, UNC5, Down's syndrome cell adhesion molecule (DSCAM), CD146 and A2B adenosine receptor (A2BAR) [19–22].

It has been demonstrated that Netrin–1, the most studied guidance cue, triggers attraction effect through DCC and neogenin, or repulsion effect via UNC5 A-D [15]. In the central nerve system, Netrin 1 is secreted by ventricular zone neural progenitors and floor-plate cells in the ventral embryonic spinal cord [23, 24]. In the process of peripheral neural injury and regeneration, increased levels of Netrin–1 are mainly produced by Schwann cells [25]. Netrin–1 expression was increased in hypoxia conditions [22], inflammation and various diseases such as obesity [26], type 2 diabetes [27], acute lung injury [28], atherosclerosis [29] and abdominal aortic aneurysm (AAA) [30]. However, it is not clear whether Netrin–1 is involved in the pathogenesis of endometriosis.

Co-localization of Netrin–1 and CD68, a marker of macrophage, is identified in atherosclerotic plaques [29], adipose tissues [26] and inflamed aortic vessel wall [30], suggesting that macrophages may participate in inflammation by secreting Netrin–1. In fact, the inflammatory microenvironment of endometriosis can promote macrophage infiltration [31, 32], which play a crucial role in the etiology and pathogenesis of this disease including inflammatory response [33], angiogenesis [32], proliferation [34] and neurogenesis [35]. The interaction between macrophages and nerve fibers contributes to neuroinflammation and pain generation in endometriosis [35]. A large number of up-regulated molecules released by nerve fibers in endometriotic lesions such as monocyte chemotactic protein–1 (MCP–1), colony-stimulating factor 1 (CSF–1) and leukemia inhibitory factor (LIF), are responsible for the recruitment of macrophages from vessels within the lesions [36, 37]. On the other hand, infiltrated macrophages in the lesions in turn mediate neurogenesis by secreting neurotrophins, semaphorins, and vascular epithelial growth factor (VEGF) [35, 37, 38]. Although macrophage activation is closely related to endometriosis, yet, macrophages differentiate into classically activated macrophages (pro-inflammatory, M1) or alternatively activated macrophages (anti-inflammatory, M2) [39], and the polarization of M1/M2

macrophages in endometriosis remains highly debated [31, 32, 40, 41], Therefore, it is necessary to explore the role of macrophage-mediated Netrin-1 in the pathogenesis of endometriosis.

In the present study, we aimed to investigate the role of Netrin-1 mediated by macrophages in endometriosis-associated pain. To do so, we firstly detected expressions of Netrin-1 and its receptors in endometriotic lesions. Secondly, we localized Netrin-1 and macrophages in endometriotic lesions. Thirdly, we determined polarization phenotypes of peritoneal macrophages. Finally, we observed the expression and secretion of Netrin-1 after macrophage M1 polarization was induced *in vitro*.

Methods

Patients

A total of 60 women with (case group, n=37) and without endometriosis (other benign gynecologic diseases, control group, n=23) undergoing laparoscopic surgery at the Women's Hospital between January 2018 and June 2018 were recruited in this study. In endometriosis group, 17 women were at stage I-II while 20 of them were at stage III-IV, according to the Revised American Fertility Society Scoring system. Pain symptoms were observed in 48.6% (n = 18) and 0% (n = 0) of the endometriosis and non-endometriosis group, while infertility was 24.3% (n = 9) and 17.4% (n = 4), respectively. None of participants have received hormone therapy 6 month before surgery.

Samples Collection

Peripheral blood samples were obtained 1 day before the surgery while endometriotic lesions and endometrial tissues were collected during the surgical procedure. The bloods or cell supernatants were centrifuged at 1000 g for 10 minutes, and the supernatants were transferred into 1.5 mL tubes and stored at -80°C until processing. Half of the endometrial tissue was frozen in -80°C for mRNA detection, and the other half was immersed in formalin (Solarbio) for further immunohistochemical and immunofluorescence staining.

ELISA

The concentrations of Netrin-1 in serum or culture supernatants were quantified by ELISA kits (Cusabio) according to the manufacturer's instructions.

qRT-PCR and Western blot analyses

Total RNA was extracted from endometrial samples or cells with TRIzol reagent (Takara) and reversed by PrimeScript Reverse Transcription (RT) reagent kit (Takara) according to the manufacturer's recommendations. SYBR Premix Ex Taq kit (Takara) was used to quantitative polymerase chain reaction (PCR) and the fold change was determined through $2^{-\Delta\Delta Ct}$ method. The primers for Netrin-1, receptors and inflammatory factors were synthesized from Generay and the sequences were listed in Table 1 and Table 2. Western blot analysis was used to test Netrin-1 protein expression levels with Netrin-1 antibody (Abcam) in THP-1 and NR8383 cells in M1 polarization process.

Immunohistochemical Staining

Specific antibody of Netrin-1 (Abcam), DCC (Bioss), UNC5B (Bioss) and A2BAR (Invitrogen) were used to access the expression levels and localization of Netrin-1 and its receptors in endometrial tissues by immunohistochemistry (IHC). The sum of the percentage (0-3) and intensity scores (0-3) were represented as IHC scores to show the expression levels of molecule. Detailed immunohistochemical process and scoring were described before [10].

Double Immunofluorescence Staining

Slides of endometriotic lesions were incubated with primary antibody of Netrin-1 and CD68 (Abcam), and then were rinsed in PBS before mounted with corresponding fluorescent secondary antibody (Abcam).

Flow cytometric analysis

Peritoneal macrophages were washed with erythrocyte lysis buffer and incubated with Human Fc Block (BD Biosciences) on ice. Subsequently, the cells were incubated PE-CyTM7-conjugated anti-CD86 (BD Biosciences), and PE-conjugated anti-CD163 (BD Biosciences) antibody for 15 min. For intracellular staining, the cells were fixed and permeabilized in Fixation and Permeabilization Solution (BD Biosciences) for 20 min, and incubated with FITC-conjugated anti-CD68 (BD Biosciences) antibody for 1 h. The samples were then analyzed with a FACS Verse system and analyzed with BD FACS DIVA software (BD Biosciences, United States). PE-CyTM7-conjugated IgG1, PE-conjugated IgG1 and FITC-conjugated IgG2b (BD Biosciences) antibody served as control antibodies. CD68+CD86+CD163- macrophages were identified as M1 macrophages, while CD68+CD86-CD163+ cells were identified as M2 macrophages.

Cells Intervention

Human monocytic cell line THP-1 and rat alveolar macrophage-derived cell line NR8383 cells were purchased from Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured with Dulbecco's modified Eagle Medium/F-12 containing 15% fetal calf sera (Gibco). THP-1 cells were induced to macrophages (M0) by phorbol-12-myristate-13-acetate (PMA, 20 ng/mL, Sigma). Then, differentiated human THP-1 macrophages and NR8383 cells were treated with lipopolysaccharide (LPS, 20 ng/mL, Sigma) and interferon gamma (IFN- γ , 20 ng/mL, Peprotech) to further induce M1 phenotype macrophages. Then expression levels of M1 phenotype markers tumor necrosis factor alpha (TNF)- α , interleukin (IL)-1 β , IL-6, MCP-1 and nitric oxide synthase 2 (iNOS) were assessed at 1 h, 3 h, 6 h, 12 h, 24 h and 48 h after stimulation. Meanwhile, mRNA and protein expression levels of Netrin-1 as well as secreted Netrin-1 levels in cell supernatants were measured using qRT-PCR, Western blot and ELISA respectively.

Statistics

Data were analyzed using SPSS Version 24.0 (IBM). The continuous data variables were quantified as mean \pm SEM. Differences in variables between two groups and multiple groups were analyzed using unpaired Student t test and one-way ANOVA, respectively. Nonparametric testing was used where sample sizes were insufficient to confirm normality of data distribution. Statistical tests (χ^2 and Mann-Whitney U test) were performed to compare the frequency and median among groups. *P* values of $<.05$ were considered statistically significant.

Results

Characteristics of the Patient

There were no significant differences between endometriosis group and non-endometriosis group with respect to their age, gravidity, parity, abortion, infertility, or cycle stage, except for pain symptoms (Table 3).

Netrin-1 concentrations in serum is associated with endometriosis pain in the patients

The serum concentrations of Netrin-1 in endometriosis women were significantly higher than those from women without endometriosis ($P<.05$, Fig. 1A), and were correlated with endometriosis pain ($P<.05$, Fig. 1B).

Netrin-1 was over-expressed in endometriotic lesions

Netrin-1 was not only expressed in epithelial and interstitial vascular endothelial cells, but also expressed in endometrial stromal cells in endometriotic lesions from endometriosis women with (Fig. 1E) or without (Fig. 1F) pain, and in endothelial cells in eutopic endometrium from women with (Fig. 1G) or without (Fig. 1H) endometriosis. The mRNA expression levels of Netrin-1 in endometriotic lesions were significantly higher than those in eutopic endometrium from women with ($p<.01$, Fig. 1C; $p<.01$) or without endometriosis ($p<.01$, Fig. 1C; $p<.00001$), and both were correlated with endometriosis pain ($p<.01$, Fig. 1D; $p<.01$). Moreover, Netrin-1 was co-expressed with CD 68 in endometriotic lesions (Fig. 1I-L).

Endometriosis polarizes peritoneal macrophages towards the M1 phenotype

CD68+ cells were recognized as peritoneal macrophage in women with endometriosis (Fig. 2 A, C) or without endometriosis (Fig. 2 B, D). Flow cytometry plots revealed a significantly higher proportion of M2 macrophages (CD86-CD163+) in non-endometriosis women compared to endometriosis women (Fig. 3C). The proportion of M1 macrophages (CD68+CD163-) was high in endometriosis women, but it did not reach statistical significance (Fig. 3A). In short, the proportion of CD86+ macrophages significantly increased in endometriosis patients compared with those without endometriosis, while the proportion of CD163+ macrophages did not differ (Fig. 3E, F).

Netrin-1 was secreted by Macrophages

PMA treatment induced significantly higher mRNA expression levels of IL-1 β ($P<.01$), IL-6 ($P<.05$), iNOS ($P<.01$) and lower TNF- α ($P<.01$) mRNA expression levels and unchanged MCP-1 ($P>.05$) and Netrin-1 ($P>.05$) mRNA expression levels in human THP-1 macrophages compared with untreated monocytic THP-1 cells. Then, mRNA expression levels of M1 phenotype markers TNF- α , IL-1 β , IL-6, MCP-1 and iNOS were all significantly elevated after combined stimulation of LPS and IFN- γ within 48 h, reaching their peaks at 1 h ($P<.05$; Fig. 4A), 1 h ($P<.01$; Fig. 4B), 6 h ($P<.01$; Fig. 4C), 24 h ($P<.01$; Fig. 4D) and 48 h ($P<.01$; Fig. 4E), respectively. The mRNA, protein expression levels and supernatant concentrations of Netrin-1 in PMA-differentiated human THP-1 macrophages were significantly higher at 12 h ($P<.05$; Fig. 4F), 24 h ($P<.05$; Fig. 4G) and 48 h ($P<.01$; Fig. 4H) respectively after M1 polarization.

In NR8383 cell line, the mRNA expression levels of TNF- α , IL-1 β , IL-6, MCP-1 and iNOS all increased with 48 h after LPS and IFN- γ treatment, reaching their peak at 6 h ($P<.01$; Fig. 4I), 6 h ($P<.05$; Fig. 4J), 3 h ($P<.05$; Fig. 4K), 6 h ($P<.05$; Fig. 4L) and 12 h ($P<.05$; Fig. 4M), respectively. Meanwhile, the mRNA and protein levels as well as supernatant concentrations of Netrin-1 in NR8383 cells were significantly elevated at 12 h ($P<.05$; Fig. 4N), 24 h ($P<.05$; Fig. 4O) and 48 h ($P<.0001$; Fig. 4P) respectively after M1 polarization.

Netrin-1 receptors was more expressed in endometriotic tissues

The mRNA expression levels of DCC and A2BAR in endometriotic lesions were significantly higher than those in eutopic endometrium from women with ($P<.01$; $P<.05$) or without ($P<.01$; $P<.01$) endometriosis (Fig. 5A, I). On the contrary, UNC5B, UNC5C and DSCAM mRNA expression levels in endometriotic lesions were significantly lower than those in eutopic endometrium from women with ($P<.01$; $P<.0001$; $P<.01$) or without ($P<.0001$; $P<.0001$; $P<.01$) endometriosis (Fig. 5D, E, G). Eutopic endometrium from endometriosis women also showed a significantly higher UNC5C mRNA expression levels than those from non-endometriosis women ($P<.05$; Fig. 5E). No statistical differences in UNC5A, UNC5B or CD146 mRNA expression levels among endometriotic lesions and eutopic endometrium from women with or without endometriosis were found (Fig. 5C, F, H).

Immunohistochemistry results showed that DCC was mainly expressed in epithelial and stromal cells and interstitium in endometriotic lesions (Fig. 6A-C), and showed a higher IHC score in endometriotic lesions than those in eutopic endometrium from women with or without endometriosis ($P<.0001$). UNC5B was mainly expressed in glandular epithelial cells (Fig. 6D-F) and was less expressed in endometriotic lesions than those in eutopic endometrium from women with ($P<.01$) or without ($P<.05$) endometriosis. A2BAR was widely expressed in endometriotic lesions (Fig. 6G-I), and the IHC scores were higher than those in eutopic endometrium from endometriosis ($P<.05$) and non-endometriosis ($P<.05$) women.

Discussion

This study demonstrated that Netrin-1 increased in serum and endometriotic lesions in endometriosis women. Since Netrin-1 is an axon guide molecule, we speculate that Netrin-1 mediates endometriosis pain by promoting nerve fiber infiltration in endometriotic lesions. Actually, Netrin-1 has bi-functionality on axonal guidance. It has been reported that Netrin-1 leads to axon attraction binding to DCC receptor or repulsion binding to UNC5A-D receptors in the same cells [15]. Neogenin, DSCAM and CD146 also show promoting effect on axon extension [18]. The bi-functionality on axon guidance is based on the crystal structure of Netrin-1 and its receptors [42]. Besides, cross-link with different receptor types and the charge changes on Netrin-1 and receptors also determine promotion or inhibition of Netrin-1 in axon guidance [18]. Our study demonstrated that endometriotic lesions showed a significantly higher expressions levels of DCC, neogenin and lower expression levels of UNC5B and UNC5C compared with eutopic endometrium, which further supported that Netrin-1 is responsible for endometriosis pain by promoting nerve infiltration in endometriotic lesions. Dorsal root ganglion (DRG) neurons express DCC, neogenin and UNC5A-D receptors [43]. In a rat model of sciatic nerve transection, the expression of DCC receptor is up-regulated while the expression of UNC5B and UNC5C is down-regulated in sensory neurons [43]. Transplantation of Netrin-1 overexpression bone marrow mesenchymal stem cells promotes axon regeneration and functional recovery of the sciatic nerve after crush injury [44]. However, Netrin-1 treatment (500 ng/mL) inhibits neurite outgrowth of adult DRG neurons in explant and dissociated cultures, which may be mediated by UNC5A-C receptors on DRG [45]. Thus, the effect of Netrin-1 on Nerve outgrowth is complex and the mechanism of Netrin-1 involved in endometriosis pain remains to be further studied.

M1 macrophages, activated by IFN- γ , LPS or TNF- α , participate in tissue injury, inflammatory and immune responses by producing pro-inflammatory cytokines and chemokines [46]. In contrast, M2 macrophages can be activated by IL-4, IL-10, IL-13, or transforming growth factor- β (TGF- β), thus participating in tissue repair tumor angiogenesis and vessel normalization [47]. In endometriosis, several studies have reported that the macrophages in women with endometriosis are predominantly of M2 phenotype (CD163+/CD206+), which play an important role in the development of endometriosis [31, 41, 48–50]. As endometriosis is an estrogen-dependent disease, it has been reported that estrogen promotes M2 polarization through activation of the signal transducers and activators of transcription (STAT3) and P38-mitogen activated protein kinases (MAPK) pathway [51, 52]. However, another study shows opposed result that 17 β -estradiol represses suppressor for M2 polarization via inhibiting the JAK1-STAT6 signaling pathway [53]. Takebayashi et al. have reported that macrophage population slants toward M1 in the endometrium of endometriosis patients due to significantly lower ratio of the number of CD163+ or CD206+ macrophages to CD68+ macrophages [40]. In this study, we found that the peritoneal macrophages of endometriosis were mainly of CD86+CD163+ type, while those of non-endometriosis women were mainly of CD86-CD163+ type (M2). The percentage of CD86+ macrophages in endometriosis women was significantly higher than that in control group, which displayed a unique M1/M2 polarization signature that was skewed towards the classical M1 activation phenotype. This was consistent with the subsequent results of experiments *in vitro* that M1 polarization induced up-regulation of Netrin-1 synthesis and secretion in human and rat cell line. A recent study also has reported that Netrin-1 enriched macrophages are those that highly expressed pro-inflammatory markers as Netrin-1 mRNA expression levels are increased in CD68+/CD206- pro-inflammatory phenotypes rather than CD68+/CD206+ samples [30]. Actually, polarization is a dynamic process as the signals are temporally and dynamic, and the use of terms M1 and M2 is confusing due to the lack of specific phenotypic scoring criteria [54, 55]. Many physiological or pathological macrophages did not show a clear M1 or M2 phenotype [56], or macrophages with combinations of M1 and M2 markers can be found during M1/M2 polarization [57, 58]. New methods and technical advances are needed to reassess activation and classification of macrophage.

Netrin-1 gene is a direct transcriptional target of nuclear factor (NF)- κ B, which up-regulates Netrin-1 in colorectal carcinoma and mammary epithelial cells in response to inflammation [59]. However, another study has reported that activation of NF- κ B represses Netrin-1 expression levels in adenocarcinomic alveolar epithelial cells and dermal microvessel endothelial cells [28]. Actually, macrophages from endometriosis patients show a statistically significant higher proportion of NF- κ B nuclear translocation, and release various cytokines, growth factors and angiogenic factors to participate in endometrial fragment adhesion, proliferation and neovascularization [33]. Since we only tested cell lines *in vitro*, primary macrophages from endometriosis women are more valuable depending on the amount of cytokine, time of exposure, and the competition for cytokine [54]. Thus, the regulatory mechanism of Netrin-1 in endometriosis macrophages needs to be further studied.

Netrin-1 also plays a role in angiogenesis, cell migration, cell proliferation and cell survival. The present study confirmed the higher expression of Netrin-1, constant expression of CD146 and lower expression

of UNC5B in endometriotic lesions. Netrin-1 enriched macrophages also highly express pro-angiogenic markers [30] and treatment of Netrin-1 with low doses (50–200 ng/mL) on endothelial cells promotes proliferation, migration and tube formation via binds to high affinity receptor CD146 [19]. However, high concentrations of Netrin-1 (1000–2000 ng/mL) inhibit the above effects, possibly via UNC5B signaling pathway [19]. In endometriosis, nerve fibers are accompanied by immature blood vessels within endometriotic lesions [13]. Netrin-1 also dose-dependently regulates cell migration of Schwann cell, endothelial cell via activating or inhibiting MAPK pathway by CD146 or UNC5B receptor [19, 60, 61]. In inflammatory, it has been reported that Netrin-1 in endothelial cells inhibits inflammatory cell migration, such as leukocyte and macrophage [30, 62, 63]. This may be an anti-inflammatory response in the body, but it can lead to the accumulation of inflammatory cells in the lesions, resulting in AAA or atherosclerosis [29, 30]. In endometriosis, macrophage retention in endometriotic lesions increases the local concentration of Netrin-1, which may play a role in the infiltration of nerve fibers. In tumorigenesis, Netrin-1 promotes cell survival, proliferation, invasion and migration in different types of cancer, such as prostate carcinoma, hepatocellular carcinoma, gastric cancer and breast cancer [64–67]. Although endometriosis is a benign gynecological disease, it has malignant behaviors in adhesion, proliferation, invasion, metastasis and recurrence [68–71]. Thus, elevated Netrin-1 may be also involved in the growth of endometriotic lesions.

Conclusions

In summary, the present study indicated that increased Netrin-1 in women with endometriosis may participate in endometriosis pain. Target therapy toward Netrin-1 and macrophages may not only interfere with the process of endometriosis pain, but also inhibit the progression of endometriosis.

Abbreviations

LPS: Lipopolysaccharide; IFN- γ : interferon gamma; NGF: nerve growth factor; BDNF: brain derived neurotrophic factor; NEGF2: neurite growth factor 2; GAP-43: Growth-associated protein 43; DCC: deleted in colorectal cancer; UNC5: uncoordinated; DSCAM: Down's syndrome cell adhesion molecule; A2BAR: A2B adenosine receptor; AAA: abdominal aortic aneurysm; MCP-1: monocyte chemotactic protein-1; CSF-1: colony-stimulating factor 1; LIF: leukemia inhibitory factor; VEGF: vascular epithelial growth factor; TNF- α : tumor necrosis factor alpha; IL: interleukin; iNOS: nitric oxide synthase 2; DRG: dorsal root ganglion; TGF- β : transforming growth factor- β ; MAPK: mitogen activated protein kinases.

Declarations

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Authors' contributions

S. D., J.W and X. Z. designed research studies. S. D. and X. G. performed the majority of the experiment. L. Z., T. L. and Q. Y. acquired and analyzed data. S. D. and X. G. wrote the manuscript. J. W. and X. Z. edited the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Human Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (no. 20190012).

Consent for publication

Written informed consent was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Primer sequences of Netrin-1 receptors.

Primer	Forward (5'-3')	Reverse (5'-3')
ctin (h*)	AGAAGGATTCTATGTGGCG	GGATAGCACAGCCTGGATAGCA
etrin-1 (h)	CGACCCCAAGAACGGCGACCCGCC	CCTCCTGCTCGTTCTGCTTG
CC (h)	AGCAGGGAGCTCTATGTCCA	ACTGACTTCTTCCTGCTCCG
eogenin (h)	CAGCCTGTGATTAGTGCCA	TCATAGGTGGAGGTCCTGG
NC5A (h)	CAAGGTTGCTGAGCTGCTG	GTCCAGGTGGAGTTCTGGG
NC5B (h)	TGTGCATGCAAATGCTGGAG	TGTCTGTGTCGAAGTCACGG
NC5C (h)	GCAAATGCTCGTGCTACCTG	TCAATGCTCACTTCCGGAC
NC5D (h)	TCAATGGTGGGCCTTTGT	ATTCGCTCCACACTTCCCAG
SCAM (h)	CAAGAGGTAGTGTGTTGCCAGC	AGACGACAGTGATGTACGCC
D146 (h)	CCCTCACACCAGACTCCAAC	GTTCGCTTTACGAGACGGG
2BAR (h)	CTGCAGACGCCACCAACTA	ATTCGTGGTCCATCCCAGG

* h=huam.

Table 2. Primer sequences of Netrin-1 and M1 phenotype markers.

Primer	Forward (5'-3')	Reverse (5'-3')
APDH (h*)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
etrin-1 (h)	CGACCCCCAAGAAGGCGCACCGCCC	CCTCCTGCTCGTTCTGCTTG
NF- α (h)	CGAGTGACAAGCCTGTAGCC	TGAAGAGGACCTGGGAGTAGAT
-1 β (h)	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
-6 (h)	ACTCACCTCTCAGAACGAATTG	CCATCTTGGAAAGGTTCAGGTTG
[CP-1 (h)	AAACTGAAGCTCGCACTCTCGC	AGGTGACTGGGCATTGATTG
JOS (h)	TTCAGTATCACAAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
apdh (r $\#$)	CTCATGACCACAGTCCATGC	TTCAGCTCTGGATGACCTT
etrin-1 (r)	CGTTACGCTCACTCTGTCGC	GCCTCCTGCTCGTTCTGCT
NF- α (r)	ACCATGAGCACGGAAAGCAT	AACTGATGAGAGGGAGCCA
-1 β (r)	AGTGAGGAGAATGACCTGTT	CGAGATGCTGCTGTGAGATT
-6 (r)	GCCAGAGTCATTAGAGCAATA	GTTGGATGGTCTTGGTCCTAG
[CP-1 (r)	TCGGCTGGAGAACTACAAGA	GCTGAAGTCCTTAGGGTTGA
JOS (r)	CTGCTTGTGCGGAGTGT	ATTCTTCCTGATAGAGGTGGT

* h=huam; # r=rat.

Table 3. Characteristics of patients.

Parameters	Endometriosis (n = 37)	Controls (n = 23)	P
Age (years), mean ± SEM	33.5 ± 1.0	30.6 ± 1.2	0.07
Gravidity, median (rang)	3 (0-5)	2 (0-5)	0.16
Parity, median (rang)	1 (0-2)	1 (0-4)	0.94
Abortion, median (rang)	2 (0-4)	1 (0-3)	0.06
Menstrual cycle phase, n (%)			0.40
Proliferative	29 (78.4)	20 (87.0)	
Secretory	8 (21.6)	3 (13.0)	
Pain symptoms, n (%)	18 (48.6)	0 (0)	<.0001
Infertility, n (%)	9 (24.3)	4 (17.4)	0.53
r-AFS			
I-II, n (%)	17 (45.9)	-	
III-IV, n (%)	20 (54.1)	-	

Figures

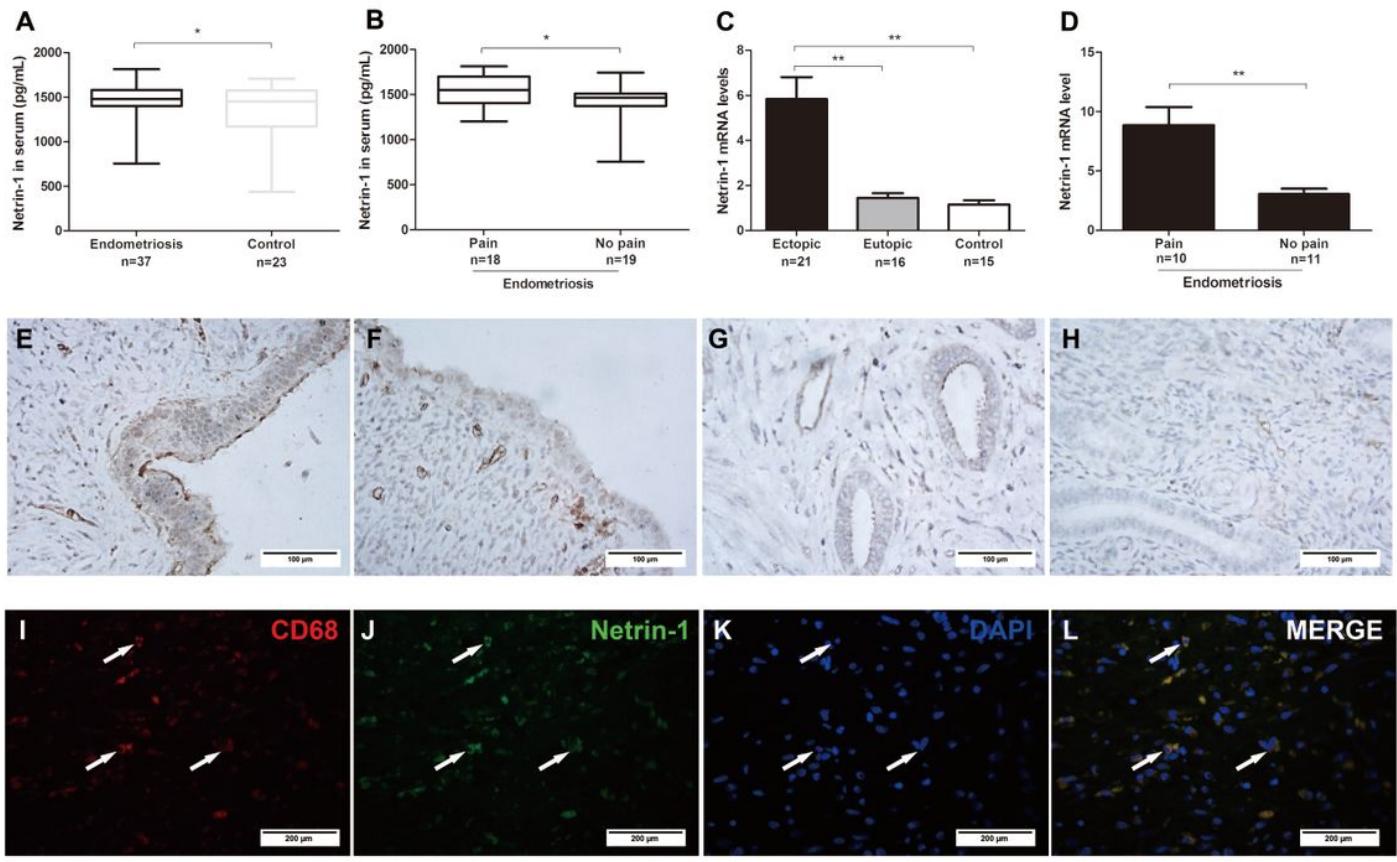
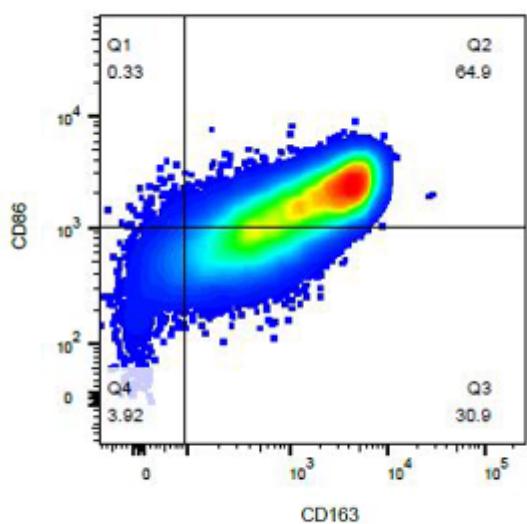
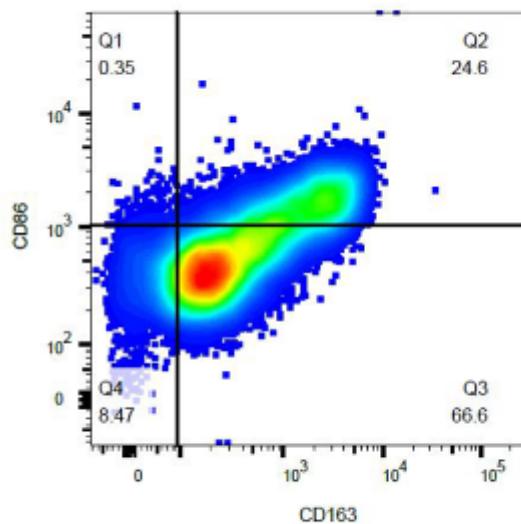
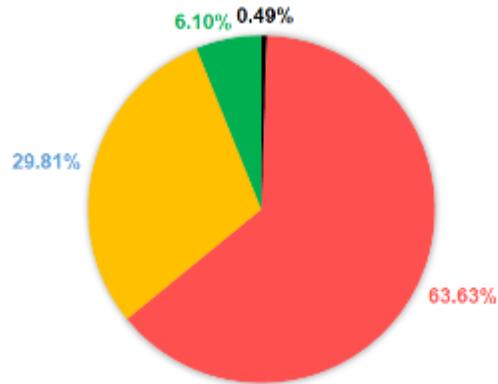
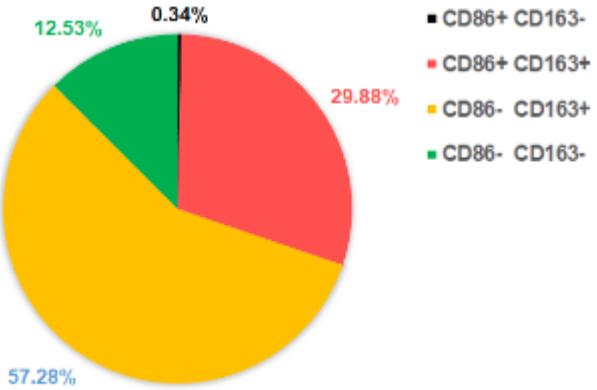


Figure 1

Netrin-1 levels in women with endometriosis. (A-D) The Netrin-1 concentrations in serum (A, B) and mRNA expression levels in endometrial tissues (C, D) were tested using RT-qPCR and ELISA respectively. (E-H) The location and expression levels of Netrin-1 in endometriotic lesions (E, F) eutopic endometrium with (G) or without (H) endometriosis were analyzed by immunohistochemical staining. (I-K) The location of Netrin-1 and CD68 in endometriotic lesions were tested by immunofluorescence double staining. Error bars show mean \pm SEM. * P<.05, ** P<.01, *** P<.0001. (One way ANOVA and Student t test).

A**B****C****D****Figure 2**

Polarized phenotypes of peritoneal macrophages in women with or without endometriosis.

Representative dot plot and statistical chart of CD68+ cells in peritoneal fluids from endometriosis (A, C, n=8) and non-endometriosis (B, D, n=6) women by flow cytometric analysis using CD86 and CD163 markers.

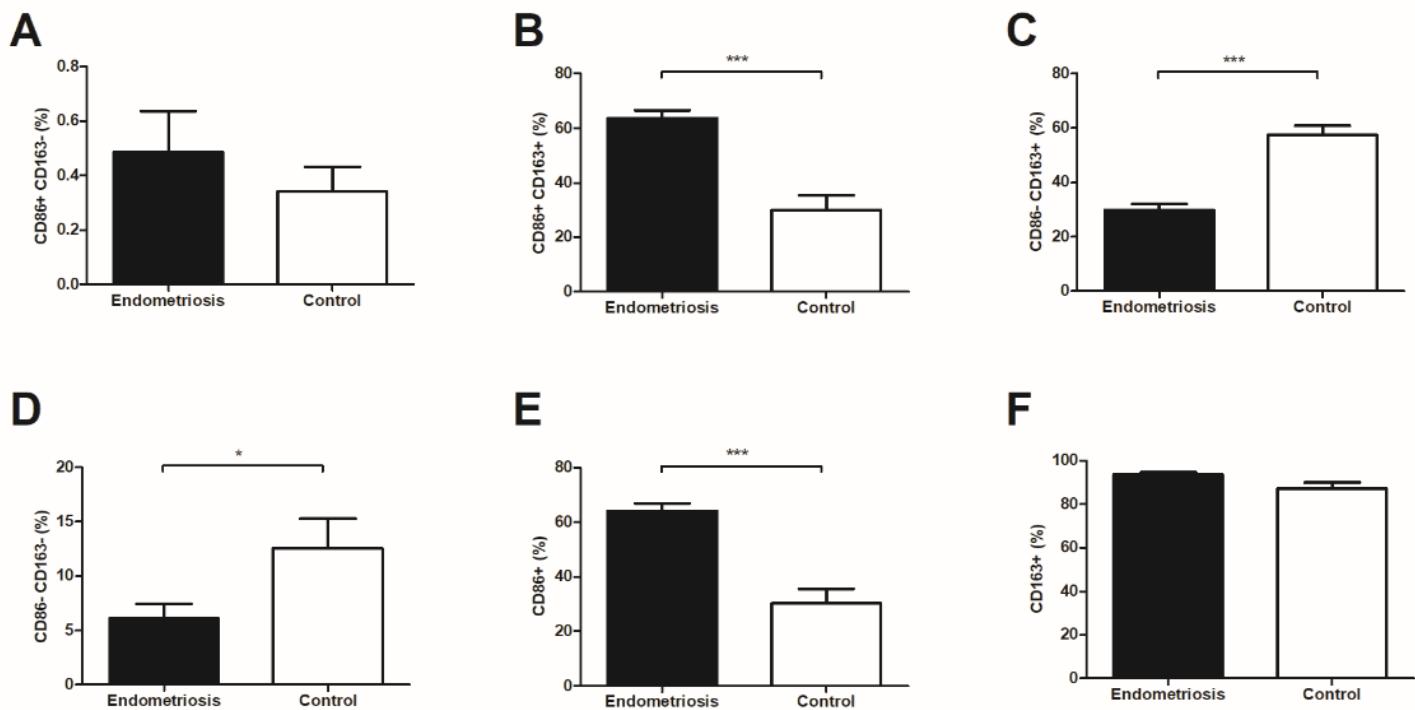


Figure 3

Different polarized phenotypes of peritoneal macrophages in endometriosis and non-endometriosis women. The percentages of CD86+CD163- (A), CD86+CD163+ (B), CD88-CD163+ (C), CD86-CD163- (D), CD86+ (E), and CD163+ (F) cells in each group. * P<.05, *** P<.0001. (Student t test).

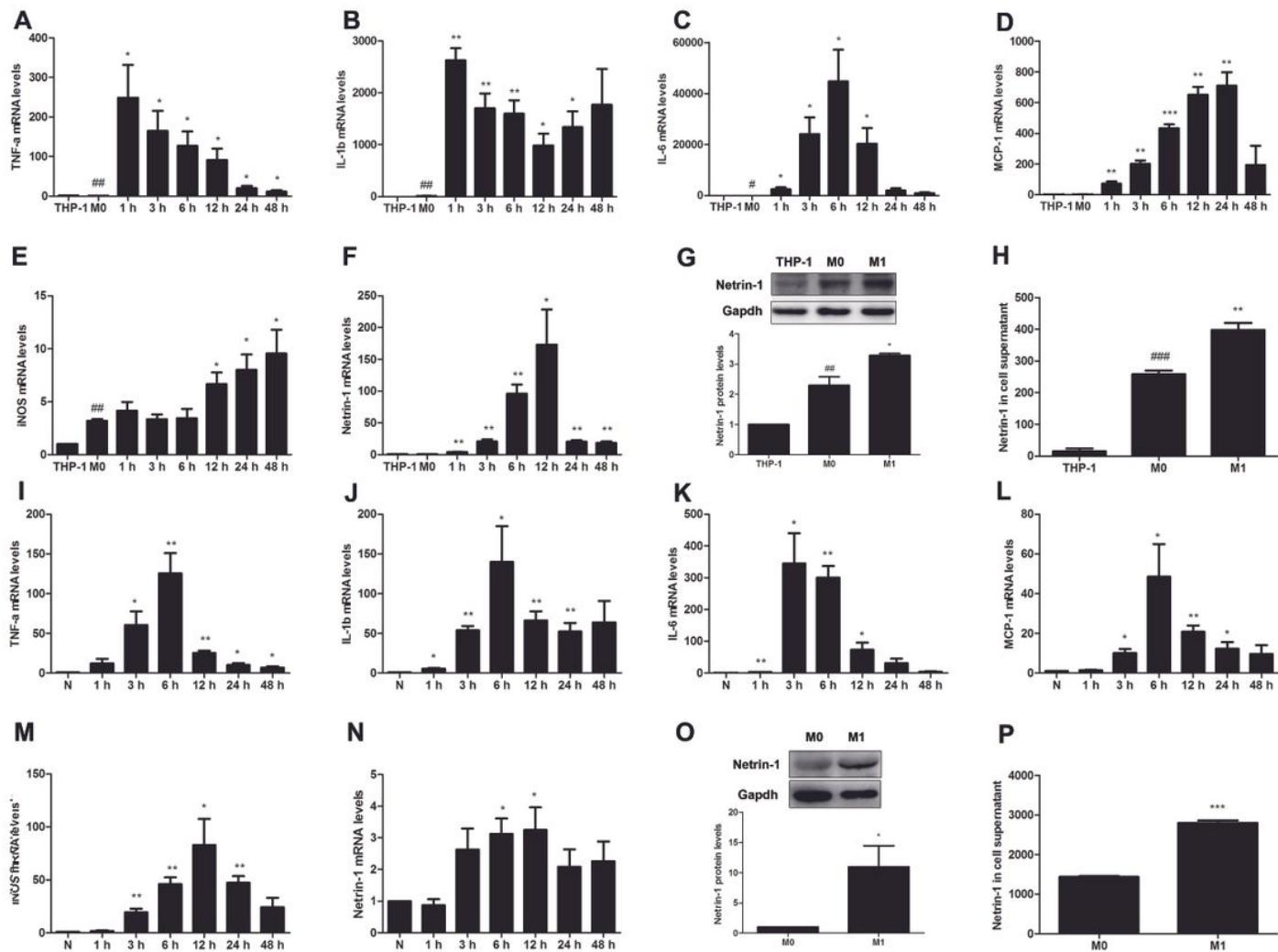


Figure 4

Expression levels of macrophage M1 phenotype markers and netrin-1 after M1 polarization in vitro. (A-H) The mRNA expression levels of TNF- α (A), IL-1 β (B), IL-6 (C), MCP-1 (D) and iNOS (E) in THP-1 cells were tested using RT-qPCR after PMA stimulation and combined stimulation of LPS and IFN- γ ; The mRNA (F) and protein levels (G) as well as supernatant concentrations (H) of Netrin-1 in THP-1 were measured using RT-qPCR, Western Blot and ELISA, respectively. (I-P) The mRNA expression levels of TNF- α (I), IL-1 β (J), IL-6 (K), MCP-1 (L) and iNOS (M) in NR8383 cells were tested using RT-qPCR after combined stimulation of LPS and IFN- γ ; The mRNA (M) and protein levels (O) as well as supernatant concentrations (P) of Netrin-1 in NR8383 cells were measured using RT-qPCR, Western Blot and ELISA, respectively. Error bars show mean \pm SEM. # Compared with THP-1, # P<.05, ## P<.01, ### P<.0001; * Compared with macrophage M0 phenotype, * P<.05, ** P<.01, *** P<.0001. (Student t test).

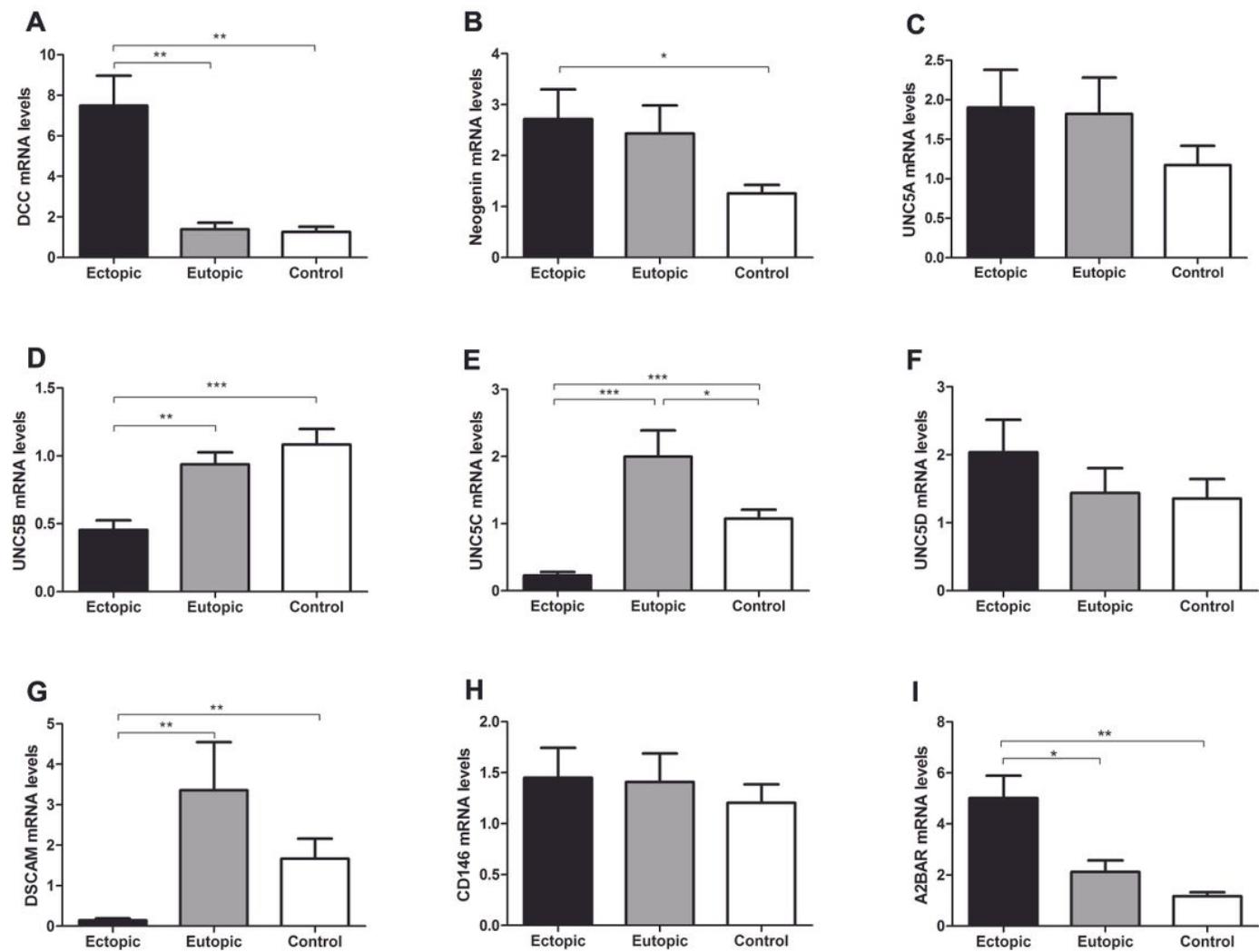


Figure 5

Expression levels of Netrin-1 receptors in endometrial tissues. The mRNA expression levels of DCC (A), Neogenin (B), UNC5A (C), UNC5B (D), UNC5C (E), UNC5D (F), Dscam (G), CD146 (H) and A2BAR (I) in endometriotic lesions and eutopic endometrium from women with or without endometriosis were tested using RT-qRCR. Error bars show mean \pm SEM. * P<.05, ** P<.01, *** P<.0001. (One way ANOVA and Student t test).

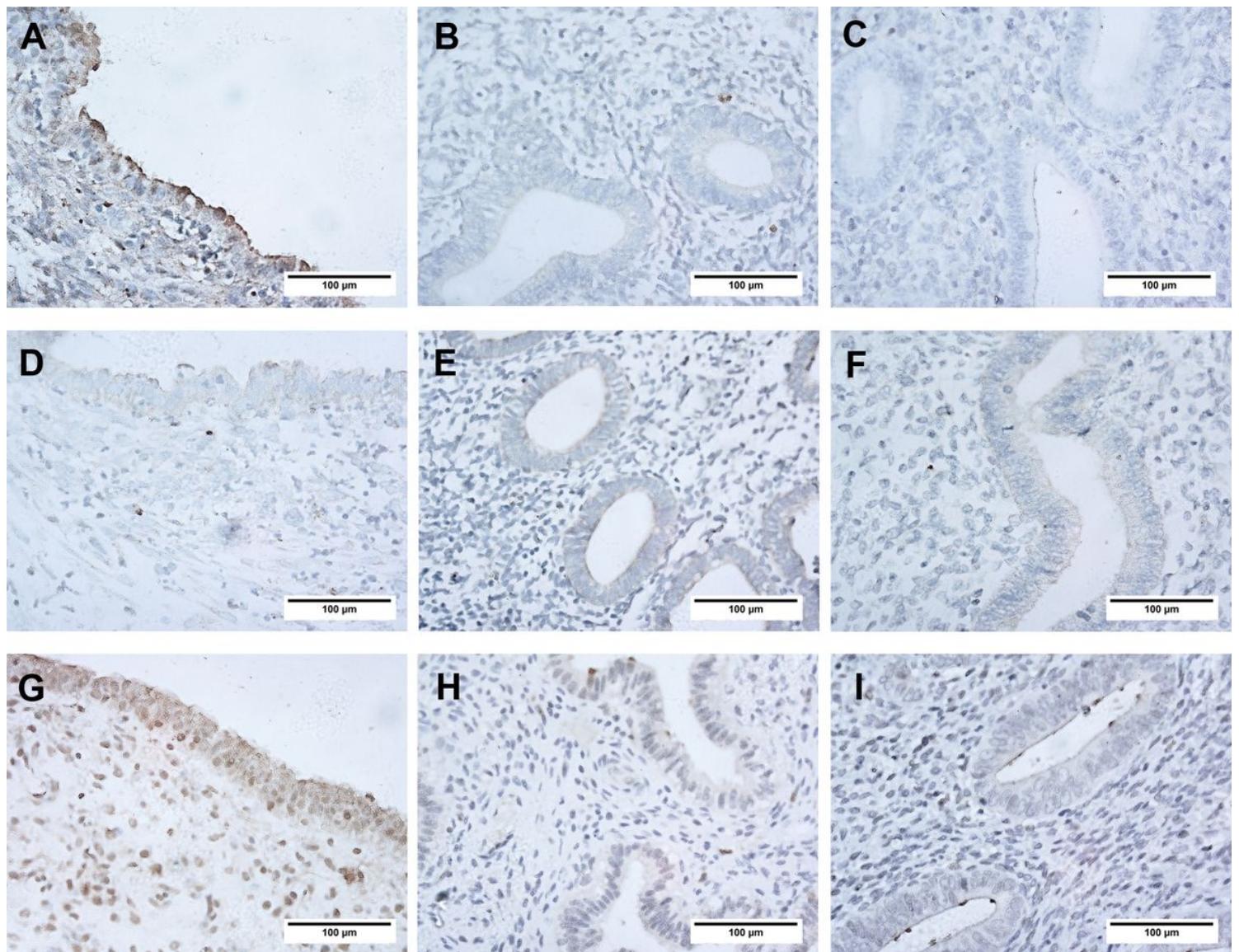


Figure 6

DCC, UNC5B and A2BAR immunoreactive staining in endometrial tissues. The locations and expression levels of DCC (A-C), UNC5B (D-F) and A2BAR (G-I) in endometriotic lesions (A, D, G) and eutopic endometrium from women with (B, E, H) and without (C, F, I) endometriosis were measured using immunohistochemistry analysis.