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Estrogen is an important mediator of mast cell activation in ovarian endometriomas

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Short title: E2 activates MC in ovarian endometriomas

Abstract

Endometriosis is an estrogen-dependent disease. Previous research has shown that abnormal enzymes associated with estrogen (E2) metabolism and an increased number of mast cells (MCs) in endometriomas are implicated in pathogenesis of endometriosis. However, it remains unclear how MCs mediate the role of E2 in endometriosis. Accordingly, we investigated whether E2 was associated with the number of MCs, and the rate of degranulation, in local ovarian endometriomas, as well as the role of E2 on MCs during the pathogenesis of endometriosis. Using enzyme-linked immunosorbent assay and immunohistochemistry, we found that concentrations of E2, and the number and activity of MCs, were significantly higher in ovarian endometriomas than in controls, and that these parameters were correlated with the severity of endometriosis-associated dysmenorrhea. By measuring the release of hexosaminidase, we found that the rate of RBL2H3 cell degranulation increased after E2 treatment. Furthermore, activation of RBL2H3 cells by E2 was found to trigger release of biologically active nerve growth factor, which promotes neurite outgrowth in PC12 cells and also sensitizes dorsal root ganglion cells via up-regulation of *Nav1.8* and transient receptor potential cation channel (subfamily V member 1) expression levels. When treated with E2, endometriotic cells could promote RBL2H3 cell recruitment by up-regulating expression levels of stem cell factor, transforming growth factor-β, and monocyte chemoattractant protein-1; these observations were not evident with control endometrial cells. Thus, elevated E2 concentrations may be a key factor for degranulation and recruitment of MCs in

ovarian endometriomas, which play a key role in endometriosis-associated dysmenorrhea.

Introduction

Endometriosis is characterized by the presence of functional endometrium outside of the uterine cavity, resulting in dysmenorrhea, dyspareunia, pelvic pain, and infertility (Eskenazi & Warner 1997). Endometriosis is considered as not only a chronic inflammatory disorder but also an estrogen-dependent disease (Burney & Giudice 2012, Xiong *et al*. 2015). Although serum estrogen (E2) levels are similar when compared between women with endometriosis and those without (Huhtinen *et al.* 2012), E2 concentrations in endometriotic lesions are known to be elevated (Rizner 2009). These elevated concentrations of E2 are often attributed to the up-regulated expression of aromatase (CYP19) and the down-regulated expression of 17β-hydroxysteroid dehydrogenase (17BHSD) type 2 and sulfate transferase in endometriotic lesions (Zeitoun *et al.* 1998, Zeitoun & Bulun 1999, Rizner 2009, Ferrero *et al.* 2014). Interestingly, the number of mast cells (MCs) and activated MCs is also increased in endometriotic lesions (Sugamata *et al.* 2005). Furthermore, stem cell factor (SCF), also known as MC growth factor, is also known to increase in the peritoneal fluid of women with endometriosis (Osuga *et al.* 2000). Basing on these findings, we hypothesized that E2 may be involved in the pathogenesis of endometriosis by mediating MCs.

MCs are key molecules in the immune system, particularly in terms of allergic reactions. In women with endometriosis, MCs accumulate around blood vessels and

in endometriotic lesions, are located extremely close to nerve fibers (Matsuzaki *et al.* 1998a, Anaf *et al.* 2006). In our previous animal studies, the number of MCs, and that of activated MCs in endometriotic lesions were associated with the levels of serum E2 and tumor necrosis factor-α (TNFA) and the expression of nerve growth factor (NGF) (Lin *et al.* 2015). Estrogen receptors are expressed on MCs (Zaitsu *et al.* 2007) and researches have shown that activated MCs release both TNFA and NGF (Church & Levi-Schaffer 1997, Kleij & Bienenstock 2005). These results suggest that MCs may mediate the role of E2 in the pathogenesis of endometriosis, although the direct effect of E2 upon TNFA and NGF cannot be excluded.

Recently, ultra-micronized palmitoylethanolamide (PEA, an endogenous fatty acid amide that has the capacity to stabilize MCs, thereby controlling inflammation associated with MC activation), was used to treat a rat endometriosis model (Iuvone *et al.* 2016). The results of this study showed that PEA-treated rats experienced less pain and had small endometriotic cysts, a low number of MCs, and low levels of both NGF and vascular endothelial growth factor in their endometriotic lesions (Iuvone *et al.* 2016). Our previous animal studies also showed that the number of activated MCs, and the ratio of degranulation/total number of MCs in endometriotic lesions, as well as serum TNFA levels, considerably decreased when model rats were treated with sodium cromoglycate, an MC stabilizer that inhibits the release of histamine (Zhu *et al.* 2015). These findings further support the fact that E2 promotes the growth of endometriotic lesions and triggers pain by activating MCs, which subsequently release a variety of mediators. However, how E2 recruits and activates MCs to

promote the growth of endometriotic lesions and thus trigger the pain associated with endometriosis requires further investigation.

In the present study, we aimed to investigate the effects of local E2 upon the recruitment and degranulation of MCs in endometriotic lesions and determine whether these cells are involved in the pain associated with endometriosis and the growth of endometriotic lesions. First, we determined the concentrations of E2, the number of activated MCs, and the ratio of degranulation/total number of MCs in ovarian endometriotic lesions. We also determined the correlations between local E2 levels, the number of degranulated MCs, and a variety of clinical parameters. Secondly, we investigated the effects of E2 on the degranulation of RBL-2H3 cells and then induced neurite outgrowth of PC12 cells, along with the peripheral sensitivity of dorsal root ganglion (DRG) cells, in response to high concentrations of E2, a condition similar to that seen in endometriosis. Finally, we observed the migration of RBL2H3 cells using a trans-well migration assay and detected the release of MC-associated mediators in ovarian endometriotic cells *in vitro*.

Materials and methods

Patients

We recruited 80 women undergoing laparoscopic surgery for ovarian endometriosis, uterine leiomyoma, and infertility in our hospital between January 2015 and October 2016. These patients were classified into two groups depending on surgical findings (Chapron *et al.* 2011): an endometriosis group (n = 41) consisting of subjects with histologically-proven endometriosis and a control group (n = 39) consisting of

subjects who did not show any macroscopic signs of endometriosis following meticulous exploration of the abdominal cavity during surgery. Endometriosis was graded according to the revised American Fertility Society (r-AFS) classification (Canis *et al*. 1997). Patient characteristics are shown in **Table 1**, which demonstrates clinical heterogeneity between different cases. The severity of pain was documented by using a standardized questionnaire with a visual analog scale (VAS) which was measured on a scale of 0–10; a score of 1–3 was considered mild, 4–6 as moderate, and > 6 as severe pain (Priya *et al.* 2016). The VAS score was self-assessed by each patient prior to treatment. All study subjects had a regular menstrual cycle, and none had received hormonal therapy for at least three months before surgery. In addition, we excluded all patients who suffered from other apparent systemic or local pain conditions, except for endometriosis-related dysmenorrhea, and those diagnosed with autoimmune diseases (Sinaii *et al*. 2002).

Tissue collection

Ectopic and eutopic endometrial samples were acquired from women with ovarian endometriomas who were undergoing hysteroscopy and laparascopy. Samples of normal endometrium were collected from patients undergoing hysterectomy with uterine leiomyoma or hysteroscopy with infertility. We routinely collected endometrial samples during or immediately after the surgical procedure. Specimens from women with or without endometriosis were fixed immediately in 10% neutral-buffered formalin for 24 h before processing and embedding in paraffin for immunohistochemistry in accordance with a standard protocol. Of these, specimens

from 17 women with endometriosis and 18 women without endometriosis were immersed in liquid nitrogen and stored at −80°C for enzyme-linked immunosorbent assay (ELISA). A number of specimens were also placed in Dulbecco's Modified Eagle Medium/F-12 (Sigma) at 4°C for endometrial cell culture. Endometrial histology was dated according to the general classifications described before (Kelm Junior *et al.* 2008). Each patient provided informed consent to participate in the study, which was approved by the Human Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University.

Isolation and culture of human ectopic and normal endometrial cells

Endometriotic cyst walls and normal endometria in proliferative and secretory phases were collected from women with or without endometriomas at the time of laparoscopy and hysteroscopy. The phase of the menstrual cycle was determined by menstrual history and confirmed histologically. Samples were collected, washed with 1× phosphate-buffered saline (PBS) and transferred to the laboratory on ice. Fresh samples were dissected into small pieces and digested with type I collagenase (Life Technologies, Carlsbad, USA) for 60-90 min. Debris was removed by 100 μm apertures sieves. Endometrial cells were then resuspended in DMEM/F12 medium containing 10% (V/V) fetal bovine serum and cultured at 37ºC in a humidified 5% CO_2 in air (V/V).

Cell lines

A rat basophilic leukemia (RBL2H3) cell line, a mucosal mast cell analog (Barsumian *et al*. 1981), was purchased from American Type Culture Collection (ATCC, USA)

and grown in minimum Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin. PC12 cell line was selected as a neuronal model and purchased from the Chinese Academy of Sciences (Shanghai, China); this model has originally described by Greene and Tischler (Greene & Tischler 1976). PC12 cells were cultured in a complete medium consisting of 85% F-12 medium (Sigma), 10% heat-inactivated horse serum (Gibco), and 5% fetal calf serum (Gibco). In addition, we purchased a rat DRG cell line from the European Collection of Cell Cultures (Public Health England, UK). This was cultured in DMEM/F12 medium (Sigma), supplemented with 20% fetal bovine serum (Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin. All these three cell lines were maintained at 37ºC in a humidified incubator with a 5% CO_2 atmosphere (V/V).

Determination of endometrial E2 concentration

The concentration of E2 in endometrial tissues was determined by an ELISA kit, which was used in accordance with the manufacturer's instructions (CSB-E05108h, CUSABIO, China). In brief, 100 mg of endometrial tissue was rinsed with 1× PBS, homogenized in 1 mL of 1× PBS, and stored overnight at −20ºC. Then, two freeze-thaw cycles were performed to break the cell membranes and the resulting homogenates were centrifuged at 5000 × g, 2–8ºC, for 5 min. The resultant supernatant was then removed and assayed immediately in accordance with the manufacturer's instructions. After 10 minutes, the optical density for each well was determined with a microplate reader set to 450 nm.

Immunohistochemical staining

Tissue blocks were prepared and sectioned at 4 μm using routine deparaffinization and rehydration procedures. Sections were incubated with anti-mouse tryptase primary antibody (dilution 1:800, ab2378, Abcam, Cambridge, MA, USA) and anti-rabbit c-kit primary antibody (dilution 1:200, ab32363, Abcam, Cambridge, MA, USA) for 60 min at room temperature. After washing with 1× PBS, the sections were incubated with Envision-labeled polymer-alkaline phosphatase mouse/rabbit (Envision/HRP/Mo, GK400105; Envision/HRP/Rb, GK400305/15, Novocastra, Newcastle upon Tyne, UK) for 60 min. The antigen–antibody reaction was then visualized using diaminobenzidine as a chromogen (GK346810, Novocastra, UK). After washing, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with a mounting medium. Tonsils were used as a positive control, and HeLa-cultured cells were used as a negative control.

Quantitation of degranulated MCs

Degranulated MCs were counted under a light microscope as previously described (Sugamata *et al.* 2005). In brief, the degranulated MCs possessed an irregular shape with an uneven color and a non-complete cell membrane surface, while the granulated MCs were round or oval with uniform color and an intact cell membrane surface. In each sample, the number of cells per field (×20 objective, ×10 ocular) were counted in a total of five fields. Results were then expressed as a mean and standard error of the mean (SEM). All slides were counted by two blinded observers.

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

The specific steps for RNA isolation, cDNA synthesis, and real-time RT-PCR were performed as previously described (Zenclussen *et al.* 2005). Real-time PCR was performed with an Applied Biosystems 7900HT system (Applied Biosystems, Foster City, CA, US) using SYBR Premix Ex TaqTM kit (Takara Bio, Inc.). Specific primers used for amplification were synthesized by Generay (Shanghai, China) (**Table 2**). For each sample, an average cycle threshold (Ct) value was calculated from triplicate wells and the fold change was determined using the $2^{-\Delta\Delta Ct}$ method.

Measurement of RBL2H3 cell degranulation

RBL2H3 cell degranulation was measured through the release of hexosaminidase (hex) (Dastych *et al.* 1999). First, RBL2H3 cells were distributed across 96-well flat bottom plates (5×10^4 cells /well), cultured for two days in estrogen-free medium, and then stimulated with different concentrations of E2 (E2759, Sigma, USA) (0, 1, 10, 100, 500, 1000, 10^4, 10^5 pmol/L) at different times (5, 10, 15, 30, 60, 120 min). Subsequently, degranulation was evaluated by the release of hex according to the protocol (Kuehn *et al.* 2010). Finally, plate absorbance was read at 405 nM with a reference filter at 620 nm, and the proportion of hex activity present in the supernatant was calculated as a percentage.

Observation of neurite outgrowth of PC12 cells

A bioassay using the PC12 cell line is commonly used to detect and measure biologically active NGF, which stimulates neurite outgrowth of PC12 cells (Woo *et al.* 1995). To detect and quantify the biological activity of NGF in RBL2H3 cell

supernatants after stimulation with E2, PC12 cells were re-plated onto 24-well culture plates at a concentration of 2×10^4 cells/well, and RBL2H3 cell culture supernatants (1×10^6 cells/mL), or serial dilutions of NGF as controls, were added to the wells. After 24 h, the number of PC12 cells showing neurite outgrowth was counted on a dark field inverted microscope (Nikon). Cells with at least two neurites which were more than 50 μm long, were judged as neurite outgrowth-positive cells. All samples were tested in duplicate, and quantification was performed in a blinded manner.

Co-culture of RBL2H3 cells and DRG cells *in vitro*

RBL2H3 cells were cultured in 0.4 μm filter inserts (Corning 3412, USA) in DMEM-F12 + 10% FBS until 70–80% confluency. For experimentation, the RBL2H3 cell-bearing inserts were rinsed with DMEM-F12 and placed in wells so as to overlay each DRG cell-derived culture. The total amount of medium used was 4 mL per well for 6-well plates (1.5 mL per insert, and 2.5 mL per well). For DRG monoculture, cells were cultured directly in wells with or without E2 stimulation (500 pmol/L). For the co-culture system, cultures were treated with either E2 or ICI182780 (S1191, Selleck, USA), as indicated. Then, 24 h after treatment, total RNA was extracted from DRG cells, and real-time RT-PCR was performed to detect whether MCs mediate the role of E2 in the peripheral sensitization of DRG cells.

Chemotactic movement of RBL2H3 cells

First, peritoneal fluid was collected from patients with or without endometriosis. Then, ovarian endometriotic cells and endometrial cells were cultured *in vitro*, and the cell supernatant collected after 24 h culture with E2 stimulation. The migration of

RBL2H3 cells was evaluated using the trans-well migration assay (8 μm, Corning 3422, USA). In the upper chamber, RBL2H3 cells were evenly-spread using serum-free medium (Opti-MEM, Gibco, USA) and 500 μl of either peritoneal fluid or cell supernatant was added to the lower chamber through the side wall. After incubation for different time periods, cells were stained with 0.1% crystal violet. Digital images were then acquired and five views per hole were quantified.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (San Diego, CA). All experiments were performed at least three times. For normally distributed data, we used the Student's t-test and one-way analysis of variance (ANOVA), followed by Scheffe's test, for group comparisons. Data that was not normally distributed was analyzed with the Mann-Whitney U test. Analysis of real-time PCR data was performed using transformed values. Correlations were assessed by Pearson correlation and statistical significance was defined as when $P < 0.05$.

Results

High concentrations of E2 in ovarian endometriotic lesions are positively correlated with endometriosis-related dysmenorrhea

No significant difference in age was observed between women with endometriosis and those without ($P = 0.694$). The concentrations of E2 were 1560.7 ± 90.2 pg/mL in ectopic lesions ($n = 17$), 1870.5 ± 269.0 pg/mL in eutopic endometrium ($n = 17$), and 622.9 ± 193.0 pg/mL in normal endometrium ($n = 18$). Ectopic lesions and eutopic endometrium had significantly higher E2 concentrations compared with normal

endometrium ($P < 0.001$). Moreover, the difference in E2 concentrations between ectopic and eutopic endometrium had statistical significance ($P < 0.001$). During the proliferative phase, eutopic and normal endometrium E_2 concentrations were higher ($n = 11$, 2060.7 ± 214.9 pg/mL; $n = 13$, 759.4 ± 175.6 pg/mL) than those in the secretory phase ($n = 6$, 1680.3 ± 195.0 pg/mL, $P = 0.003$; $n = 5$, 486.4 ± 148.7 pg/mL, $P = 0.007$), but there was no significant difference in E2 concentrations between the proliferative ($n = 9$, 1624.5 ± 274.5 pg/mL) and secretory phase ($n = 8$, 1496.9 ± 180.5 pg/mL) in ectopic lesions ($P = 0.282$). In addition, during the proliferative and secretory phase, E2 levels in ectopic lesions or eutopic endometrium were significantly higher than those of normal endometrium ($P < 0.001$; **Table 3**).

CYP19 expression was significantly higher in ectopic lesions when compared with either eutopic endometrium or control endometrium ($P < 0.001$), whereas 17BHSD2 expression was significantly higher in normal endometrium compared with eutopic endometrium ($P < 0.001$) or ectopic lesions ($P < 0.001$). Moreover, sulfate transferase expression was statistically higher in normal endometrium than in eutopic endometrium ($P = 0.002$), although the difference between normal and ectopic endometrium did not reach statistical significance ($P = 0.053$; Supplementary Figure 1).

Next, we investigated whether the concentrations of E2 in serum or ectopic lesions were associated with the severity of dysmenorrhea; Pearson correlation confirmed that E2 concentrations in ectopic lesions were moderately ($r = 0.575$, $P =$

0.016) correlated with the degree of dysmenorrhea, whereas the concentrations of E2 in serum were not (r = 0.339, P = 0.183**; Fig 1).**

A high number of MCs and the rate of degranulation in ovarian endometriotic lesions are positively correlated with endometriosis-related dysmenorrhea but not the size of ovarian endometriotic cysts

Tryptase-positive MCs were noted around blood vessels and the interstitium with fibrosis. The ratio of granulated/degranulated/total MCs (×20 objective, ×10 ocular, mean ± SEM) in ectopic lesions (2.6 ± 0.1/4.8 ± 0.3/7.4 ± 0.2) was greater than that in eutopic endometria (1.7 ± 0.1/0.6 ± 0.1/2.3 ± 0.2, P < 0.001) and normal endometria (1.0 ± 0.1/0.4 ± 0.04/1.4 ± 0.1, P < 0.001). Furthermore, the number of degranulated MCs was also significantly higher than granulated MCs in ectopic lesions (P < 0.001; **Fig 2A**).

C-kit (CD117) was expressed by almost all degranulated MCs and showed a similar distribution of tryptase. The number of degranulated MCs in ovarian endometriotic lesions (mean ± SEM, 4.6 ± 0.2) was also significantly higher when compared with endometriotic endometrium (mean ± SEM, 0.8 ± 0.1, P < 0.001) and control endometrium (mean ± SEM, 0.5 ± 0.1, P < 0.001; **Fig 2B**). Combined with clinical information, the number of MCs, and the rate of degranulation, were not significantly related with the menstrual cycle (P > 0.05).

Next, we investigated whether degranulated MCs were associated with clinical parameters; Pearson correlation showed that the total number of MCs, and the rates of degranulated/total MCs, were correlated with the severity of dysmenorrhea (r = 0.542,

299 $P < 0.001$; $r = 0.879$, $P < 0.001$), but not with the size of ovarian endometriotic cysts
300 ($r = -0.014$, $P = 0.933$; $r = 0.165$, $P = 0.302$; **Fig 3**).

301 **Increased rate of RBL2H3 cell degranulation in high E2 concentrations**

302 High concentrations of E2, a high number of MCs, and high rates of degranulation are
303 all significantly higher in ovarian endometriotic cysts than in controls. To determine
304 whether E2 concentration was correlated with MC degranulation rate, RBL2H3 cells
305 were cultured *in vitro* and treated with different concentrations of E2. Various
306 concentrations of E2 were shown to stimulate RBL2H3 cell degranulation ($P < 0.001$).
307 The maximum MC degranulation rate of RBL2H3 cells occurred at 500 pmol/L of E2
308 (**Fig 4A**), and although different E2 concentrations could trigger MC degranulation
309 within a 5 min time period, maximal rates of degranulation occurred at 15 min (**Fig**
310 **4B**).

311 **Activation of RBL2H3 cells by E2 can trigger the release of biologically active**
312 **NGF**

313 As MC degranulation was correlated with endometriosis-related dysmenorrhea, PC12
314 cells were then cultured *in vitro* and treated with the supernatant of RBL2H3 cells that
315 had been previously treated with 500 pmol/L E2. As shown in **Fig 5A**, 21.4% of PC12
316 cells cultured with medium alone expressed at least two neurites with sizes of ≥ 50
317 μm. At a concentration of 2ng/mL, NGF produced striking neurite outgrowth in PC12
318 cells, resulting in 58% of cells expressing neurites (**Fig 5F**). When the supernatants of
319 RBL2H3 cells incubated with 500 pmol/L E2 for 24 h were added to PC12 cells
320 instead of medium, the number of neurite-positive cells increased significantly (50.4%

± 5.0%, P < 0.001; **Fig 5D**). By contrast, supernatants from RBL2H3 cells incubated with medium alone (**Fig 5C**), or with an NGF receptor blocker (**Fig 5E**), did not promote neurite outgrowth. Thus, supernatants from RBL2H3 cells incubated with E2 are likely to contain biologically active NGF that can stimulate neurite elongation in PC12 cells.

Activation of RBL2H3 cells by E2 can promote the peripheral sensitization of DRG cells

We used a co-culture of RBL2H3 cells and DRG cells *in vitro*, which was stimulated with E2 (500 pmol/L) or ICI182780, to investigate whether E2 mediates the role of MCs in the peripheral sensitization of DRG cells. As shown in **Fig 6**, the expression levels of *Nav1.8* in DRG cells were up-regulated after E2 treatment during co-culture with RBL2H3 (P = 0.015). This up-regulated level of expression was partially blocked by ICI182780, although this effect was not statistically significant (P =0.121). Furthermore, the expression of transient receptor potential cation channel subfamily V member 1 (*Trpv1*) in DRG cells was also up-regulated after treatment with E2 (P = 0.020) and was blocked completely by ICI182780 (P = 0.015).

Endometriotic cells can promote RBL2H3 cell recruitment by up-regulating the expression levels of related cytokines

Although E2 concentrations in endometriotic lesions were correlated with the rate of MC degranulation, the relevance of high local concentrations of E2, the total number of MCs, and the effect of E2 on MC recruitment in endometriotic lesions remains unclear. Therefore, we created a micro-environment of high local concentration of E2,

which was similar to that seen in endometriotic lesions. The supernatant from ovarian endometriotic cells (**Fig 7D**) in the lower chamber space that was previously stimulated with 10^{-7} mol/L E2 for 24 h recruited more MCs compared with the control ($P < 0.001$; **Fig 7C**). Moreover, peritoneal fluid from patients with endometriosis (**Fig 7F**) also recruited more MCs than from patients without endometriosis ($P < 0.001$; **Fig 7E**).

MCs were recruited under the influence of E2 in endometriotic lesions, to investigate the release of key active mediators released from endometrial cells, we determined the mRNA levels of SCF, transforming growth factor-β (TGFB), and monocyte chemoattractant protein-1 (MCP1) by using RT-PCR in endometrial cells, which were induced by E2. The levels of SCF mRNA expression in both control endometrial cells and ovarian endometriotic cells increased gradually with increased intervention time, reaching maximal levels 24 h after intervention with E2. However, the expression level of SCF in ectopic endometrial cells was significantly higher than that of control endometrial cells ($P = 0.042$). Similar to SCF, the expression levels of TGFB and MCP1 mRNA also increased with increasing intervention time but reached a maximum at 12 h and then decreased. Nevertheless, following E2 treatment, the mRNA expression levels of SCF, TGFB, and MCP1 in ectopic endometrial cells were higher than that of control endometrial cells (**Fig 8**).

Discussion

Our previous research proved that the activity of MCs in endometriotic lesions was associated with serum E2 levels and also the expression levels of TNFA in the serum,

and NGF in endometriotic lesions (Lin *et al*. 2015). In this present study, we found that high levels of E2 in ovarian endometriomas could recruit and activate MCs, which were then able to release NGF and promote nerve growth and the sensitization of nerve fibers which may play a role in endometriosis-associated dysmenorrhea.

Our results showed that CYP19 expression in endometriotic lesions was increased, whereas the expression levels of 17BHSD2 and sulfate transferase in ovarian endometriotic lesions were reduced when compared with control endometrium. The aberrant expression of estrogen-metabolizing enzymes led to elevated E2 concentrations in local ovarian endometriomas. Combined with clinical parameters, we further confirmed that local high levels of E2 levels in endometriosis lesions were positively related to pain symptoms in patients with endometriosis; in contrast, the concentrations of E2 in serum were not. Several studies have demonstrated that high levels of E2 can not only promote ectopic endometrial cells to secrete a series of cytokines to participate in pain symptoms (Rizner 2009, Ferrero *et al*. 2014), but an also act on immune cells accumulating in the lesions to accelerate the disease process (Garzetti *et al*. 1993, Greaves *et al*. 2015). Interestingly, our study demonstrated that the number and activity of MCs were higher in ovarian endometriotic lesions compared with controls, as reported by other studies (Matsuzaki *et al.* 1998b, Sugamata *et al.* 2005). Moreover, a high number of MCs, and the rate of degranulation in ovarian endometriotic lesions, were also positively correlated with the extent of the associated dysmenorrhea but not with the size of ovarian cysts. Since MCs have been reported to express E2 receptors (Zaitsu *et al*. 2007), suggest that

local high levels of E2 may have an effect on the activation of MCs that are involved in endometriotic pain.

In our RBL2H3 cell culture experiments, we found that the degranulation of MCs increased swiftly after the cells were treated with E2; moreover, this effect did not require IgE cross-linking, suggesting that E2 treatment stimulates MCs to release mediators in a direct manner. Furthermore, the optimum concentration of E2 was equivalent to the local concentration of E2 in ovarian endometritic lesions, which may partly explain the high rate of degranulation in local endometriotic lesions.

Our results further provide evidence that E2 can trigger RBL2H3 cells to release biologically active NGF that can stimulate neurite elongation in PC12 cells, which is consistent with previous studies which reported that MCs can synthesize, store, and release NGF (Leon *et al*. 1994). On the one hand, NGF acts as a chemoattractant, thereby causing an increase in the number of MCs, as well as their degranulation (Horigome *et al*. 1993, Marshall *et al*. 1999, Rizner 2009), on the other hand, NGF can promote nerve growth and induce the expression of neuropeptides and lower the threshold of neurons for firing (Lindsay & Harmar 1989). As previously reported, MCs are located close to nerve fibers, which makes them an ideal candidate for modulating neural activity and nociception (Anaf *et al*. 2006, Aich *et al*. 2015). Additionally, using co-cultures of RBL2H3 cells and DRG cells *in vitro*, our results showed that the stimulation of MCs by E2 can up-regulate the expression of *Nav1.8* and *Trpv1* in DRG cells. Both of these mechanisms contribute to peripheral sensitization, a particular form of stimulus-evoked functional plasticity of the

409 nociceptor, to reduce its firing threshold and increase responsiveness (Zhuang *et al*.
410 2004). *Nav1.8* contributes the most to sustaining the depolarizing stage of action
411 potentials in nociceptive sensory neurons (Renganathan *et al*. 2001, Blair & Bean
412 2002), while *Trpv1* predominantly transmits heat and pain sensation and plays a role
413 in interactions between the inflammatory environment, pain and hyperalgesia
414 (Koerber *et al*. 2010). The former has been extensively reported to play a role in
415 neuropathic pain (Lai *et al*. 2003), while the latter has been reported to participate in
416 the process of pain in patients with endometriosis (Liu *et al*. 2012) or rat models of
417 endometriosis (Lian *et al*. 2017). The up-regulated expression of *Nav1.8* and *Trpv1*
418 under a co-culture environment of RBL2H3 cells under the stimulation of E2
419 indicated that E2 can stimulate MC degranulation to participate in the process of pain,
420 which may provide a partial explanation for the fact that local high levels of E2, and
421 the activity of MCs, have a positive correlation with endometriosis-related
422 dysmenorrhea. Furthermore, our data showed that *Nav1.8* and *Trpv1* were important
423 targeting molecules of MC-DRG cell interaction, which can also provide new
424 treatments for relieving endometriosis pain in addition to stabilizing MC in
425 endometriotic lesions (Joshi *et al*. 2009).

426 As previously documented, E2 can regulate the expression of the chemokine
427 receptors CCR4 and CCR5 in the HMC-1 cell line, as well as CCR3 and CCR5 in
428 BMMCs (Jensen *et al*. 2010), thus indicating an involvement in the recruitment of
429 MCs. Here, we tentatively propose that a micro-environment with high levels of E2
430 can promote the recruitment of MCs by up-regulating factors such as SCF, TGFB,

and MCP1 in ectopic endometrial cells, which may partly explain the higher number of MCs in endometriotic lesions than controls. Moreover, peritoneal fluid from patients with endometriosis can also recruit more MCs than patients without endometriosis, which is accordance with the discovery of increased levels of SCF, a multi-functional growth factor which plays an important role in the recruitment of MCs, in the peritoneal fluid of women with endometriosis compared those without (Osuga *et al*. 2000)..

In summary, our experiment proved that high local concentrations of E2 are associated with the number of MCs, and the rate of degranulation in local ovarian endometriotic lesions, and play a role in the pathogenesis of endometriosis-associated dysmenorrhea. Ectopic endometrial cells stimulated with E2 can promote RBL2H3 cell recruitment by up-regulating the expression levels of SCF, TGFB, and MCP1. Furthermore, high local levels of E2 can directly stimulate the degranulation of MCs, which can trigger the release of biologically active NGF and promote the peripheral sensitization of DRG cells, a process which may also play a role in endometriosis-related dysmenorrhea. (**Fig 9**).

While we believe that high local concentrations of E2 can induce the activation of MCs, which play a role in endometriosis pain, our study features some limitations which should be taken into account when interpreting our findings. First, we used an MC line as a target cell; these do not behave in the exact same manner as MCs in patients with endometriosis. As there are no known methods which can be used to separate primary MCs from specific phenotypes in patients with endometriosis, future

studies should be carried out using primary MCs from the peritoneal fluid of a rat model of endometriosis. Secondly, because our experimental ideas are based on previous animal experiments published in 2015 (Lin *et al*. 2015), this study draw conclusions mainly through the analysis of tissue samples from patients and experiments using different rat cell lines *in vitro* but without animal experiments. As this is a continuous study, we will further validate and deepen our research in the next animal experiments.

Endometriosis is widely viewed as an estrogen-dependent disease (Eskenazi & Warner 1997, Rizner 2009, Ferrero *et al.* 2014). Our present data highlights the importance of high local concentrations of E2 in mediating the degranulation and recruitment of MCs in ovarian endometriotic lesions, which are hypothesized to play an important role in endometriosis-related dysmenorrhea. Based on this point of view, we provide clinically-relevant evidence that the reduction of E2 in local endometriotic lesions could alleviate pain in patients, and further suggest that membrane stabilizers that inhibit MC degranulation may hold promise for endometriosis treatment in the future.

Declaration of interest

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

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625

Figure legends

Figure 1. High concentrations of E2 in ovarian endometriotic lesions are positively correlated with endometriosis-related dysmenorrhea. (A) Correlation between concentrations of E2 in the serum and the degree of dysmenorrhea in patients with endometriosis. (B) Correlation between concentrations of E2 in endometriotic lesions and the degree of dysmenorrhea in patients with endometriosis. Each dot represents data from an individual patient. The Pearson coefficient of correlation and the significance of this correlation are shown in the lower right corner.

Figure 2. Immunohistochemistry and statistical analysis of MCs in different endometrial tissues following staining with tryptase (A) and c-kit (B). The graph represents the number of MCs counted in five fields (×20 objective, ×10 ocular) for each patient, with error bars representing SEM. Ec: ectopic endometrium; Eu: eutopic endometrium; Nm: normal endometrium. Scale bars = 500 μm. ***P < 0.001.

Figure 3. A high number of MCs and the rate of degranulation in ovarian endometriotic lesions were positively correlated with endometriosis-related dysmenorrhea but not the size of ovarian endometriotic cysts. (A) Correlation between the number of MCs and the degree of dysmenorrhea. (B) Correlation between the rate of MC degranulation and the degree of dysmenorrhea. (C) Correlation between the number of MCs and the size of ovarian endometriotic cysts. (D) Correlation between the rate of MC degranulation and the size of ovarian endometriotic cysts. Each dot represents data from an individual patient. The Pearson

coefficient of correlation and the significance of this correlation are shown in the lower right corner.

Figure 4. Increased rate of RBL2H3 cell degranulation in high E2 concentrations. (A) Rates of degranulation after RBL2H3 cells were stimulated with various concentrations of E2 at 5, 10, 15, 30, 60, 120 min; the most suitable E2 concentration of RBL2H3 cells degranulation was 500 pmol/L (indicated by the arrow). (B) Rates of degranulation after RBL2H3 cells were stimulated with 500 pmol/L E2 over 120 min; peak degranulation rate occurred at 15 min (indicated by the arrow). For all experiments, n = 3. ***P < 0.001.

Figure 5. Activation of RBL2H3 cells by E2 can trigger the release of biologically active NGF. (A) PC12 cell complete culture medium. (B) PC12 cells cultured for 24 h with 500 pmol/L E2. (C) Supernatants from RBL2H3 cells (1×10^6 cells/mL) cultured for 24 h without E2 stimulation. (D) Supernatants from RBL2H3 cells (1×10^6 cells/mL) cultured for 24 h with 500 pmol/L E2. (E) Replicate activated RBL2H3 cell supernatants with 100 ng/mL of anti-NGF antibody. (F) 2 ng/mL NGF. After 24 h, the number of PC12 cells with at least two neurites that were approximately 50 μm or longer was determined on a dark field inverted microscope. Data are shown as mean ± SEM. For all experiments, n = 5. Scale bars = 200 μm. ***P < 0.001.

Figure 6. Activation of RBL2H3 cells by E2 can promote the peripheral sensitization of DRG cells. (A) *Nav1.8* (B) *Trpv1*. RQ: relative quantification. Data show mean ± SEM. For all experiments, n = 3. *P < 0.05, **P < 0.01.

Figure 7. Endometriotic cells with E2 treatment, and peritoneal fluid collected from patients with endometriosis, can promote RBL2H3 cell recruitment. (A) (Negative control) Endometrial cell complete culture medium. (B) (Standard control) Supernatants from normal endometrial cells cultured for 24 h without E2 stimulation. (C) Supernatants from normal endometrial cells cultured for 24 h with 10^{-7} mol/L E2. (D) Supernatants from ectopic endometrial cells cultured for 24 h with 10^{-7} mol/L E2. (E) Peritoneal fluid from patients without endometriosis. (F) Peritoneal fluid from patients with endometriosis. For all experiments, n = 3. Scale bars = 200 μm. **P < 0.01, ***P <0.001.

Figure 8. Up-regulation of the expression levels of SCF (A), TGFB (B), and MCP1 (C) in normal endometrial cells or ovarian endometriotic cells following E2 treatment. RQ: relative quantification. For all experiments, n = 3. *P < 0.05.

Figure 9. Schematic diagram of key findings. This study showed that high levels of E2 in ovarian ectopic lesions can promote MC recruitment by up-regulating the expression levels of SCF, TGFB, and MCP1 in ectopic endometrial cells. Additionally, high levels of E2 could directly trigger MC degranulation leading to the release of biologically- active NGF, which can promote nerve growth and the sensitization of nerve fibers by up-regulating the expression of *Nav1.8* and *Trpv1*.

Supplementary Figure 1. The aberrant expression of estrogen-metabolizing enzymes in different endometrial tissues. (A) CYP19 (B) 18BHSD2 (C) Sulfate transferase. RQ: relative quantification. Data show mean ± SEM. For all experiments, n = 3. **P < 0.01, ***P < 0.001.

65

Table 1 Characteristics of recruited patients

Patient characteristics	Endometriosis (n = 41)	Controls (N = 39)	P
Age (years)	Mean = 35.2 SD = 6.7	Mean = 34.7 SD = 5.7	0.694[t]
Serum E2 level	Median = 371.5 IQR = 541.0	Median = 250.5 IQR = 246.9	0.536[U]
Menstrual cycle (n, %)			
Proliferative phase	31(75.6%)	31(79.5%)	
Secretory phase	10(24.4%)	8(20.5%)	
rAFS stage (n, %)			
I	0	NA	
II	0	NA	
III	15 (36.6%)	NA	
IV	26 (63.4%)	NA	
Severity of dysmenorrhea (n, %)			
None	11 (26.8%)	34 (87.2%)	
Mild	8 (19.5%)	4 (10.3%)	
Moderate	9 (22.0%)	1 (2.6%)	
Severe	13 (31.7%)	0	
Cyst size (diameter, cm)	Mean = 5.2 SD = 1.7	NA	
Co-occurrence of peritoneal endometriosis			
No	9 (22.0%)	NA	
Yes	32(78.0%)	NA	
Co-occurrence of deep infiltrating endometriosis			
No	38 (92.7%)	NA	
Yes	3 (7.3%)	NA	

SD, standard deviation; IQR, interquartile range; rAFS, revised American Fertility Society; [t]Student's t-test; [U]Mann-Whitney U test

Table 2 List of primers used in this study

Gene	Species	Forward primer	Reverse primer
GAPDH	Human	GCCATCAATGACCCCTTCATT	TGACGGTGCCATGGAATTT
GAPDH	Rat	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
CYP19	Human	TGGACACCTCTAACACGCTCTTC	GAGCTTGCCATGCATCAAAA
17βHSD2	Human	AGGAATTGCGAAGAACCTGCT	CGGCCATGCATTGTTTGTAGT
SULT1E1	Human	TGCAGAAAAGAAAACCTCATGAAT	ATGACCAGCCACCATTAGAAA
SCF	Human	AACCCAGGTGCTTTGAGAAG	CAATGCCACACACTGAGACA
TGFB	Human	TAGACCCTTTCTCCTCCAGGAGACG	GCTGGGGGTCTCCCGGCAAAAGGT
MCP1	Human	AGGAAGATCTCAGTGCAGAGG	AGTCTTCGGAGTTTGGGTTTG
Nav1.8	Rat	TGAGACCTGGGAGAAGTTCG	AGCAGCGACCT CATCTTCAT
Trpv1	Rat	GACATGCCACCCAGCAGG	TCAATTCCCACACACCTCCC

SULT1E1, sulfotransferase family 1E member 1; SCF, stem cell factor; TGFB, transforming growth factor-β; MCP1, monocyte chemoattractant protein-1; Trpv1, transient receptor potential cation channel subfamily V member 1.

Table 3 Concentration of E2 in endometrium (pg/mL)

Group	Number	Mean ± SD	Proliferative phase (n)	Secretory Phase (n)
Ec	17	1560.7 ± 90.2***	1624.5 ± 274.5 (n = 9)***	1496.9 ± 180.5 (n = 8)***
Eu	17	1870.5 ± 269.0***	2060.7 ± 214.9 (n = 11)***	1680.3 ± 195.0 (n = 6)***#[1]
Nm	18	622.9 ± 193.0	759.4 ± 175.6 (n = 13)	486.4 ± 148.7 (n = 5)#[2]

*** indicates $P < 0.001$ for comparisons of E2 in each phase with normal endometrium while #[1] indicates $P = 0.003$ and #[2] indicates $P = 0.007$ for comparisons of E2 between the proliferative and secretory phase in one group. Ec: ectopic endometrium; Eu: eutopic endometrium; Nm: normal endometrium.

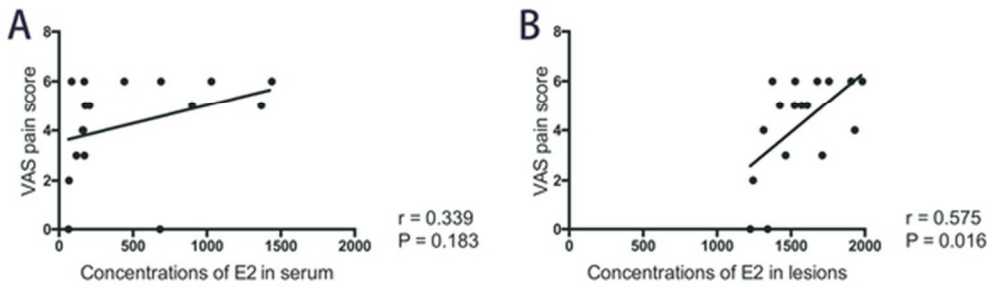

Figure 1. High concentrations of E2 in ovarian endometriotic lesions are positively correlated with endometriosis-related dysmenorrhea.

50x15mm (300 x 300 DPI)

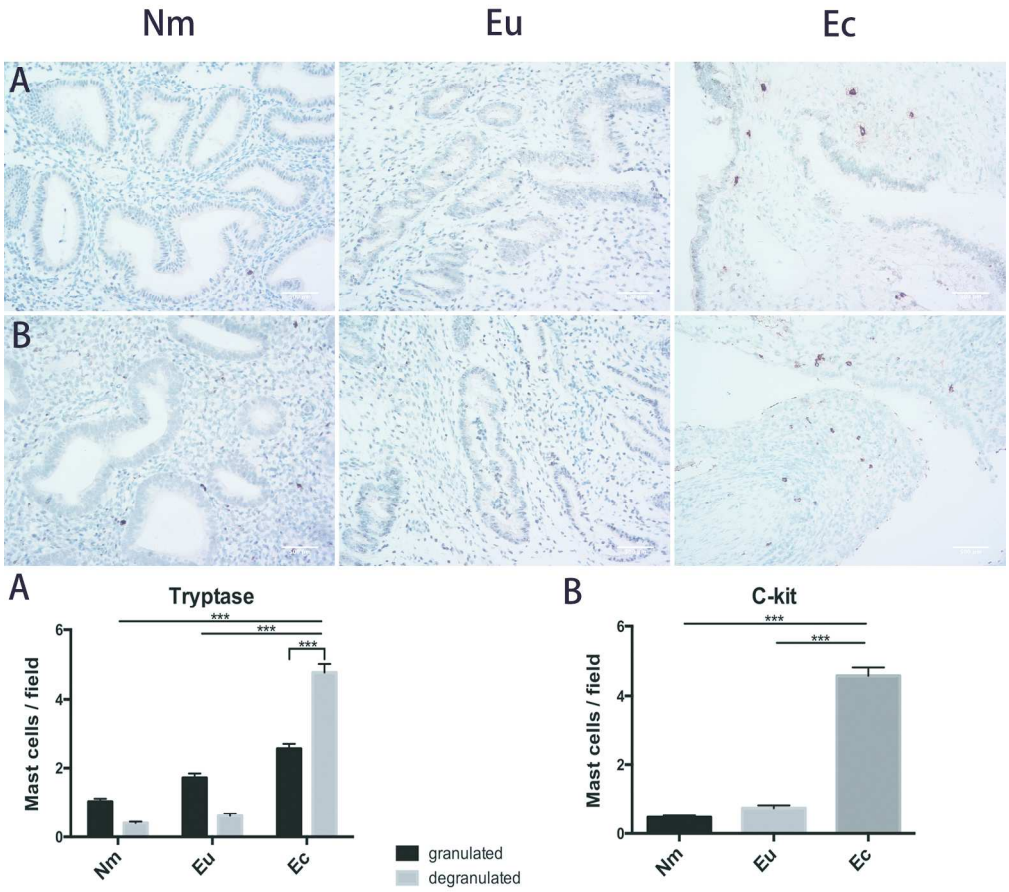

Figure 2. Immunohistochemistry and statistical analysis of MCs in different endometrial tissues following staining with tryptase (A) and c-kit (B).

209x195mm (300 x 300 DPI)

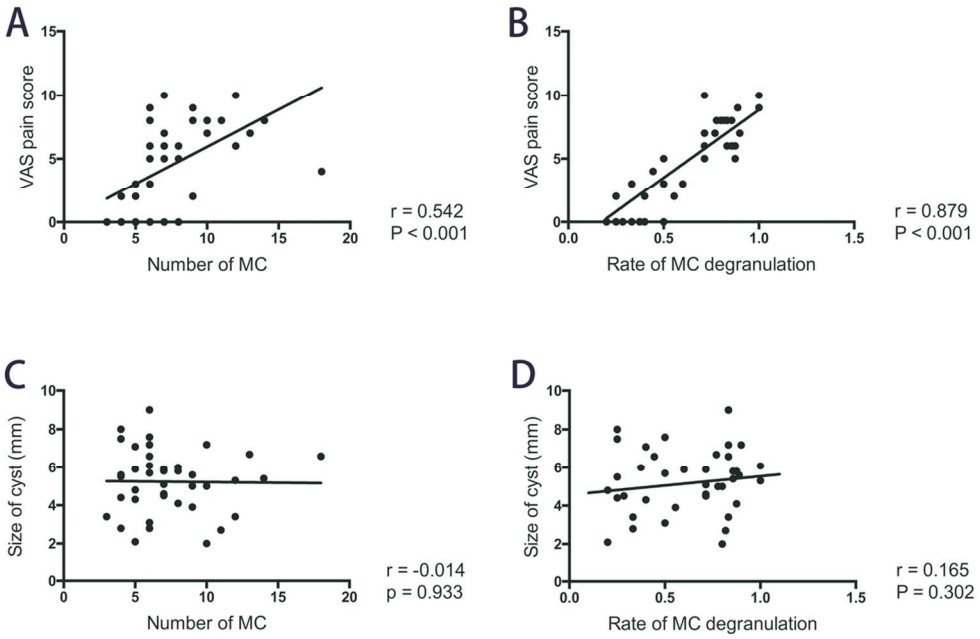

Figure 3. A high number of MCs and the rate of degranulation in ovarian endometriotic lesions were positively correlated with endometriosis-related dysmenorrhea but not the size of ovarian endometriotic cysts.

108x70mm (300 x 300 DPI)

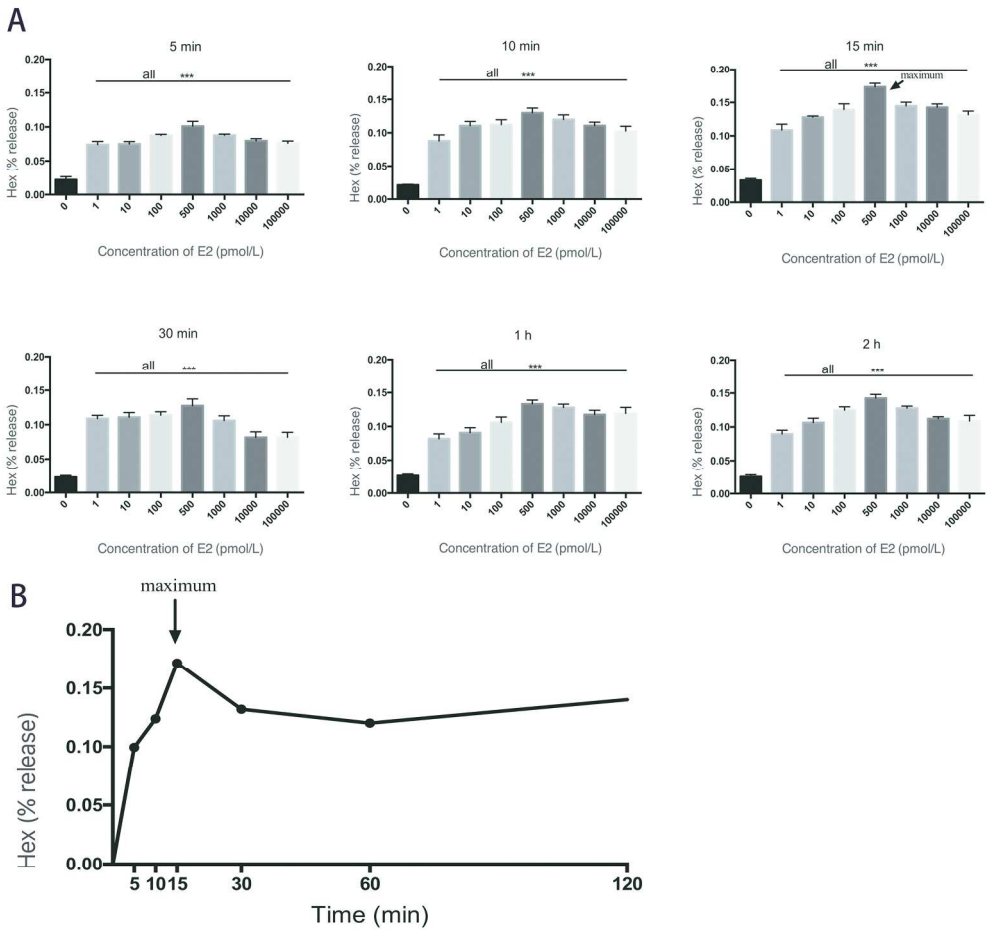

Figure 4. Increased rate of RBL2H3 cell degranulation in high E2 concentrations.

209x208mm (300 x 300 DPI)

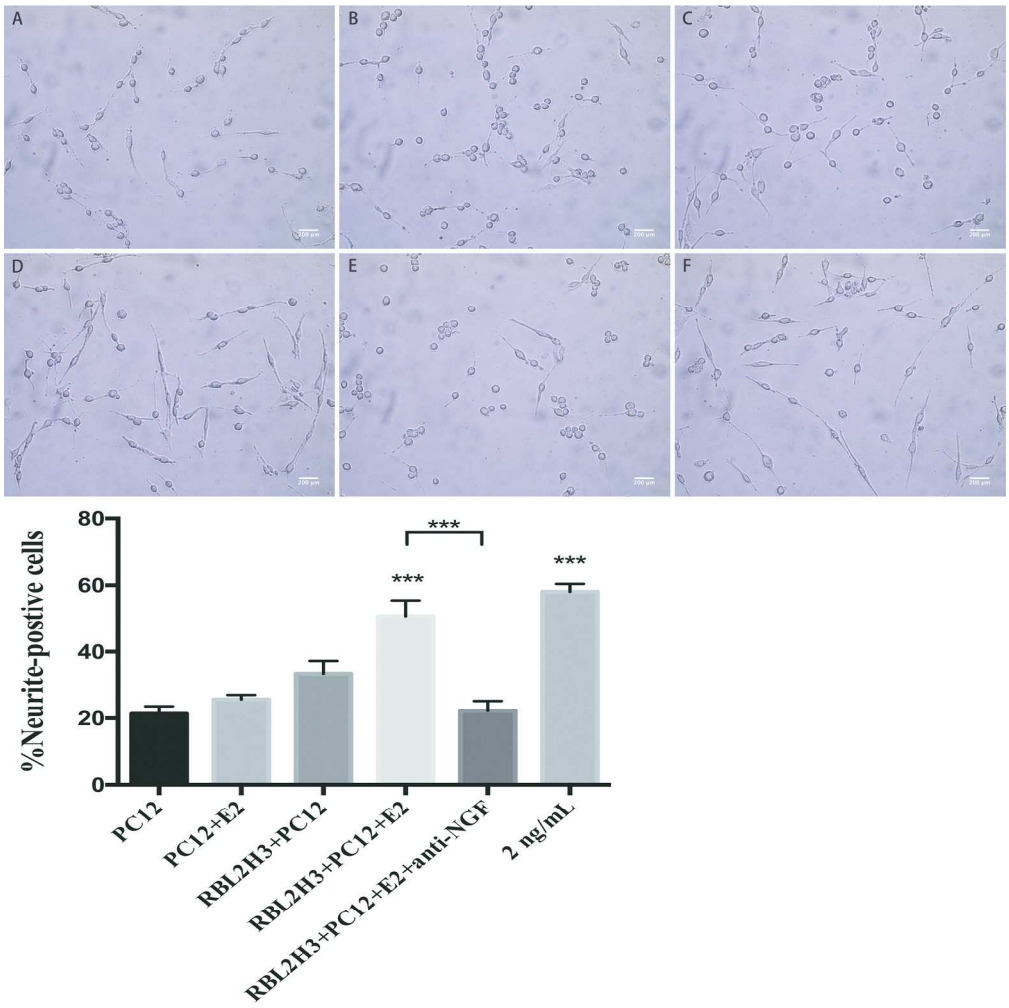

Figure 5. Activation of RBL2H3 cells by E2 can trigger the release of biologically active NGF.

209x210mm (300 x 300 DPI)

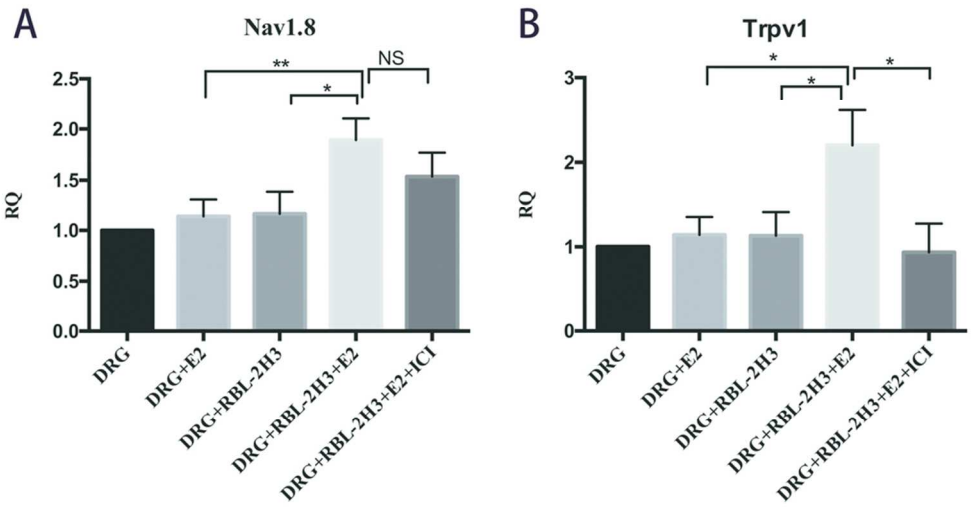

Figure 6. Activation of RBL2H3 cells by E2 can promote the peripheral sensitization of DRG cells.

88x46mm (300 x 300 DPI)

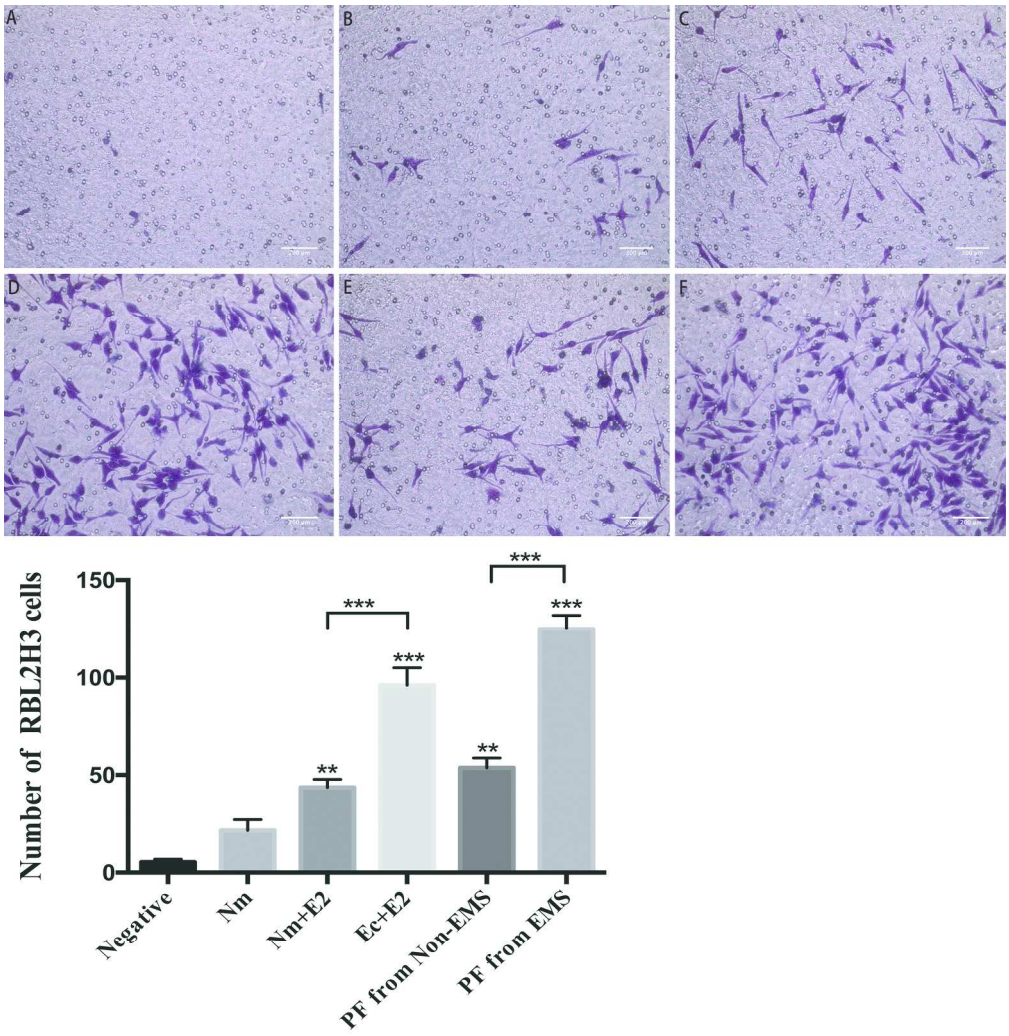

Figure 7. Endometriotic cells with E2 treatment, and peritoneal fluid collected from patients with endometriosis, can promote RBL2H3 cell recruitment.

209x218mm (300 x 300 DPI)

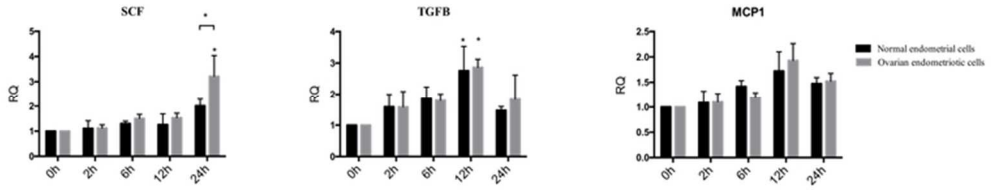

Figure 8. Up-regulation of the expression levels of SCF (A), TGFB (B), and MCP1 (C) in normal endometrial cells or ovarian endometriotic cells following E2 treatment.

32x6mm (600 x 600 DPI)

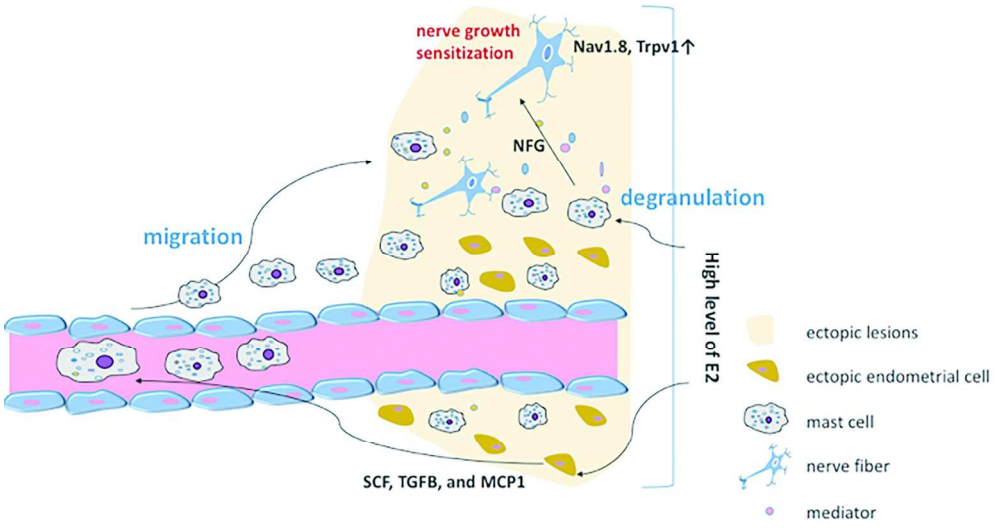

Figure 9. Schematic diagram of key findings.

254x142mm (300 x 300 DPI)