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Endometrial stromal cell attachment and matrix homeostasis in abdominal wall endometriomas

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STUDY QUESTION: How does progesterone alter matrix remodeling in abdominal wall endometriomas compared with normal endometrium?

SUMMARY ANSWER: Progesterone may prevent attachment of endometrial cells to the abdominal wall, but does not ameliorate abnormal stromal cell responses of abdominal wall endometriomas.

WHAT IS KNOWN ALREADY: Menstruation is a tightly orchestrated physiologic event in which steroid hormones and inflammatory cells cooperatively initiate shedding of the endometrium. Abdominal wall endometriomas represent a unique form of endometriosis in which endometrial cells inoculate fascia or dermis at the time of obstetrical or gynecologic surgery. Invasion of endometrium into ectopic sites requires matrix metalloproteinases (MMPs) for tissue remodeling but endometrium is not shed externally.

STUDY DESIGN SIZE, DURATION: Observational study in 14 cases and 19 controls.

PARTICIPANTS /MATERIALS, SETTING, METHODS: Tissues and stromal cells isolated from 14 abdominal wall endometriomas were compared with 19 normal cycling endometrium using immunohistochemistry, quantitative PCR, gelatin zymography and cell attachment assays. P values < 0.05 were considered significant and experiments were repeated in at least three different cell preps to provide scientific rigor to the conclusions.

MAIN RESULTS AND THE ROLE OF CHANCE: The results indicate that MMP2 and MMP9 are not increased by TGFβ1 in endometrioma stromal cells. Although progesterone prevents attachment of endometrioma cells to matrix components of the abdominal wall, it does not ameliorate these abnormal stromal cell responses to TGFβ1.

LARGE SCALE DATA: N/A.

LIMITATIONS REASONS FOR CAUTION: Endometriomas were collected from women identified pre-operatively. Not all endometriomas were collected. Stromal cells from normal endometrium were from different patients, not women undergoing endometrioma resection.

WIDER IMPLICATIONS OF THE FINDINGS: This work provides insight into the mechanisms by which progesterone may prevent abdominal wall endometriomas but, once established, are refractory to progesterone treatment.

STUDY FUNDING/COMPETING INTEREST(S): Tissue acquisition was supported by NIH P01HD087150. Authors have no competing interests.

Key words: endometriosis / transforming growth factor beta / MMP2 / MMP9 / progesterone / fibronectin / progesterone receptors

Introduction

Endometriosis is a chronic disease defined as the presence of endometrium-like tissue outside of the uterine cavity. Endometriosis is one of the most common causes of infertility and chronic pelvic pain affecting 1 in 10 women of reproductive age (Eskenazi and Warner, 1997; Giudice, 2010; Giudice and Kao, 2004). After initial attachment to its ectopic location, endometriotic cells implant and invade the extracellular matrix (ECM) where they proliferate and form a dense inflammatory reaction (van der Linden, 1996; Witz et al., 1999). As the disease develops, these endometriotic cells need to establish cell–cell and cell–ECM interactions to survive. Endometriomas involving the abdominal wall represent an unusual phenomenon resulting from direct inoculation of the abdominal wall with endometrial cells during surgical intervention which are subsequently stimulated by estrogen to produce painful endometriomas. The ECM of the abdominal wall contains collagen types I and IV, tenascin, vitronectin, fibronectin and laminin, all of which may be the potential binding targets of endometriotic cells (Harrington et al., 1999; Griffith et al., 2010).

Matrix metalloproteinase (MMP) enzymatically digest certain ECM proteins and therefore play an important role in tissue remodeling processes (Emonard and Grimaud, 1990). The ability of MMP2 (gelatinase A) and MMP9 (gelatinase B) to degrade type IV collagen and fibronectin has been firmly established (Aznavoorian et al., 1993). In the endometrium, it has been reported that some MMPs play important roles in endometrial physiologic characteristics (Rawdanowicz et al., 1994). Several proteases are upregulated in late secretory endometrium (Talbi et al., 2006) including MMP2.

Upregulation of proteases coincides with increased transforming growth factor β (TGF β)-responsive genes during the late luteal phase, a time in which progesterone levels decrease substantially. In several progesterone receptor (PR)-responsive cells, TGF-β1 further compromises expression of PR (Kane et al., 2008) and progesterone action by inhibiting PR-mediated gene transcription leading to induction of inflammatory response pathways and activation of NFκB, a transcription factor that further antagonizes PR function (Allport et al., 2001) (Kalkhoven et al., 1996; Davies et al., 2004). Here, we tested the hypothesis that TGFβ1 and progesterone differentially regulate matrix proteins, cell adhesion and secretion of MMPs in stromal cells from normal endometrial stromal cell (NESC) and endometriomas (Ecto-ESCs).

Materials and Methods

Ethical approval for use of endometrial tissue and endometriosis tissue

Normal human endometrial tissues ($n = 19$) were obtained from hysterectomy specimens conducted for benign non-endometrial pathology (e.g. premalignant disease of the cervix, uterine prolapse and leiomyomas) with informed consent under a protocol approved by the Institutional Review Board at the University of Texas Southwestern Medical Center from 2011 to 2016. Abdominal wall endometriomas ($n = 14$ from 13 subjects) were obtained at the time of endometrioma resection. All patients were parous, 23–48 years of age, menstruating regularly and were free of any hormone treatment for >30 days prior to surgery. In cases of normal endometrium, histopathologic examination excluded pathology and identified the corresponding day of the menstrual cycle.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues (three proliferative controls and four endometriomas) were sectioned at 4 μ and mounted on adhesive slides, along with multi-tumor sandwich block sections containing over 50 different normal and tumor tissues for external positive and negative controls (Miller and Groothuis, 1991). Details of the primary antibodies used are shown in Table I. For estrogen receptor α (ERα), PR and integrin α5 stains, antigen retrieval was by steam in 1 mM EDTA, pH 8.5 × 30 min. For Pax8, Integrin β1 and CD68, slides were placed in 0.25 mM Tris base buffer, pH 9.0, in a pressure cooker. Negative controls were comprised of all treatments but without primary antibody.

ESC isolation procedure and cell culture conditions

Endometrioma nodules were dissected from surrounding fat, fascia and surrounding tissue. NESC ($n = 6$) and endometrioma SCs ($n = 5$) were separated from epithelial glands by digesting the tissue fragments with collagenase, as previously described (Nasu et al., 1998). Cultured cells from the endometriomas were elongated and fibroblast in appearance and could not be distinguished microscopically from ECSs of the endometrium. After one passage, cells were > 99% pure as analyzed by immunostaining for vimentin (V9, Dako, Copenhagen, Denmark), cytokeratin (Dako), factor VIII (Dako) and leukocyte common antigen (2B11 + PD7/26, Dako). Cells isolated from each individual patient were used for one experiment at a time in triplicate and repeated in at least three different cell preps.

Table I Antibodies for immunohistochemistry.

Antigen	Clone	Dilution	Source	Species
Estrogen receptor α	SP1	1:25	Spring Bioscience, Pleasanton, CA	Rabbit
Pax8	NA-Polyclonal	1:1600	Proteintech, Rosemont, IL	Rabbit
Progesterone receptor	SP2	1:400	LifeSpan Biosciences	Rabbit mAb
Smad3	C67H9	1:1000	Cell Signaling	Rabbit mAb
Phosphor-Smad3	C25A9	1:1000	Cell Signaling	Rabbit mAb
CD68	PGM1	1:100	Invitrogen	Mouse mAb
Integrin α5	NA	1:250	Abcam	Rabbit mAb
Integrin β1	NA	1:500	Abcam	Rabbit mAb

TGF-β1 and progesterone treatment

Pre-confluent cells (passage 1) were placed in serum-free medium for 24 h at 37°C before treatment. Thereafter, cells were treated with vehicle, various concentrations of TGFβ1 (0.1–5 ng/ml), progesterone (1–100 nM) or both. Experiments were performed in triplicate plates three times.

Gelatin zymography

MMP2 and MMP9 activity in the culture media was analyzed by zymography ($n = 3$ cell preps each for hESCs and endometriomas). Quantitative gelatin zymography was performed as described (Wieslander *et al.*, 2009).

RNA extraction from endometrial tissue and quantitative real-time PCR

Total cellular RNA was extracted from cultured human stromal cells from normal endometrial and endometriosis or frozen tissue using RNAqueous-4PCR Kit (Ambion, USA), according to the manufacturer's protocol ($n = 6$ per group). Reverse transcription reactions were conducted with 1 μg total RNA in a reaction volume of 20 μl. For real-time PCR, primer sequences to amplify MMP2 were ^{5}TTGATGGCATCGCTCAGATC^{3} (sense) and ^{5}TGTCACGTGGCGTCACAGT^{3} (antisense); MMP9, ^{5}CCACCACAAC ATCACCTATTGG^{3} (sense) and ^{5}GCAAAGGCGTCGTCAATCA^{3} (antisense); EDA (Extra Domain A of fetal fibronectin (Fn)), ^{5}GGAATTCC ATATGAACATTGATCGCCCTAAAGGACT^{3} (sense) and ^{5}ATAAGAA TGCGGCCGCTGTGGACTGGGTTCCAATCAGGGG^{3} (antisense); III_{11} (Fn-non-EDA), ^{5}GGAATTCCATATGGAAATTGACAAACCATCCCA^{3} (sense) and ^{5}ATAAGAATGCGGCCGCGGTTACTGCAGTCTGAACCA^{3} (antisense); and GAPDH, ^{5}GGAGTCAACGGATTTGGTCGTA^{3} (sense) and ^{5}CAACAATATCCACTTTACCAGAGTTA^{3} (antisense). Primers were chosen so that the resulting amplicons would cross an exon junction, thereby eliminating false-positive signals from genomic DNA contamination. To differentiate Fn mRNA which contains EDA (Fn-EDA) from Fn mRNA which does not contain EDA (Fn-non-EDA), we used EDA-specific primers. As EDA is located between exons III_{11} and III_{12}, Fn-EDA forward primer was designed to span the junction of exon III_{11} and EDA, so this primer set specifically detect EDA-containing Fn mRNA. Fn-non-EDA reverse primer was designed to span the junction of exons III_{11} and III_{12}, so this primer set is not able to detect EDA-containing Fn, and it only detects Fn mRNA that does not contain EDA. Gene expression was normalized to expression of GAPDH. A pre-programmed dissociation protocol was used after amplification to ensure that all samples exhibited a single amplicon. Levels of mRNA were determined using the ddCt method (Applied Biosystems, USA).

Immunoblotting analysis

Pre-confluent cultures were placed in serum-free medium for 24 h before treatment. The optimal time for stimulation was determined by a time course study performed as background experiments. After treatment, cells were washed with phosphate buffered saline (PBS), and whole cell extracts were prepared as described previously (Kishore *et al.*, 2014).

Assessment of cell adhesion to ECM protein *in vitro*

Cell adhesion was evaluated using the CytoSelect™ 48-well cell adhesion assay (ECM Array, Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol. At first, progesterone treatment was conducted in 6-well plates. Pre-confluent ESCs (passage 1) were placed in serum-free medium for 24 h at 37°C before treatment. Thereafter, cells were treated with serum-free medium containing vehicle and progesterone. After 48 h, cells were harvested in serum-free DMEM containing 0.5% bovine serum albumin (BSA) (0.7×10^{6} cells/ml). Cell suspensions were added to each well coated with fibronectin, collagen type I, collagen type IV, laminin, fibrinogen or BSA in a volume of 150 μl and incubated at 37°C for 90 min. Cells were then washed four times with 250 μl of PBS. Cell stain solution (200 μl, Cell Biolabs) was added to each well and incubated for 10 min at room temperature. Cells were washed four times with 500 μl of deionized water. After air drying for 20 min, 200 μl of extraction solution (Cell Biolabs) was added to each well and placed on an orbital shaker for 10 min. The absorbance of the extracted samples was measured at 560 nm. Results were normalized by subtracting absorbance of negative controls.

Statistical analysis

Values were expressed as means ± SEM or median with range as appropriate. Experimental data were analyzed by unpaired Student's *t*-test whereas imaging and surgical size were compared by Wilcoxon signed rank or paired *t*-tests. A one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test was used for multiple comparisons. *P* values of <0.05 were regarded as statistically significant.

Results

Abdominal wall endometriomas–histomorphology

Characteristics of patients with abdominal wall endometriomas are presented in Table II. For studies involving cultured endometrioma stromal cells (Ecto-ESCs), all samples were obtained in the proliferative phase. Our examination of 14 samples retrieved by informed consent from 2011 to 2016 revealed a mean age of 31 years, and all patients presented with a history of cesarean section (C/S) with one after cesarean hysterectomy (Table II). Two cases were recurrent resections. Although the time from C/S to resection of endometrioma varied widely (from 11 months to 12 years), the average time between these surgical events was 6 years (median 6 [5, 8.7, 25%, 75%]). Duration of symptoms was only 15 months suggesting that the tumors are dormant for long periods of time prior to bothersome symptoms. In this series of patients, imaging was conducted with MRIs in five or computerized tomography (CT) in eight. Of these, eight scans underestimated endometrioma size from slight underestimates of 20% to large underestimated sized (12.9-fold) with an average underestimate of 3.9 ± 0.36-fold. Two scans overestimated endometrioma volume 40–80% and four scans were concordant. Imaging was very accurate for predicting fascial involvement (eight predicted fascial invasion with seven confirmed at surgery).

Endometriomas differed considerably in size (from 2 to 10.5 cm diameter) with various degrees of fat and fibrotic reaction (Fig. 1). Histologically, stroma cells and epithelium were surrounded by dense fibrotic stroma (Fig. 2A). The epithelial cells were confirmed to be of MÜllerian origin using PAX8 immunostaining (Fig. 2B). Most samples had 'stromal nodules' of a dense inflammatory infiltrate and high concentrations of CD68-positive macrophages (Fig. 2C). Macrophages were also distributed throughout the entire stroma of the endometrioma (Fig. 2D). Both stromal and glandular endometrial cells were positive for nuclear estrogen receptor α and progesterone receptor

Table II Characteristics of women from whom abdominal wall endometriomas were sampled.

Patient characteristics		Comments
Age, y, mean ± SEM	31 ± 1.5	
Gravidity, median (range)	3 (1, 4)	
C/S parity, median (range)	2 (1,4)	
C/S incision	9 Pfannenstiel 4 vertical	1 Prior vertical C/S had primary and recurrent resections
Years after C/S, mean ± SEM, range	6.0 ± 0.87, 11 months–12 years	1 Prior cesarean hysterectomy for placenta previa
Cycle phase	9 Proliferative, 4 luteal, 1 unknown	
Imaging size		
Volume, cc, mean ± SEM	21.5 ± 5.6	
Largest diameter, cm	3.4 ± 0.3	
Surgical size		
Volume, cc, mean ± SEM	69.6 ± 18*	
Largest diameter, cm	5.1 ± 0.7**	
Duration of symptoms, mo, mean ± SEM	15.5 ± 3.9	

*$P = 0.006$ compared with imaging size, Wilcoxon signed rank.
**$P = 0.06$ compared with imaging diameter, paired t-test.
C/S, cesarean section.

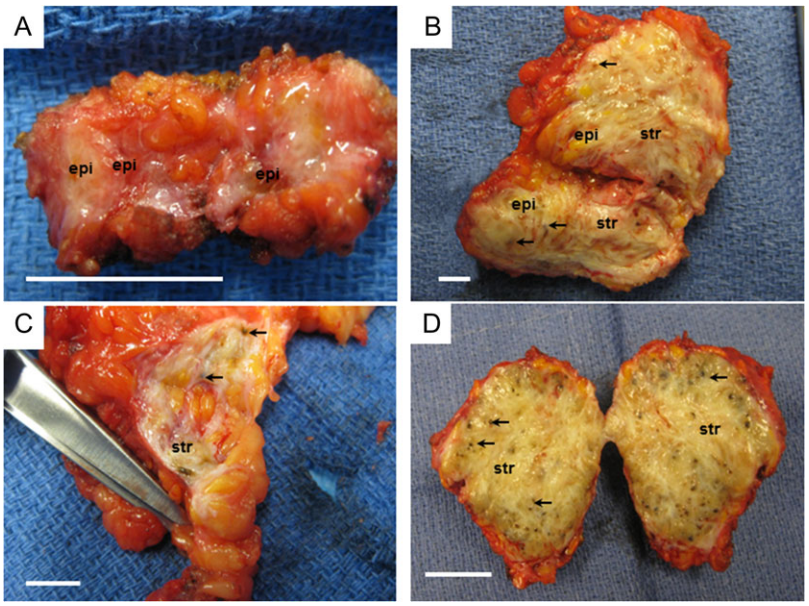

Figure 1 Gross pathology of abdominal wall endometriomas. Four endometriomas are shown illustrating differences in size and appearance. Resected endometriomas were bisected to illustrate varied tissue architecture. epi, endometrial surface epithelium; str, endometrial stroma; arrows indicate endometrial nodules. Bar = 1 cm.

(Fig. 2E, F). Interestingly, ERα was localized to glandular epithelium and the immediate surrounding stromal cells whereas stromal cells remote from the glands were predominantly ER negative (Fig. 2E). In contrast, although nuclear localization of PR was also predominantly in glandular epithelium and periglandular stroma, PR was also expressed throughout stroma remote from epithelium including the dense fibrotic stromal cells of the endometrioma pseudocapsule (Fig. 2F). Relative gene expression of total PR and PR-B (Fig. 2G) revealed that the PR-B isoform was decreased significantly 4-fold in endometriomas relative to normal proliferative endometrium. Total PR was decreased 7-fold.

Expression of matrix molecules in endometrioma tissues and endometrioma stromal cells in culture

Expression of two major integrins of endometrium (ITG β1 and α5) was increased significantly in endometriomas relative to normal

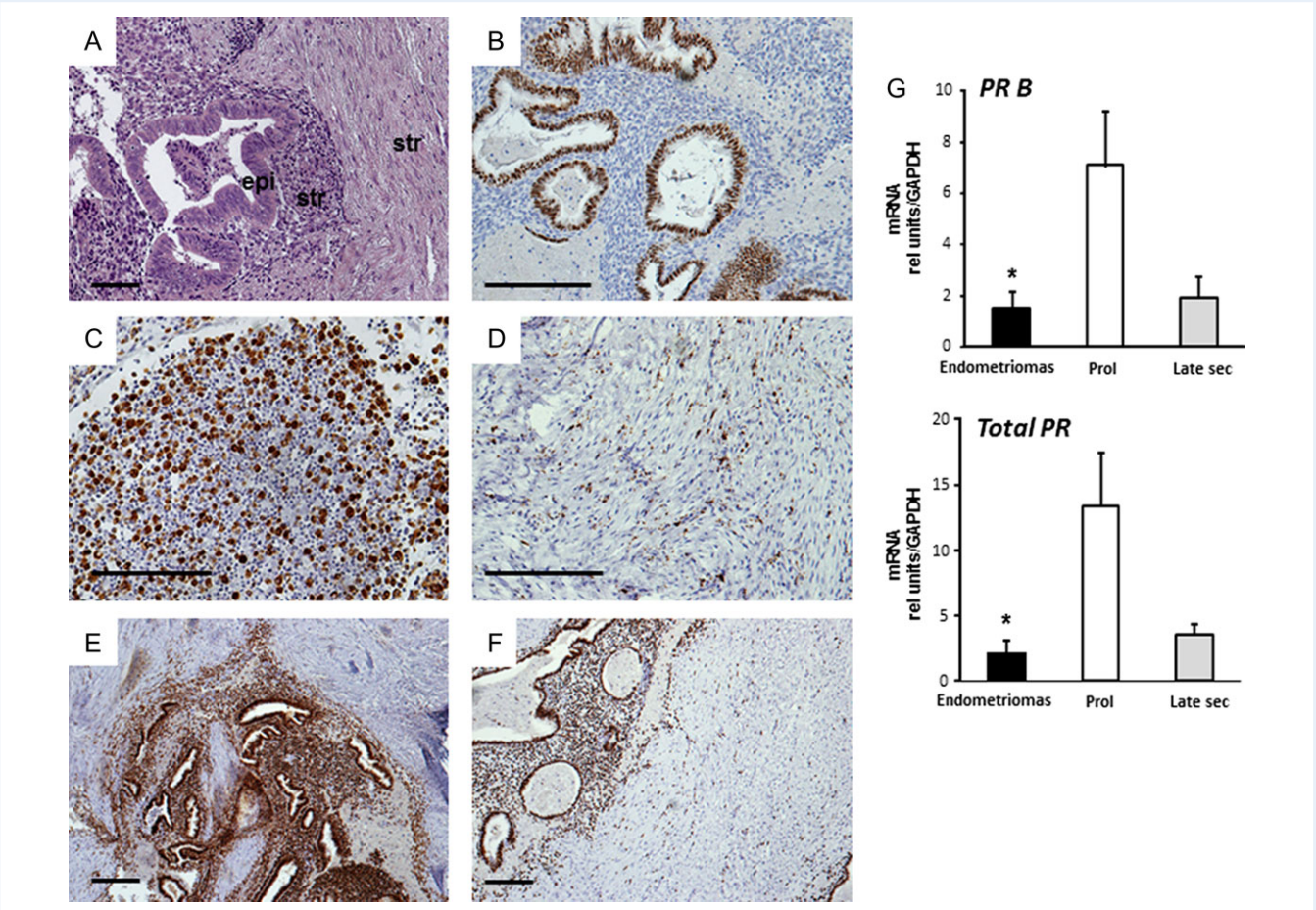

Figure 2 Histomorphology of abdominal wall endometriomas. (**A**) Hematoxylin and eosin staining reveals dense stroma surrounding glandular epithelium. Note inflammatory cell infiltrate, stromal nodules and fibrotic reaction. (**B**) Immunostaining of PAX8 in endometrial epithelial glands of abdominal wall endometrioma. (**C**, **D**) Immunostaining of CD68+ macrophages in endometriomas. (**C**) Dense accumulation of macrophages in an endometrial nodule. (**D**) Stromal macrophages surrounding endometrioma glandular epithelial cells. (**E**) Estrogen receptor α (ERα) immunostaining in endometriomas. (**F**) Immunohistochemistry for progesterone receptor (PR) in endometrioma. Note PR positive cells throughout the periglandular and remote dense stromal cells. Results represent three different endometriomas. Bars for panels (**A**–**F**) = 200 μm. (**G**) Relative gene expression of total *PR* and *PR-B* in endometriomas (all proliferative phase, $n = 3$), proliferative endometrium ($n = 7$) and secretory endometrium ($n = 6$). *$P < 0.05$, ANOVA.

endometrium regardless of menstrual day (Fig. 3). Expression of the proteoglycan versican was increased in four of five endometriomas. E-Selectin was also increased significantly in endometriomas.

In contrast with tissue gene expression, ITGβ1 was decreased modestly in stromal cells from endometriomas suggesting that upregulation of ITGβ1 in endometrioma tissue may be due to dysregulation in glands or immune cells or a compensatory mechanism. Versican and selectin, on the other hand, were upregulated in endometrioma tissues and endometrioma-derived stromal cells (Fig. 3B). Progesterone did not alter expression of these matricellular molecules.

Gene expression levels may not reflect the proteins and may be upregulated to compensate for decreased protein expression. Thus, normal endometrium and endometrioma tissues were immunostained for ITGA5 and ITGB1 subunits (Fig. 3C). ITGA5 was highly expressed in normal ESCs and the intensity of staining increased dramatically in the secretory phase. In endometriomas (all proliferative phase), ITGA5 was positive in the ESCs, but observational differences from normal endometrium were not apparent. Interestingly, the reactive fibrous connective tissue surrounding the endometrioma nodules was negative for ITGA5 as were endometrial glands. ITGB1 staining was weak in endometriomas with sporadic weak staining in endometrial fibroblasts.

Endometrioma stromal cells differ from normal stromal cells

MMP2 and MMP9 increase during the late secretory phase in response to progesterone withdrawal resulting in matrix destruction and detachment of endometrial tissue. Previously, we found that TGFβ1 induced increases in MMP2 and MMP9 in NESCs (Itoh *et al.*, 2012). Since endometriosis tissue appears to survive progesterone withdrawal (i.e. remains intact and does not shed externally during the menstrual cycle), we sought to determine if endometrioma-derived stromal cells responded differently to TGFβ1 (Fig. 4). In contrast with NESCs, TGFβ dose-dependently downregulated MMP

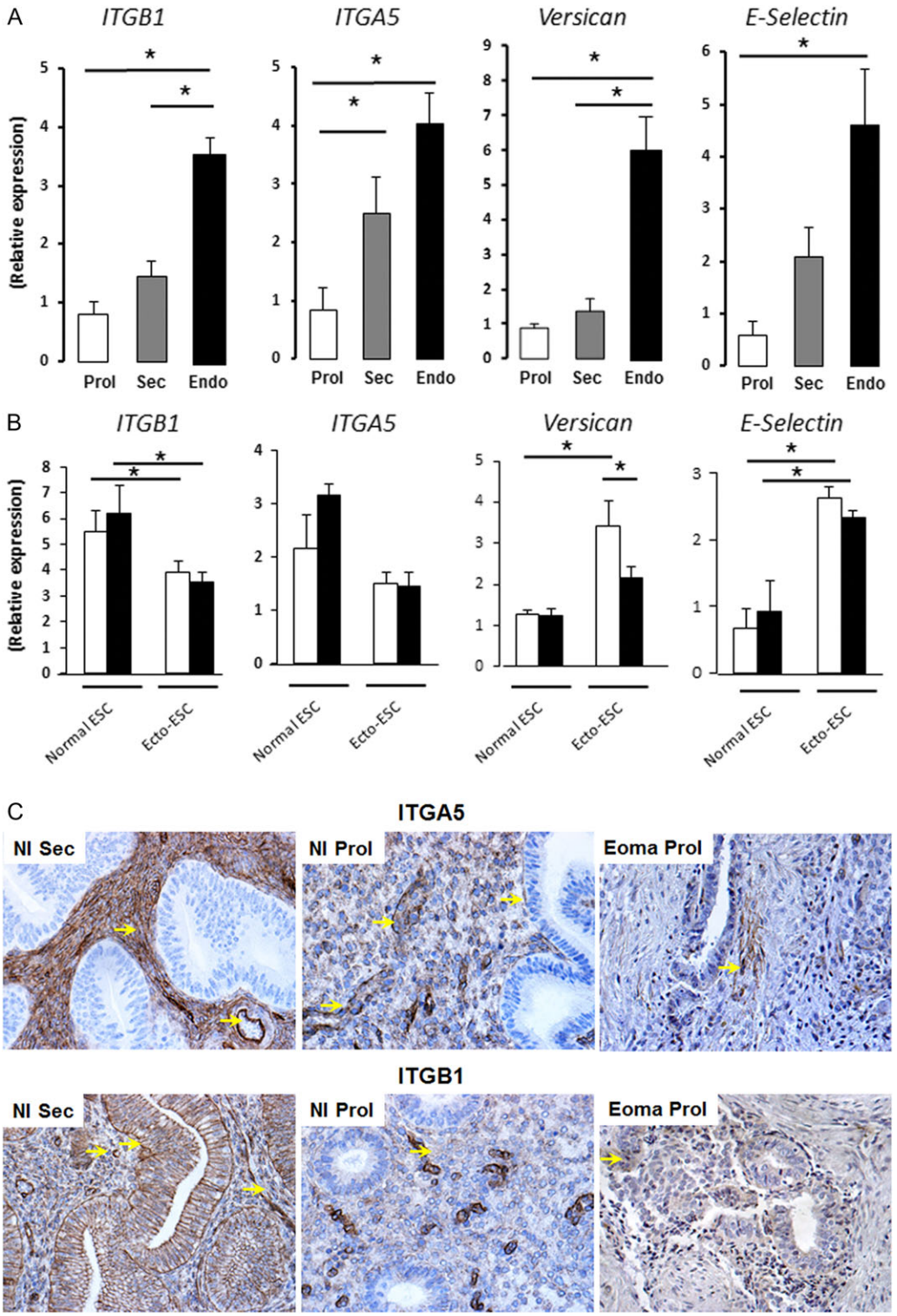

Figure 3 Expression of integrins and cell adhesion matrix molecules in endometrial and abdominal wall endometrioma tissues and cells. (**A**) Expression of matrix molecules in proliferative phase endometrium (Prol, $n = 7$), secretory phase endometrium (Sec, $n = 6$) or endometriomas (Endo, all proliferative phase, $n = 5$). *ITGB1*, integrin beta 1; *ITGA5*, integrin alpha 5. (**B**) Expression of matrix molecules in endometrial stromal cells of normal endometrium (Normal ESC) or endometrioma stromal cells (Ecto-ESC) treated with vehicle (open bars) or progesterone (10^{-7} M, solid bars). Data represent mean ± SEM of four preps from each cell type. Quantitative RT-PCR was used to determine the relative levels of gene expression normalized to the housekeeping gene GAPDH. *$P < 0.05$ ANOVA or t-test as appropriate. (**C**) Immunostaining of ITGA5 (upper panel) and ITGβ1 (lower panel) in normal secretory (NI Sec) and proliferative (NI Prol) endometrium and endometrioma tissues (Eoma Prol). Note that ITGA5 is positive in stromal and endothelial cells (arrows) but absent in glands. ITGβ1 is positive in endometrial stromal cells and microvessels and secretory glands. Staining in endometriomas is weak. Data represent consistent results in three tissues per group. Magnification ×200.

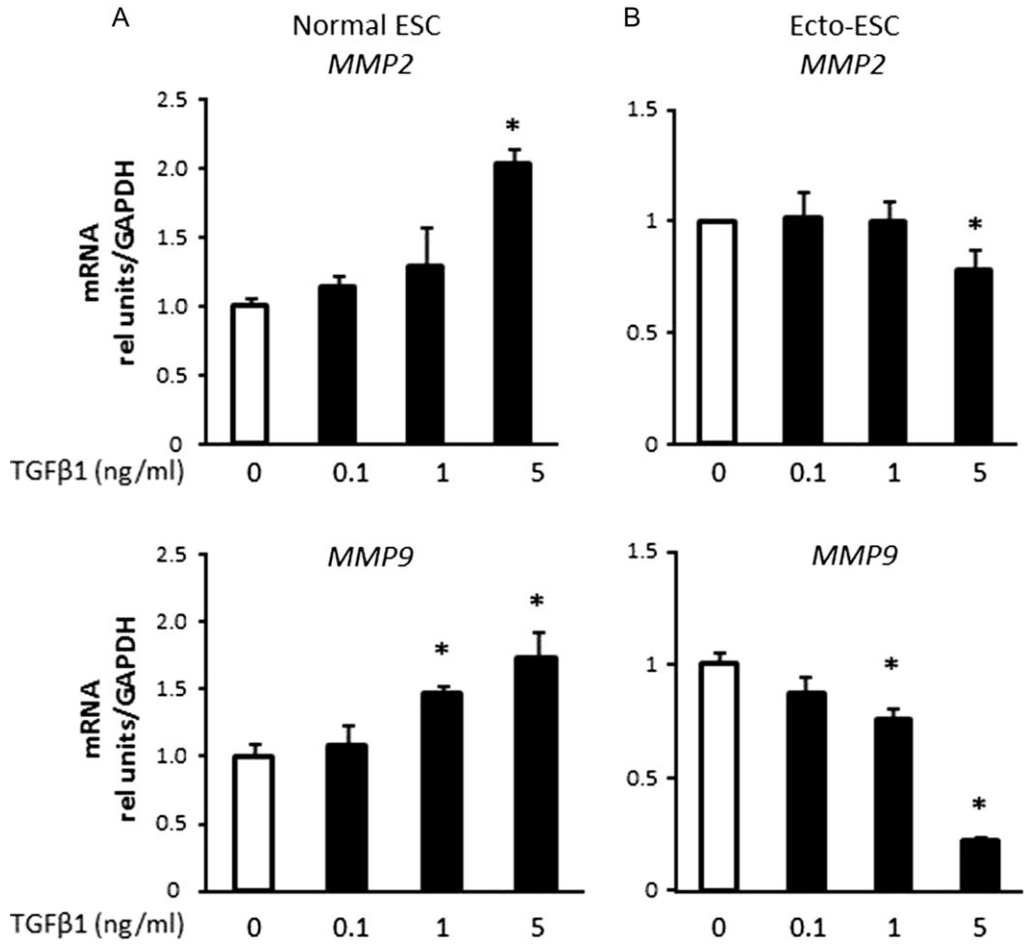

Figure 4 TGF-β1 differentially regulates *MMP2* and *MMP9* mRNA in endometrioma and endometrial stromal cells. *MMP2* and *MMP9* mRNA expression of normal ESC (**A**) or Ecto-ESC (**B**) with treatment of different doses of TGF-β1 for 48 h. Error bars, SEM. $n = 4$ in each group. *$P < 0.05$ compared with vehicle (0 ng/ml).

gene expression in endometrioma-derived stromal cells (Ecto-ESCs, Fig. 4).

TGFβ-mediated responses are downregulated by progesterone (Itoh, Kishore, Lindqvist, Rogers and Word, 2012). To determine if these responses were maintained in Ecto-ESCs, cells were treated with vehicle, progesterone, TGFβ1 or progesterone + TGFβ1 for 48 h. In contrast with NESCs (Fig. 5A), endometrioma cells demonstrated progesterone resistance in that progesterone did not alter TGFβ1-induced decreases in MMP2. Likewise, in Ecto-ESCs, TGFβ1 decreased baseline levels of *MMP9* and progesterone did not alter this effect (Fig. 5B). Results using gelatin zymography of conditioned media confirmed these results (Fig. 5C–E).

Adhesion of normal ESC and endometrioma-ESC to ECM molecules

Using several ECM molecules, we compared attachment of NESCs with Ecto-ESCs. Adhesion of Ecto-ESCs to several ECM components was increased significantly including fibronectin (9-fold), collagen I (8-fold), collagen IV (8-fold), laminin-1 (4.5-fold) and fibrinogen (7-fold) (Fig. 6). Interestingly, progesterone (1–100 nM) treatment for 48 h significantly inhibited attachment of Ecto-ESCs to every ECM molecule tested (Fig. 6). In contrast, progesterone did not inhibit attachment of NESCs (Fig. 6).

Fibronectin (FN) is particularly abundant in stroma of endometrium (Fazleabas *et al.*, 1997). FN exists with multiple splice variants. For example, the extra domain A (EDA) of FN is usually expressed in fetal tissues but also occurs in adult tissues undergoing remodeling and this splice variant mediates proinflammatory responses (Mogami *et al.*, 2013). FN and FN-EDA gene expression was determined (Fig. 7). FN mRNA was increased significantly in endometrioma tissue compared with endometrium regardless of menstrual stage. Although FN-EDA was upregulated ~2-fold, non-EDA-FN was upregulated 14-fold in endometriomas (Fig. 7A) Like versican, FN gene expression (predominantly non-EDA-FN) was increased significantly in ecto-ESCs (Fig. 7B), suggesting that the stromal cells are the source of increased FN in endometrioma tissues. Although progesterone inhibited adhesion of endometrioma stromal cells to fibronectin, progesterone did not alter FN or FN-EDA mRNA (Fig. 7B). Immunoblot analysis of urea-extracted matrix proteins revealed a single immunoreactive protein for FN in normal ESCs whereas Ecto-

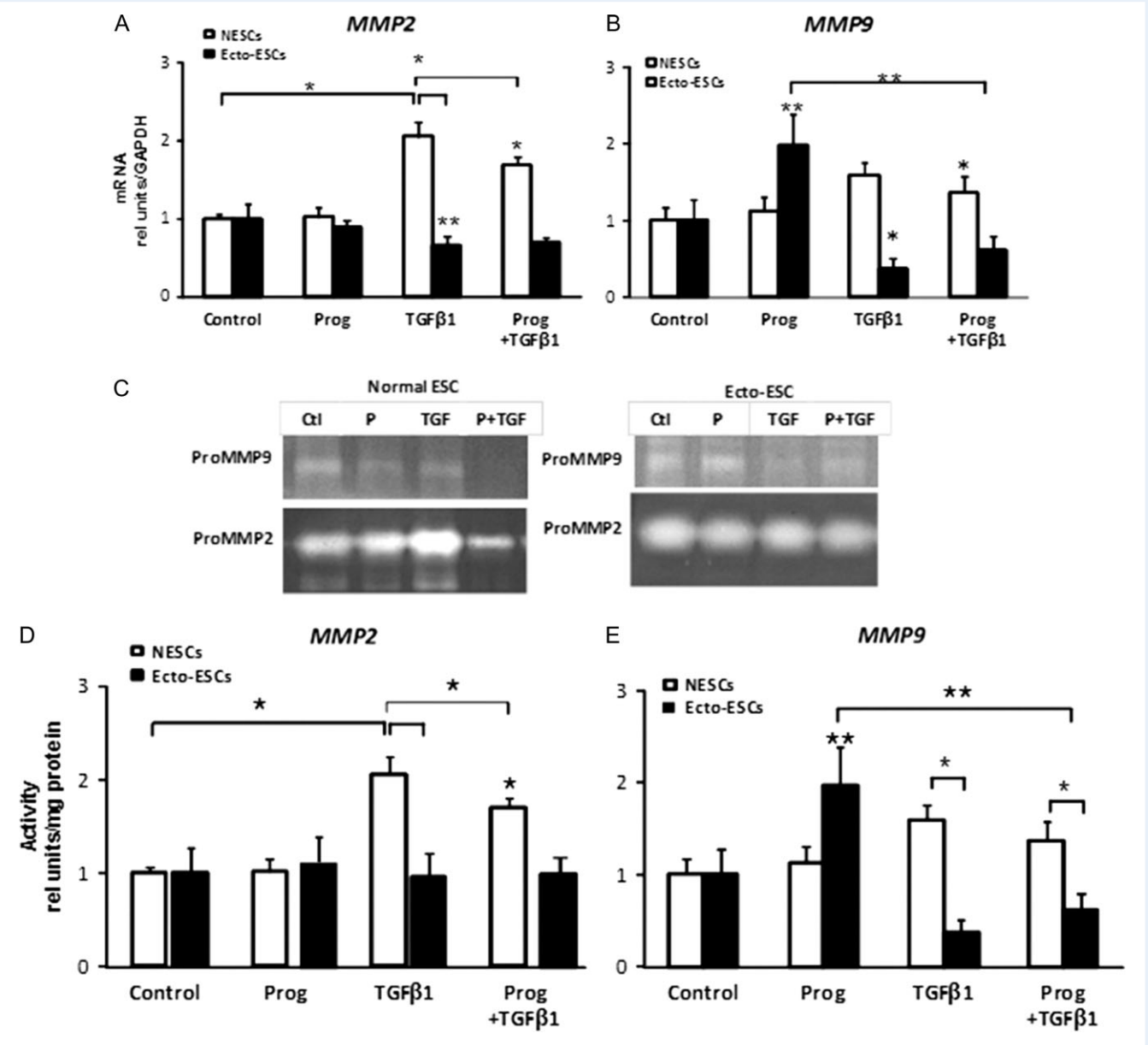

Figure 5 Effect of progesterone and TGFβ1 on *MMP2* and *MMP9* mRNA in stromal cells from normal ESCs or Ecto-ESCs. Levels of MMP2 (**A**) and MMP9 mRNA (**B**) were quantified using qPCR and expressed relative to *GAPDH* in normal ESCs (NESCs, open bar) or endometrioma stromal cells (Ecto-ESCs, solid bar). Cells were treated with progesterone (10^{-7} M), TGFβ1 (5 ng/ml), or both for 48 h (maximal effect). Data represent mean ± SEM of cells from four different samples in each group. *$P < 0.05$, **$P < 0.01$ compared with NESC, ANOVA. (**C**) Gelatin zymography of media extracts from Normal ESCs or Ecto-ESCs treated with vehicle (Ctl), progesterone (P, 10^{-7} M), TGFβ1 (TGF, 5 ng/ml) or progesterone + TGFβ1 (P + TGF) for 48 h. Results are representative of three different cell preps in each group shown quantitatively in panels (**D**–**E**).

ESCs expressed this FN as well as a differentially glycosylated isoform (Fig. 7C). This isoform was not soluble (released into the media).

Discussion

Abdominal wall endometriomas differ from classical forms of endometriosis in the peritoneal cavity or ovarian endometriomas. The pathogenesis of abdominal wall endometriomas, like endometriosis, is unknown but is believed to result from inoculation of endometrial cells at the time of gynecologic surgery. This idea is supported in our cohort of patients in whom 100% experienced cesarean section prior to surgical intervention for abdominal wall endometriomas which were characterized by dense fibrotic tissues comprised of fibroblastic stromal cells. These stromal cells, however, were unlike dermal fibroblasts in that they were PR+, suggesting that the source of these cells was endometrial stroma. Interestingly, however, abdominal wall endometriomas are notoriously insensitive to progesterone or progestin treatment (Ramesh *et al.*, 2016). In this study, we investigated expression of MMPs and adhesion molecules in abdominal wall endometriomas and progesterone responses of endometrioma stromal cells.

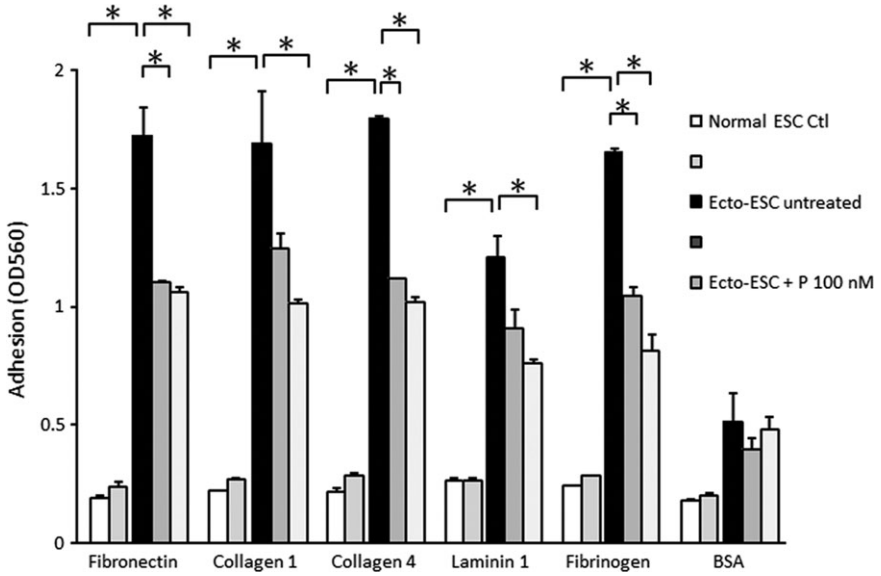

Figure 6 Adhesion of normal ESC and Ecto-ESC to ECM molecules. Stromal cells from normal human endometrium (normal ESC) or ectopic endometrium (Ecto-ESC) were treated with or without progesterone (P, 1 or 100 nM) for 48 h and adhesion to ECM-coated plates was screened. Bovine serum albumin (BSA) was used as control. Data represent mean ± SEM from three cell preps in each group. *$P < 0.05$, ANOVA.

Endometrium, matrix and menstruation

The ECM is formed from secreted proteins and glycoproteins, and forms the ground substance outside cells in all tissues. The ECM appears to play an important role in cell–cell interactions during menstruation which is characterized by hemorrhagic shedding of the superficial layer of endometrium as a result of ECM breakdown. This menstrual process is associated with expression of TGFβ-1 and MMPs (Kokorine *et al.*, 1996). High concentrations of inflammatory mediators and immune cells account for ~40% of the stromal compartment (Cousins *et al.*, 2016) and are known to play a pivotal role in shedding of the endometrium during menstruation followed by scarless healing and regrowth (Cousins et al., 2016). In endometriomas, however, dense fibrous connective tissue accumulates (scarring) with a profound increase in the ratio of stromal cells to glands. It follows, therefore, that inflammatory and protease-mediated shedding is aberrant in endometriomas leading to few glands relative to the dense matrix-producing stromal compartment.

TGFβ and endometrial MMPs

TGFβ superfamily members are closely associated with tissue remodeling events and reproductive processes. In the endometrium, TGF-β1 was found in stromal cells (Johnson *et al.*, 2005), and endometrial TGF-β1 mRNA is significantly increased in the mid and late secretory and menstrual phases compared with proliferative and early secretory phases (Casslen *et al.*, 1998). Thus, it is possible that protease activation in the late luteal phase may cleave latent TGF-β binding protein and activate TGFβ signaling in endometrial cells. Here, we found that TGFβ1 increased *MMP2* and *MMP9* in normal ESCs but decreased these MMPs in endometrioma stromal cells. TGFβ1 cell signaling through transcriptional activation of SMAD signaling, however, was intact in both cell types, suggesting that TGFβ1-induced activation of other cell pathways was impacted differentially in these cells, or that chromatin structure and coactivator/repressor complexes differed among the two cell types. This is supported by observations in variety of tissues (Edwards *et al.*, 1987) (Kerr *et al.*, 1990) (Marti *et al.*, 1994) (Braundmeier and Nowak, 2006).

In normal menstruation, TGFβ1, MMPs and immune cell infiltration increase cyclically during the premenstrual and menstrual phase. In endometriomas, however, MMPs are chronically elevated with chronic persistent macrophage infiltration. We suggest that macrophage-derived MMPs pave the way for matrix remodeling and growth of invasive endometrioma stromal cells. The lack of cyclic TGFβ-stimulated stromal cell-derived MMPs may serve to support invasion rather than shedding of endometrium thereby leading to scarring and matrix deposition.

Progesterone and endometrioma stromal cells

Progesterone suppresses many matrix molecules whereas progesterone withdrawal induces FN and ITGs during menstruation (Cao *et al.*, 2007). FN is known to induce MMP2 (Hoffmann *et al.*, 2006). Increased expression of integrin α5 has been reported in endometriotic epithelial cells (Beliard *et al.*, 1997) and integrin β1 protein expression in endometriotic stromal cells (Adachi *et al.*, 2011). In this study, we found that *ITG α5* and *ITG β1, FN, versican* and *E-selectin* mRNA were increased in endometriomas relative to normal endometrium, and that increases in *versican*, *E-selectin* and FN carried over into cultured stromal cells. Unlike normal ESCs, progesterone did not alter increased expression of these matrix molecules in endometrioma-derived SCs. Some of this insensitivity to progesterone may be due to a number of factors including decreased PR levels, increased progesterone metabolism (Bulun *et al.*, 2006, 2010), inflammation-induced dysregulation of PR binding sites (Al-Sabbagh *et al.*, 2012; Pabona *et al.*, 2012; Zelenko *et al.*, 2012) or

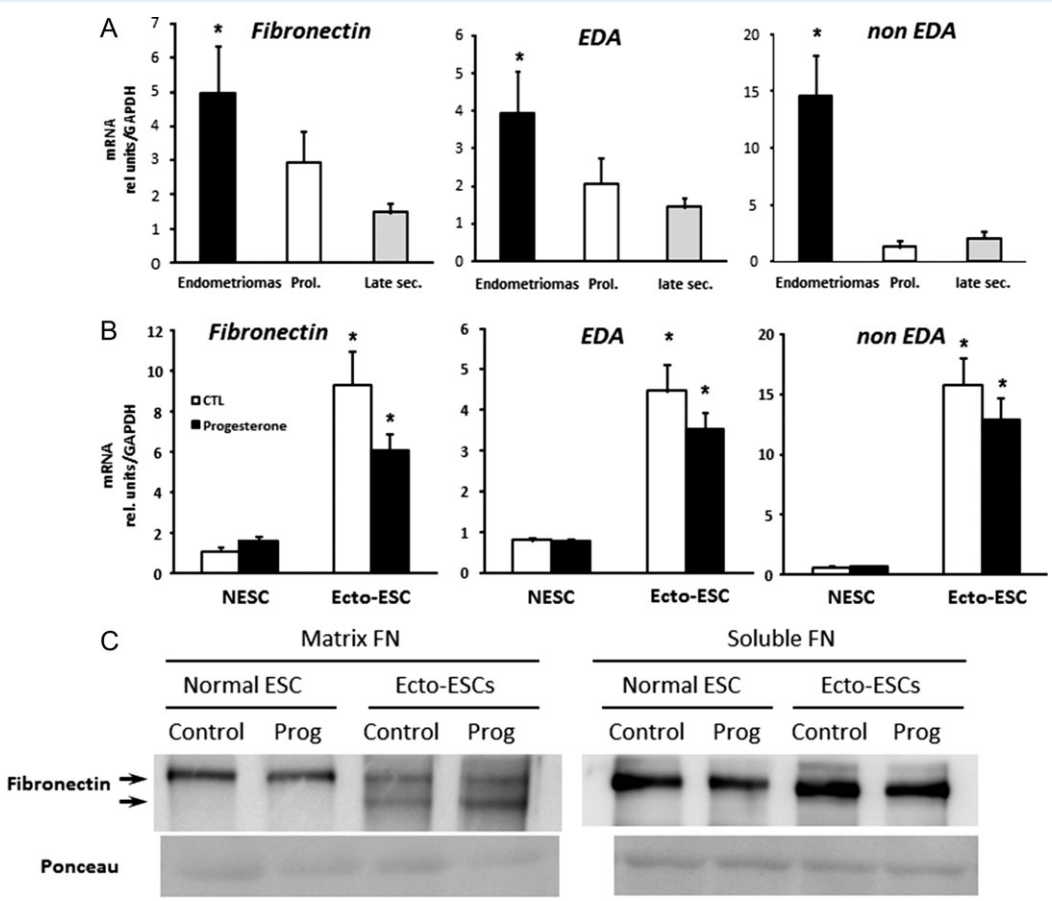

Figure 7 Fibronectin in endometriomas and endometrioma stromal cells. (**A**) Relative mRNA levels of total fibronectin, EDA-FN and non-EDA-FN in endometrioma tissues ($n = 3$), proliferative endometrium ($n = 4$) or late secretory (d18–25) endometrium ($n = 6$). *$P < 0.05$ compared with normal endometrium of either phase, ANOVA. (**B**) Effect of progesterone (10^{-7} M × 48 h) on relative mRNA of fibronectin, EDA-FN and non-EDA-FN in stromal cells from normal endometrium or endometriomas. *$P < 0.05$ compared with NESC. Progesterone resulted in no significant effects. (**C**) Immunoblot analysis of urea-extracted or soluble (media) FN in normal ESCs or endometrioma-derived ESCs (Ecto-ESCs). Ponceau staining is shown to illustrate equal loading of urea extracts and concentrated media.

changes in cell-specific coactivators/repressors (Suzuki *et al.*, 2010; Shi *et al.*, 2014; Zelenko *et al.*, 2012).

One of the most dramatic findings of this study was the increased adhesiveness of endometrioma stromal cells to purified matrix molecules including FN, collagen types I and 4, laminin and fibrinogen. Our data indicate that increased adhesiveness of endometrioma stromal cells to multiple matrix components is not due to α5β1 but is likely due to other matrix attachment proteins such as syndecans, versican, FN and E-selectin. Interestingly, although progesterone did not alter expression of the matrix molecules, progesterone dose-dependently inhibited adhesion of the cells to purified matrix. Adhesion of cells treated with high-dose progesterone (100 nM) remained increased relative to normal ESCs. Progesterone may have a clear role in prevention of ESC adhesion, but may not treat already established disease.

Limitations of this study include the limited number of endometrioma cell preps, lack of a comprehensive evaluation of matrix adhesion molecules and the absence of endometriomas in the secretory phase. Nonetheless, these studies indicate that abdominal wall endometriomas are characterized by numerous PR+ stromal cells engulfed in a dense ECM. Endometrial glandular epithelial cells are dispersed intermittently throughout the tumor, all of which are PR, Pax8 and ERα positive. Endometrioma stromal cells express increased matrix molecules and significant increases in matrix adhesion. Although expression of versican, FN and selectin-E were refractory to progesterone, progesterone resulted in decreased adhesive properties of stroma cells which may be used to prevent further development but not progression of ectopic endometriomas.

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Authors' roles

H.I. participated in study design, execution, analysis and critical discussion. H.M. participated in writing of the manuscript and analysis of data. L.B.N. conducted analysis of patient data. L.W. and D.R.

conducted tissue dissection and identification of patients, R.M. performed immunohistochemistry and R. A. W. participated in study design, execution, analysis, critical discussion and writing of the article.

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Conflict of interest

The authors declare no conflicts of interest.

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