Different macrophages equally induce EMT in endometria of adenomyosis and normal

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Abstract

Endometrial cells and microenvironment are two important factors in the pathogenesis of adenomyosis. Our previous study demonstrated that macrophages can induce eutopic epithelial cells of adenomyosis to suffer from epithelial–mesenchymal transition (EMT). The aim of this study is to detect whether macrophages interacting with epithelial cells equally induce the EMT process in normal and eutopic endometria of healthy and adenomyotic patients; and whether macrophages parallelly polarize to M2. We investigated the expression levels of epithelial cadherin (E-cadherin), neural cadherin (N-cadherin), cytokeratin7 (CK7), vimentin, transforming growth factor- β 1 (TGFB1), SMAD3 and pSMAD3 using immunohistochemistry and western blot, and then estimated the genetic levels of *CD163*, *IL10* and *MMP12* using real-time quantitative polymerase chain reaction (RT-PCR) in macrophages. Eutopic and normal endometrial tissues were obtained from 20 patients with adenomyosis and 11 control patients without adenomyosis, respectively. The immunohistochemical analysis shows distinct EMT in eutopic endometria in secretory phase; the expression levels of TGFB1, SMAD3 and pSMAD3 that indicate signal pathway of EMT were also higher in secretory phase. Macrophages can induce EMT process in primary endometrial epithelial cells derived from normal and eutopic endometria. After co-culturing, THP-1-derived macrophages polarized to M2. Compared with the eutopic endometrium group, further polarization to M2 was observed in the normal endometrium group. These results indicate that adenomyosis may be promoted by the pathologic EMT of epithelial cells, which is induced by macrophages that incapably polarize to M2.

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Introduction

Adenomyosis, an estrogen-dependent inflammatory disease, is defined as the presence of endometrial glands and stroma deep within the myometrium; two distinct forms of adenomyosis, namely, diffuse and focal, have been described (Ferenczy 1998). The main clinical features of adenomyosis are dysmenorrhea and menorrhagia, which are significantly associated with peritoneal endometriosis in infertile patients at reproductive age (Kunz *et al.* 2000, 2005). However the pathogenesis of adenomyosis remains unclear. The only difference between adenomyosis and endometriosis is the site of endometriotic tissues, that is, inside or outside the uterus (Ota *et al.* 1998). Thus, knowledge on the endometriotic cell origin is indispensable for the development of preventive and targeted treatment strategies for adenomyosis. The most widely accepted theory on the pathogenesis of adenomyosis is the downgrowth and invagination of the endometrium into the myometrium (Bergeron *et al.* 2006); however the possible mechanism of gland invagination from the endometrium deep into the underlying myometrium is unknown.

Epithelial–mesenchymal transition (EMT) and its converse, mesenchymal–epithelial transition (MET), were defined decades ago (Alcorn *et al.* 1999). Once epithelial cells become competent to respond to EMT-inducing signals, these signals can promote the disruption of the intercellular adhesion complexes and the loss of the apicobasal polarity of the epithelial cells, a prime feature crucial for cells to leave the epithelium and achieve migration potentiality (Khan *et al.* 2015). Many studies indicated that EMT is a crucial process in adenomyosis and endometriosis lesions (Chen *et al.* 2010, Matsuzaki & Darcha 2012), and the endometrium and inner myometrium are closely apposed without any intervening basement membrane; thus, EMT events might occur here.

A hallmark of EMT is the downregulation of epithelial cadherin (E-cadherin) to reinforce the destabilization of adherens junctions. Specifically, the downregulation of E-cadherin is balanced by the increased expression of mesenchymal neural cadherin (N-cadherin), which results in a ‘cadherin switch’ that alters cell adhesion (Wheelock *et al.* 2008). Alterations in the expression of genes encoding cytoskeletal and polarity complex

proteins also contribute to EMT. Keratin and vimentin filaments regulate the trafficking of organelles and membrane-associated proteins, but show differences in the proteins that they target to the membrane (Lamouille *et al.* 2014). Thus, N-cadherin, E-cadherin, vimentin and cytokeratin7 (CK7) are markers for EMT and play different roles during the EMT process.

Macrophages have been known to play important roles in the adenomyosis process (Ota *et al.* 1998). One study reported an increased stromal macrophage population in the functional layer of the endometrium in patients experiencing diffuse and focal adenomyosis (Tremellen & Russell 2012). Another study indicated that, after treating adenomyosis patients with the gonadotropin-releasing hormone (GnRH) agonist, the infiltration of CD68-positive macrophages is significantly decreased in the endometrium of adenomyotic women (Khan *et al.* 2010). Although macrophages have been known to be involved in the adenomyosis process by enhancing cell growth and proliferation through the amplification of cytokine secretion (Chen *et al.* 2003, Shao *et al.* 2016), the number and identity of factors that act on this regulatory process are still unknown.

Abnormal levels of macrophages, which are important components of immune cells, have been largely reported in adenomyosis (Ota *et al.* 1998, Zhihong *et al.* 2016). Increased knowledge on the immune aspects of the pathogenesis of adenomyosis is needed for this debilitating condition. To our knowledge, macrophages, which play important roles in innate and acquired immunity, together with natural killer cells and cytotoxic T-lymphocytes in healthy women, can destroy misplaced endometrial cells (Dmowski *et al.* 1998). The EMT process induced by macrophages has been investigated systematically in tissue repair, remodeling, fibrosis (Scotton & Chambers 2007), and tumor progression (Galdiero *et al.* 2013). Alternatively activated (M2) macrophages are the major type associated with the tumor EMT process. Like tumor progression, adenomyosis also exhibits the EMT process.

Our previous study showed that THP-1-derived macrophages induced EMT process in endometrial epithelial cells of patients with adenomyosis (Min *et al.* 2017). A recent study involving EMT of endometrial epithelial cells was performed on human primary endometrial epithelial cells, which were isolated from normal endometrial tissues (Xiong *et al.* 2016). Therefore we want to investigate if the macrophages can induce normal epithelial cells to undergo EMT, and if macrophages interacting with epithelial cells equally induce the EMT process in normal and eutopic endometria of healthy and adenomyotic patients. Also, in our previous study (Min *et al.* 2017), after co-culturing with eutopic epithelial cells of adenomyosis, THP-1-derived macrophages polarized to M2. Thus, we also want to estimate if the macrophages parallelly polarize

to M2 between eutopic endometrium and normal endometrium.

Given that macrophages and EMT are all involved in the adenomyosis process, and the definite mechanism by which macrophages promote the development of adenomyosis is vague, macrophages are presumed to induce endometrial epithelial cells to undergo EMT because macrophages can induce EMT in many other diseases. The co-culture system was used in this study to test the EMT process of epithelial cells isolated from normal endometrium and adenomyotic eutopic endometrium induced by macrophages derived from THP-1. As a predominant signaling pathway in EMT, the protein expression levels of transforming growth factor- β 1 (TGFB1) and SMAD3/pSMAD3 in epithelial cells were also evaluated. The macrophages were simultaneously collected to estimate the gene expression levels of *CD163*, *IL10* and *MMP12*, a group of classic markers for M2 in the co-culture system.

Materials and methods

Ethical approval

The study protocol was approved by the Institutional Review Board of the Qilu Hospital Authority (KYL-2015-077).

Patients and tissue samples

This study recruited 20 women with adenomyosis who were diagnosed by ultrasound doctors before operations and clinical pathologists after hysterectomy at the Qilu Hospital of Shandong University from June 2015 to March 2016. After obtaining the patients' written informed consent, eutopic endometrium tissues were collected during the operation and immediately sent to the laboratory. The tissues were separated into two parts: one was cultured *in vitro*, and the remaining was stored with 10% buffered formalin and processed for paraffin embedding. For the controls, endometrial tissue samples were collected through curettage from 11 women who exhibited fallopian tube jam but without any clinical indication or history of adenomyosis or endometriosis. The diagnosis was done by ultrasound doctors before operations, and then doctors and clinical pathologists made a definite diagnosis after laparoscopic surgery. The characteristics of patients recruited with adenomyosis and controls are shown in Table 1. Because four patients with adenomyosis had endometrial polyps, they were excluded for the present analysis.

Immunohistochemical (IHC) staining

The slides were subjected to immunohistochemical (IHC) analysis. Tissue sections were dewaxed and rehydrated in ethanol and water. Antigen retrieval was performed in citrate buffer (pH 6.0, 15 min), and endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide. The tissue sections incubated overnight at 4°C for rabbit primary antibody against human CK7 (ab68459, Abcam, 1:400),

Table 1 Characteristics of patients recruited with adenomyosis and controls.

Item	Controls (n = 11)	Adenomyosis (n = 20)	Statistical significance
Age (year)			**
Mean (s.d.)	30.0 (8.7)	40.8 (5.1)	
Median (range)	30 (20–41)	40.5 (31–49)	
Menstrual phase			NS
Proliferative	7 (63.6%)	9 (45.0%)	
Secretory	4 (36.4%)	11 (55.0%)	
Gravidity			***
0	3 (27.3%)	1 (5.0%)	
1	4 (36.4%)	2 (10.0%)	
≥2	4 (36.4%)	17 (85.0%)	
Abortion			***
0	4 (36.3%)	4 (20.0%)	
1	5 (45.5%)	3 (15.0%)	
≥2	2 (18.2%)	13 (65.0%)	
Visual analog scale on the severity of dysmenorrhea			***
Mean (s.d.)	0.5 (1.1)	5.9 (2.3)	
Median (range)	0 (0–5)	6 (0–8)	
Uterus size (cm ³)			***
Mean	72.5	344.3	
Median (range)	70.1 (52.3–80.1)	285.5 (110.1–628.2)	
Co-occurrence of endometriosis			NS
No	11 (100.0%)	18 (90.0%)	
Yes	0 (0.0%)	2 (10.0%)	
Co-occurrence of endometrial polyps			NS
No	11 (100.0%)	16 (80.0%)	
Yes	0 (0.0%)	4 (20.0%)	

VAS, visual analog scale; uterus size calculated as $\pi D1D2D3/6$, where D1 = the distance from fundus to the internal OS of the cervix, D2 = transverse diameter at the level of the cornua and D3 = anteroposterior diameter at the level of cornua. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: $P > 0.05$. Wilcoxon's rank test was used for age, VAS and uterus size while for other data Fisher's exact test was used.

E-cadherin (ab40772, Abcam, 1:400), N-cadherin (ab18203, Abcam, 1:400), vimentin (ab92547, Abcam, 1:700), TGF β 1 monoclonal antibody (ab92486, Abcam, 1:200), SMAD3 monoclonal antibody (ab40854, Abcam, 1:200), and pSMAD3 monoclonal antibody (ab52903, Abcam, 1:100). The secondary antibody kit (CWBIO, Beijing, China) was utilized to link the primary antibody. The sections were counterstained with hematoxylin, dehydrated in ethanol and xylene, and mounted in Permount mounting medium. The immunostaining results were evaluated using a previously reported method (Shen *et al.* 2015). The images were obtained using a microscope (Olympus BX53, Olympus), which was fitted with a digital camera (Olympus cellSens Standard, Olympus). A series of five images was randomly selected from several sections per tissue sample. Each image was taken for each immunostained marker to yield a mean optical density value by Image Pro-Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA). Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images, and subsequent measurement readings were obtained. Immunohistochemical parameters assessed in the area detected included (1) integrated optical density (IOD), (2) total stained area (S) and (3) the mean optical density (MOD), equivalent to the mean intensity of staining across all glands.

Primary endometrial cell culture

The isolation and culture of adenomyotic primary eutopic endometrial cells and normal endometrial cells were

conducted using a previously reported method (Chan *et al.* 2004). The endometrial tissues were minced into small pieces (1 mm³). After the minced tissues were subjected to enzymatic digestion with 0.25% (w/v) collagenase II mixed with 0.125% (w/v) collagenase IV (Worthington, Lakewood, NJ, USA) for 75 min at 37°C, the tissues were filtered initially through a 100 μ m (pore size) nylon mesh to remove debris and then through a 40 μ m (pore size) nylon mesh (Falcon cell strainers; Thermo Fisher Scientific). The epithelial cells remaining in the cell strainer were collected, resuspended in Dulbecco's modified Eagle's medium/F12 (Gibco), and plated onto six-well plates. Then, the culture medium was changed to RPMI-1640 medium (Gibco) to prepare for co-culturing with macrophages. The endometrial cells were observed through an inverted microscope, and the cellular morphology of epithelial cells is shown in Fig. 1A. The endometrial cells were verified through immunofluorescent staining by using an antibody against CK7 (ab68459, Abcam, 1:200), which is a specific marker for epithelial cells (Fig. 1B). The purity of epithelial cells isolated from endometria was greater than 95%, which was in conformity with a previous study (Kao *et al.* 2011).

Epithelial cells co-cultured with macrophages

THP-1 cells (acute monocytic leukemia) were provided by Dr Chengjiang Gao (Shandong University, School of Medicine, Department of Immunology, Jinan, Shandong, China). Macrophage differentiation of the THP-1 cells was conducted using a previously reported method (Dehai *et al.* 2014).

Figure 1 (A) Isolated and cultured primary epithelial cells as well as THP-1 cells cultured with or without PMA. (B) Epithelial cells isolated from endometrium were verified by immunofluorescent staining (nonlinear adjustment). CK7, stained in green, was used as the marker for epithelia. DAPI, stained in dark blue, was used as the marker for cell nuclei. (C) Macrophages derived from THP-1 cells were verified by immunofluorescent staining (nonlinear adjustment). CD68, stained in red, was used as the marker for macrophages. DAPI, stained in dark blue, was used as the marker for cell nuclei. (D) The sketch map of the co-culturing system.

The cells were triggered by adding 100ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for two days and washed thrice with phosphate-buffered saline (PBS) to eliminate the effect of PMA. Macrophages were observed through an inverted microscope, and the cellular morphology is shown in Fig. 1A. The macrophages were also verified through immunofluorescent staining using an antibody against CD68 (ab955, 1:200; Abcam), which is a marker for macrophages (Fig. 1C). The primary endometrial epithelial

cells were co-cultured with THP-1-derived macrophages using a standard Transwell insert (0.4 μ m; Corning) (Fig. 1D). Each well was plated with approximately 10,000 primary endometrial cells. The cells were washed after incubation for 24h with 10% fetal bovine serum medium, and the inserts with induced macrophages (7.5×10^5 cells) were added to the wells. The control group had an empty insert. Epithelial cells were harvested at different time points, that is, on days 1, 2, 4 and 6.

Immunocytochemistry

We performed immunocytochemistry staining to show morphological changes of epithelial cells with EMT-related proteins as previously described (Xue *et al.* 2013). The cultured epithelial cells were washed twice with PBS and fixed in 4% paraformaldehyde (pH 7.0) for 30 min. The cells were then washed in PBS, blocked with 10% normal goat serum for 1 h, and incubated overnight with rabbit anti-human CK7 monoclonal antibody (ab68459, 1:400; Abcam) and rabbit anti-human vimentin monoclonal antibody (ab92547, 1:600; Abcam) at 4°C. The secondary antibody kit (CWBIO, Beijing, China) was utilized to link the primary antibody. Cells were counterstained with hematoxylin before mounting.

Western blot

The EMT-like process-related proteins were examined using western blot analysis for primary endometrial cells at four different time points after co-culturing the cells with THP-1-derived macrophages. The cells were scraped and extracted in a commercial kit (BestBio, Shanghai, China) for total protein extraction. All proteins that were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (P0015; Beyotime, Shanghai, China) were heated for 5 min at 100°C. Protein samples were loaded onto 10% SDS-PAGE and electroplated onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked in 5% nonfat milk (BD, Franklin Lakes, NJ, USA) reconstituted in TBST (0.15 M NaCl, 0.05% Tween 20, and 10 mM Tris-HCl (pH 8.0)) for 70 min at room temperature and subsequently incubated overnight at 4°C with the following primary antibodies: rabbit anti-human CK7 monoclonal antibody (ab68459, 1:5,000; Abcam), rabbit anti-human E-cadherin monoclonal antibody (ab40772, 1:10,000; Abcam), rabbit anti-human vimentin monoclonal antibody (ab92547, 1:5000; Abcam), rabbit anti-human N-cadherin monoclonal antibody (ab18203, 1:1000; Abcam), rabbit anti-human TGFB1 monoclonal antibody (ab92486, 1:1000; Abcam), rabbit anti-human SMAD3 monoclonal antibody (ab40854, 1:1000; Abcam), and rabbit anti-human GAPDH polyclonal antibody (10494-1-AP, 1:20,000; Proteintech, Wuhan, China). After incubating the membranes with horseradish-peroxidase-labeled secondary antibodies for 70 min at room temperature, the signal was detected by Image Studio Digits version 4.0. Three independent experiments were performed.

Real-time polymerase chain reaction (RT-PCR)

The co-cultured cells (macrophages) were washed with cold PBS, and the total RNA was extracted with TRIzol Reagent (Invitrogen Life Technologies) in accordance with the manual of the product owner. RNA (1 µg) was reverse-transcribed into cDNA with ReverTra Ace quantitative PCR (qPCR) RT Master Mix with gDNA Remover (Code No. FSQ-301; Toyobo, Osaka, Japan). Each 20 µL of PCR product contained 1× SYBR Green PCR Master Mix (Toyobo), 30 ng of cDNA, and 300 nM of each specific primer. The primers used for each gene are listed in Table 2. Subsequently, qPCR was performed on an Applied

Table 2 Primer sequences of each gene detected in real-time RT-PCR.

CD163	Forward	GGCTTGCACTTTCCTCAAGA
CD163	Reverse	AGCTGACTCATGGGAATTTTCTG
MMP12	Forward	ACTACACATTACAGGAGGCACA
MMP12	Reverse	GTCATCAGCAGAGAGGGCGAA
IL10	Forward	AGGACTTTAAGGGTTACCTGGG
IL10	Reverse	TTCTCAGCTTGGGGCATCAC
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
GAPDH	Reverse	TGGTGAAGACGCCAGTGGA

Biosystems 7500 RT-PCR System (Applied Biosystems). Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. The mean was obtained to determine the mRNA levels by quantitative RT-PCR analysis, which was performed using the ABI 7500 RT-PCR system (Applied Biosystems). The gene expression levels for each group were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mean relative gene expression level was determined, and the differences were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Graphical and statistical analyses were performed using the GraphPad prism software. Comparative statistical analyses were performed using the Mann-Whitney test of immunohistochemical staining. And Wilcoxon matched pairs test was performed of the antigen expression in western blot between the study (co-cultured with macrophage) and control groups; between the normal endometrium and eutopic endometrium groups, the Mann-Whitney test was used. The gene expression levels of macrophages among the control, normal endometrium and eutopic endometrium groups were calculated using the one-way analysis of variance (Newman-Keuls multiple comparison test). *P* values ≤0.05 were considered significant.

Results

The different expressions of EMT-associated proteins in the normal and eutopic endometrial epithelial cells during menstrual

To investigate whether EMT process exists in adenomyotic eutopic endometrium, we compared the expression levels of CK7, vimentin, E-cadherin and N-cadherin in the endometrium with or without adenomyosis. As depicted in Figs 2 and 3, we found that the expression levels of CK7, E-cadherin, N-cadherin, TGFB1, SMAD3 and pSMAD3 have no significant difference between normal and eutopic endometrial epithelial cells in proliferative phase except vimentin, which was upregulated. While in the secretory phase, the expression levels of CK7 and E-cadherin were both downregulated in eutopic endometrial epithelial cells compared with the normal; as for vimentin, N-cadherin, TGFB1 and SMAD3, the expression levels were both

Figure 2 The expression levels of CK7, vimentin, E-cadherin, N-cadherin and several signal pathway proteins (TGFB1, SMAD3 and pSMAD3) in the endometrium of women without adenomyosis and endometriosis ($n=11$), and eutopic endometrium ($n=16$) of women with adenomyosis were analyzed by immunohistochemistry. Original magnification, $\times 400$; NP, endometrium of women without adenomyosis at proliferative phase; EuP, endometrium of patient with adenomyosis at proliferative phase; NS, endometrium of women without adenomyosis at secretory phase; EuS, endometrium of patient with adenomyosis at secretory phase.

Figure 3 Results of MOD analysis (Mann-Whitney test) using all immunostaining data. All patients were represented by points. *Normal group vs eutopic group; #proliferative phase (eutopic) vs secretory phase (eutopic); & proliferative phase (normal) vs secretory phase (normal). */#&P<0.05; **/#&P<0.01; ***/#&P<0.001. The results are expressed as the mean $\pm$ S.E.M.

high in secretory phase; the expression of pSMAD3 did not differ between eutopic and normal epithelial cells. We also compared the expression level of CK7, vimentin, E-cadherin, N-cadherin, TGFB1, SMAD3 and pSMAD3 between proliferative and secretory phase. As shown in Figs 2 and 3, in eutopic endometrium, the expression levels of TGFB1, SMAD3 and pSMAD3 in secretory phase were higher than those proliferative phase; while in normal endometrium, only pSMAD3

was upregulated; however the expression of E-cadherin downregulated in secretory phase.

Mesenchymal-like morphological changes of epithelial cells in immunocytochemistry staining after co-culture

Immunocytochemical analyses of CK7 and vimentin expression were performed to investigate the morphological changes of epithelial cells. As shown in Fig. 4, analysis using an ordinary light microscope revealed mesenchymal-like changes in epithelial cells after they were co-cultured with macrophages for 2 days. The expression level of CK7 was downregulated and vimentin was upregulated in epithelial cells. Although the changes of cell morphology were less obvious than protein expression, some epithelial cells have typical mesenchymal-like morphology.

THP-1-derived macrophages induce primary endometrial epithelial cells to undergo EMT, and compared with the normal endometrium group, the downregulation of E-cadherin is evident in the eutopic endometrium group

Co-culturing of endometrial epithelial cells and macrophages can induce endometrial epithelial cells of the adenomyotic eutopic endometrium and normal endometrium to exhibit EMT, which was indicated by the downregulation of epithelial markers (E-cadherin and CK7) and the upregulation of mesenchymal markers (vimentin and N-cadherin). After co-culturing with macrophages, the epithelial cells derived from the eutopic endometrium of adenomyosis patients exhibited EMT (Fig. 5A). Statistical analysis showed that, although the protein expression levels of CK7 and E-cadherin were downregulated during the entire experimental period, statistical significance was only observed on days 1, 4 and 6 (Fig. 5B). Moreover, the protein expression level of vimentin was significantly upregulated on day 6 (Fig. 5B). Similarly, EMT occurred in epithelial cells isolated from the normal endometrium (Fig. 5A). The time point that showed statistical significance was day 6 for vimentin expression (Fig. 5C). The protein expression level of control group, epithelial cells isolated from adenomyotic eutopic and normal endometria, was estimated first before comparing the variance (m/c; shown in Fig. 5A) of the protein expression of EMT between adenomyotic eutopic and normal endometria after co-culture with macrophages. Low expression of E-cadherin was observed in adenomyotic eutopic epithelial cells compared with the normal epithelial cells before co-culturing (Fig. 5D). After co-culturing with macrophages, there was no significance of the fold changes for CK7, E-cadherin, vimentin and N-cadherin expressions between the eutopic endometrium group and the normal endometrium group (Fig. 5E).

Figure 4 Immunocytochemical staining for vimentin and CK7 expression. The cytoplasm of cells was negative for vimentin (C) and positive for CK7 (A) before co-culture; after 2 days of co-culture, the cytoplasm of cells was positive for vimentin (D) and negative for CK7 (B). The nuclei were stained with hematoxylin. Magnification, $\times 400$.

Primary endometrial epithelial cells co-cultured with macrophages did not significantly enhance the *TGFB1/SMAD3* protein expression level; however the expression level of *pSMAD3* was upregulated

The protein expression levels of *TGFB1* and *SMAD3* in the eutopic endometrium and normal endometrium groups were insignificantly upregulated after co-culturing endometrial epithelial cells and macrophages (Fig. 6A, B, and C). The protein expression level of control group epithelial cells isolated from adenomyosis eutopic and normal endometria was also estimated. Statistical analysis indicated no difference in the protein expression levels of *TGFB1* and *SMAD3* between adenomyosis eutopic and normal endometrial epithelial cells without co-culture (Fig. 6D). However the upregulation of *pSMAD3* after co-culturing was higher on day 6 in the epithelial cells both derived from normal and eutopic endometria after co-culture (Fig. 6A, B and C); statistical analysis indicated that the protein expression levels of *pSMAD3* in eutopic epithelial cells was higher than normal (Fig. 6D). After co-culturing with macrophages, the fold changes of *TGFB1*, *SMAD3* and *pSMAD3* have no difference between the eutopic endometrium group and the normal endometrium group (Fig. 6E).

When co-cultured with endometrial cells, THP-1-derived macrophages polarized to the M2 type; compared with the eutopic endometrium group, further polarization to M2 was detected in the normal endometrium group

The interaction of macrophages and endometrial epithelial cells induced the endometrial epithelial cells to undergo mesenchymal transition-like process. Meanwhile, endometrial epithelial cells can induce macrophages to polarize. The PCR results proved

that primary endometrial epithelial cells can cause macrophages to polarize to the M2 type. The genetic level of *IL10* in the eutopic endometrium and normal endometrium groups significantly increased when compared with the control group. The expression of *IL10* was higher in the normal endometrium group on day 1 and day 4 (Fig. 7B). However the genetic level of *CD163* increased only in the normal endometrium group on day 1 and day 6. The eutopic endometrium group did not exhibit *CD163* expression in macrophages compared with the control group. Therefore in terms of induced *CD163*, the normal endometrium group exhibited higher levels than the eutopic endometrium group on day 1 and day 6 (Fig. 7B). The genetic level of *MMP12* increased in the normal endometrium group at all the time points. In contrast to the control group, the *MMP12* levels increased significantly in the eutopic endometrium group only on day 1 and day 2. The induction of *MMP12* expression was higher in the normal endometrium group than in the eutopic endometrium group, except on day 2 (Fig. 7B). Throughout the experiment, the polarization of macrophages in the eutopic endometrium group was tender, whereas that in the normal endometrium group was fluctuant: the expression levels of *CD163*, *IL10* and *MMP12* initially increased, then decreased, and increased again. Although the polarization of macrophages was definite, the morphological change of macrophages was not evident (Fig. 7A).

Discussion

The EMT process shown in eutopic endometrial epithelial cells was comparable with that of the normal epithelial cells in IHC analysis at secretory phase; however it was not obvious in proliferative phase. The primary epithelial cells derived from adenomyotic eutopic and normal endometria were successfully induced to undergo EMT by co-culturing them with macrophages. In the co-cultured system, the macrophages derived from THP-1 polarized to M2, and the polarization of macrophages was more intense in the normal endometrium group than in the eutopic endometrium group.

Eutopic endometrium has always been considered the origin of ectopic endometrium in endometriosis or adenomyosis (Benagiano et al. 2013). Research on the adenomyosis mechanism gained more attention after the following definition was provided (Bird et al. 1972): 'Adenomyosis may be defined as the benign invasion of endometrium into the myometrium, producing a diffusely enlarged uterus which microscopically exhibits ectopic non-neoplastic, endometrial glands and stroma surrounded by the hypertrophic and hyperplastic myometrium.' However the specific molecular mechanism is still unclear. In recent years, EMT has emerged in adenomyosis mechanism research. Although these studies had far-reaching significance,

Figure 5 (A) Western blot of epithelial cells isolated from normal endometrium and adenomyosis eutopic endometrium. C, control group; M, macrophages (co-culturing) group. (B) Statistical analysis of the protein expression levels of E-cadherin, CK7, N-cadherin and vimentin in eutopic epithelial cells between the control and co-culturing groups (Wilcoxon matched pairs test). (C) Statistical analysis of the protein expression levels of E-cadherin, CK7, N-cadherin and vimentin in normal epithelial cells between the control and co-culturing groups (Wilcoxon matched pairs test). (D) Statistical analysis of the protein expression levels of E-cadherin, CK7, N-cadherin and vimentin between normal and eutopic epithelial cells without co-culturing. (E) Statistical analysis of the fold changes of the protein expression levels between normal and eutopic epithelial cells after co-culturing. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The results are expressed as the mean $\pm$ S.E.M.

the EMT process in adenomyosis research should be supplemented. The analysis of IHC did not find the definite evidence of EMT in the eutopic endometrium of adenomyosis in proliferative phase. However we found an EMT phenomenon in eutopic endometrium at

secretory phase, and the expression levels of TGF β 1 and SMAD3 that indicate signal pathway of EMT were higher in secretory phase, which meant that adenomyosis eutopic endometrium epithelial cells had been abnormally activated and dysfunctional. Through the

Figure 6 (A) Western blot of epithelial cells isolated from normal endometrium and eutopic endometrium of adenomyosis. C, control group; M, macrophages (co-culturing) group. (B) Statistical analysis of the protein expression levels of TGFB1 and SMAD3/pSMAD3 in eutopic epithelial cells between the control and co-culturing groups. (C) Statistical analysis of the protein expression levels of TGFB1 and SMAD3/pSMAD3 in normal epithelial cells between the control and co-culturing groups. (D) Statistical analysis of the protein expression levels of TGFB1 and SMAD3/pSMAD3 between normal and eutopic epithelial cells without co-culturing. (E) Statistical analysis of the fold changes of the protein expression levels between normal and eutopic epithelial cells after co-culturing. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The results are expressed as the mean $\pm$ S.E.M.

Figure 7 (A) Representative photomicrographs of macrophages with and without co-culturing (original magnification, $\times 400$). (B) Gene expression of THP-1-derived macrophages co-cultured with or without normal or eutopic epithelial cells. *Normal group vs control group; #adenomyosis group vs control group; &normal group vs adenomyosis group. */&P < 0.05; **/##/&P < 0.01; ***/###/&&P < 0.001. The results are expressed as the mean $\pm$ S.E.M.

comparison during the menstrual cycle, we found that the pathway of TGF β 1/SMAD3 (pSMAD3) was activated in the secretory phase, and the expression of E-cadherin was downregulated. These phenomena were consistent with previous research (Kim *et al.* 2005, Chen *et al.* 2010). We drew two reasons for the upregulated TGF β 1/SMAD3 (pSMAD3) pathway in secretory phase of eutopic endometrium: one is progesterone, as a previous study described that the progesterone induces stromal decidualization indirectly by enhancing the expression and secretion of TGF β 1 from epithelial cells (Kim *et al.* 2005); the other is the microenvironment, which stimulated the pathway to lead to EMT.

Recently, the growing body of literature strongly suggested that macrophages play a critical role in EMT regulation (Liu *et al.* 2013, Fan *et al.* 2014, Su *et al.* 2014), but its role has not been clarified in adenomyosis yet. In this study, primary epithelial cells were induced by THP-1-derived macrophages to undergo EMT. In order to keep uniformity, we chose THP-1-derived macrophages which were the factor that induced EMT in epithelial cells (Dehai *et al.* 2014, Yang *et al.* 2016) to induce EMT process of normal and eutopic epithelial cells. The EMT process of epithelial cells was determined by the exact amount of exposure of THP-1-derived macrophages

compared with the controls. Eutopic and normal epithelial cells underwent EMT. Immunocytochemical analyses of CK7 and vimentin showed the cellular morphology. Although the changes of cell morphology were less obvious than protein expression, some epithelial cells have typical mesenchymal-like morphology, which was consistent with our previous study on the topic of EMT process of Ishikawa cells (Min *et al.* 2017). Statistical analysis of western blot showed that the changes of E-cadherin and CK7 were evident at some time points, but the upregulation of N-cadherin and vimentin was not obvious in this study. The EMT of normal epithelial cells demonstrated that the microenvironment and macrophages played important roles in the EMT of adenomyosis. Considerable research has clarified the involvement of macrophages in the adenomyosis process (Yang *et al.* 2006, Khan *et al.* 2010, Tremellen & Russell 2012). However the present study proved, from another perspective, that macrophages were indispensable in the pathogenesis of adenomyosis.

After comparing the eutopic and normal epithelial cells without co-culturing, changes in the expression of E-cadherin, CK7, N-cadherin and vimentin in epithelial cells were compared between the eutopic and normal groups after co-culturing with macrophages. Consistent with a recent study (Xiong *et al.* 2016), no significant difference in the expression levels of E-cadherin and N-cadherin was observed between normal and eutopic epithelial cells. Moreover, no significant difference in the expression levels of CK7 and vimentin was observed between the two groups. In addition, after co-culturing with macrophages, the fold changes of downregulated E-cadherin and CK7, and upregulated N-cadherin and vimentin have no difference between the eutopic endometrium group and the normal endometrium group. So, we speculate that macrophages can equally induce EMT process in eutopic and the normal epithelial cells.

The TGF β 1/SMAD3 signaling pathway is essential for the EMT process. Although the upregulation of TGF β 1 and SMAD3 was statistically insignificant, the protein expression levels of pSMAD3 were higher in the co-cultured epithelial cells than in the control. The expression levels of TGF β 1, SMAD3 and pSMAD3 in epithelial cells were compared between eutopic and normal epithelial cells without co-culturing with macrophages. Then, the expression changes of TGF β 1, SMAD3 and pSMAD3 in epithelial cells were compared between eutopic and normal groups after co-culturing with macrophages. The protein expression levels of TGF β 1 and SMAD3 between eutopic and normal endometrium groups differed insignificantly, which is inconsistent with previous studies (Johnson *et al.* 2005, Cruz *et al.* 2015). Another research reported that only weak upregulation of TGF β 1 in endometriosis eutopic endometrium occurred compared with the controls (Goteri *et al.* 2015). The present study is the first to compare the protein expression levels of TGF β 1 and

SMAD3 between adenomyotic eutopic and normal endometria, and the authors hope that this study could gain more attention from researchers involved in mechanism studies of adenomyosis. No significant difference in protein expression was observed between eutopic and normal endometrium groups without co-culturing; it was the same with the fold changes of TGF β 1 and SMAD3 after co-culturing with macrophages in the eutopic endometrium group than in the normal endometrium group. This result was not consistent with our IHC analyses, and we speculated that the lack of steroids in the *in vitro* culture medium influenced it. However it is worth noting that the expressions of pSMAD3 were upregulated both in normal and eutopic epithelial cells after co-culture. But the fold changes of pSMAD3 after co-culturing have no significance. Thus, it is proved that the stimulation and induction of EMT process in the epithelial cells of the adenomyotic eutopic endometrium and normal endometrium is parallel.

Abnormal levels of immune cells, such as macrophages, have been largely reported in adenomyosis (Ota *et al.* 1998, Zhihong *et al.* 2016). Increased knowledge on the immune aspects of the pathogenesis of adenomyosis may allow the development of novel medical therapies for this debilitating condition. To our knowledge, macrophages, which play important roles in innate and acquired immunity, together with natural killer cells and cytotoxic T-lymphocytes in healthy women, can destroy misplaced endometrial cells (Dmowski *et al.* 1998). However, during the past decades, several reports identified a decrease or impairment in the cell-mediated immunity of women with adenomyosis or endometriosis (Matarese *et al.* 2003, Guo *et al.* 2016). In this study, epithelial cells from normal and eutopic endometria can induce macrophages to polarize to the M2 type. However the inducing capability of the normal endometrium group was more intense than that of the eutopic endometrium group. CD163 was a hallmark of M2 macrophage. In our study, the expression of it was not higher in THP-1-derived macrophages after co-culture with eutopic epithelial cells. That signifies that the ratios of M2 to pan-macrophages were significantly lower in adenomyosis patients than those in normal group. It can be consistent with a previous study performed in endometriosis (Takebayashi *et al.* 2015). Khan *et al.* demonstrated that, compared to control women without endometriosis, women with endometriosis had *Escherichia coli* contamination in menstrual blood with increased levels of bacterial endotoxin in the menstrual fluid and peritoneal fluid (Berbic *et al.* 2009); this will affect the polarization of macrophages. Thus, we suspect that this phenomenon exists in adenomyosis as well. On the other hand, adenomyosis itself might be the cause of the incapable polarization of M2 macrophages in the eutopic endometrium. Given the failure of polarization to M2 in the eutopic endometrium group, the misplaced

endometrial cells might escape macrophage's engulfment and elimination. This incapacity can result from the eutopic endometrium itself or pelvic microenvironment. As the uterine cavity is connected to the pelvic cavity through the oviducts, some substances like immune cells in the pelvic cavity can flow into the uterine cavity, especially during the process of ovulation. Moreover, fluctuations in the genetic levels of CD163, IL10 and MMP12 were observed in the normal endometrium group because of the sensitive characteristics and homeostasis of macrophages. The weak activation of macrophages in the eutopic endometrium group demonstrated that the eutopic endometrium was immunotolerant.

In conclusion, endometrial cells and microenvironment are two important factors in the pathogenesis of adenomyosis. This study showed that both epithelial cells isolated from eutopic and normal endometria can be equally induced by macrophages to undergo EMT. The polarization of macrophages to M2 was less intense in the eutopic endometrium group than in the normal endometrium group. Further study is required to examine the immunity mechanism of the incapability, even failure, of polarization to M2 of inducing macrophages in eutopic endometrium of adenomyosis. Because, in eutopic epithelial cells, the expression levels of E-cadherin and pSMAD3 were higher than normal; we can attribute the incapable polarization to M2 to it. Our current findings suggest that adenomyosis may be promoted by the ability of epithelial cells derived from the eutopic endometrium to undergo EMT and the incapability of inducing macrophages to polarize to M2. Thus, immune regulation and inflammation reaction reduction may help relieve adenomyosis.

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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Authors' contribution statement

Guoyun Wang and Dong Li conceived and designed the study. Min An analyzed and interpreted data, and drafted the manuscript. Min An and Ming Yuan performed the experiments. Qiuju Li recruited patients and secured tissue samples. Every author participated in the writing and revision of the manuscript.

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