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1 # Different macrophages equally induce EMT in endometria
2 # of adenomyosis and normal

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11 # ABSTRACT

12 Endometrial cells and microenvironment are two important factors in the pathogenesis
13 of adenomyosis. Our previous study demonstrated that macrophages can induce eutopic
14 epithelial cells of adenomyosis suffering from epithelial–mesenchymal transition
15 (EMT). The aim of this study is to detect whether macrophages interacting with
16 epithelial cells equally induce the EMT process in normal and eutopic endometria of
17 healthy and adenomyotic patients; and macrophages parallelly polarize to M2. We
18 investigated expressions of epithelial cadherin (E-cadherin), neural cadherin
19 (N-cadherin), cytokeratin7 (CK7), vimentin, transforming growth factor-β1 (TGFB1),
20 SMAD3 and pSMAD3 using Immunohistochemistry and Western Blot, then estimated
21 genetic levels of *CD163*, *IL10* and *MMP12* using real-time quantitative polymerase
22 chain reaction (RT-PCR) in macrophages. Eutopic and normal endometrial tissues were
23 obtained from 20 patients with adenomyosis and 11 control patients without
24 adenomyosis respectively. The analysis of Immunohistochemistry shows distinct EMT
25 in eutopic endometria in secretory phase; the expression level of TGFB1, SMAD3 and
26 pSMAD3 that indicate signal pathway of EMT were also higher in secretory phase.
27 Macrophages can induce EMT process in primary endometrial epithelial cells derived
28 from normal and eutopic endometria. After co-culturing, THP-1-derived macrophages
29 polarized to M2. Compared with the eutopic endometrium group, further polarization to
30 M2 was observed in the normal endometrium group. These results indicate that
31 adenomyosis may be promoted by the pathologic EMT of epithelial cells which is
32 induced by macrophages that incapably polarize to M2.

33 **Key Words:** Macrophages, adenomyosis, epithelial–mesenchymal transition (EMT),
34 endometrium

35 INTRODUCTION

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

50 Epithelial–mesenchymal transition (EMT) and its converse, mesenchymal–epithelial
51 transition (MET), were defined decades ago (Alcorn *et al.* 1999). Once epithelial cells
52 become competent to respond to EMT-inducing signals, these signals can promote the
53 disruption of the intercellular adhesion complexes and the loss of the apicobasal
54 polarity of the epithelial cells, a prime feature crucial for cells to leave the epithelium

55 and achieve migration potentiality (Khan *et al.* 2015). Many studies indicated that
56 EMT is a crucial process in adenomyosis and endometriosis lesions (Chen *et al.* 2010,
57 Matsuzaki & Darcha 2012), and the endometrium and inner myometrium are closely
58 apposed without any intervening basement membrane; thus, EMT events might occur
59 here.

60 A hallmark of EMT is the down-regulation of epithelial cadherin (E-cadherin) to
61 reinforce the destabilization of adherens junctions. Specifically, the down-regulation
62 of E-cadherin is balanced by the increased expression of mesenchymal neural
63 cadherin (N-cadherin), which results in a "cadherin switch" that alters cell adhesion
64 (Wheelock *et al.* 2008). Alterations in the expression of genes encoding cytoskeletal
65 and polarity complex proteins also contribute to EMT. Keratin and vimentin filaments
66 regulate the trafficking of organelles and membrane-associated proteins, but show
67 differences in the proteins that they target to the membrane (Lamouille *et al.* 2014).
68 Thus, N-cadherin, E-cadherin, vimentin, and cytokeratin7 (CK7) are markers for
69 EMT and play different roles during the EMT process.

70 Macrophages have been known to play important roles in the adenomyosis process
71 (Ota et al. 1998). One study reported an increased stromal macrophage population in
72 the functional layer of the endometrium in patients experiencing diffuse and focal
73 adenomyosis (Tremellen & Russell 2012). Another study indicated that, after treating
74 adenomyosis patients with the gonadotrophin-releasing hormone (GnRH) agonist, the
75 infiltration of *CD68*-positive macrophages is significantly decreased in the
76 endometrium of adenomyotic women (Khan *et al.* 2010). Although macrophages have

77 been known to be involved in the adenomyosis process by enhancing cell growth and
78 proliferation through the amplification of cytokine secretion (Chen *et al.* 2003, Shao
79 *et al.* 2016), the number and identity of factors that act on this regulatory process are
80 still unknown.

81 Abnormal levels of macrophages, which are important components of immune cells,
82 have been largely reported in adenomyosis (Ota *et al.* 1998, Zhihong *et al.* 2016).
83 Increased knowledge on the immune aspects of the pathogenesis of adenomyosis is
84 needed for this debilitating condition. To our knowledge, macrophages, which play
85 important roles in innate and acquired immunity, together with natural killer cells and
86 cytotoxic T-lymphocytes in healthy women, can destroy misplaced endometrial cells
87 (Dmowski *et al.* 1998). The EMT process induced by macrophages has been
88 investigated systematically in tissue repair, remodeling, fibrosis (Scotton & Chambers
89 2007), and tumor progression (Galdiero *et al.* 2013). Alternatively activated (M2)
90 macrophages are the major type associated with the tumor EMT process. Like tumor
91 progression, adenomyosis also exhibits the EMT process.

92 Our previous study showed that THP-1derived macrophages induced EMT process in
93 endometrial epithelial cells of patients with adenomyosis (Min et al. 2017). And a
94 recent study involved EMT of endometrial epithelial cells was performed by human
95 primary endometrial epithelial cells which were isolated from normal endometrial
96 tissues (Xiong et al. 2016). Therefore, we want to investigate that if the macrophages
97 can induce normal epithelial cells to EMT, and if macrophages interacting with
98 epithelial cells equally induce the EMT process in normal and eutopic endometria of

99 healthy and adenomyotic patients. And in our previous study (Min et al. 2017), after
100 co-culturing with eutopic epithelial cells of adenomyosis, THP-1-derived
101 macrophages polarized to M2. Thus, we also want to calculate that if the macrophages
102 parallelly polarize to M2 between eutopic endometrium and normal endometrium.
103 Given that macrophages and EMT are all involved in the adenomyosis process, and
104 the definite mechanism by which macrophages promote the development of
105 adenomyosis is vague, macrophages are presumed to induce endometrial epithelial
106 cells to undergo EMT because macrophages can induce EMT in many other diseases.
107 The co-culture system was used in this study to test the EMT process of epithelial
108 cells isolated from normal endometrium and adenomyosis-derived eutopic
109 endometrium induced by macrophages derived from THP-1. As a predominant
110 signaling pathway in EMT, the protein expression levels of transforming growth
111 factor-β1 (TGFB1) and SMAD3/pSMAD3 in epithelial cells were also evaluated. The
112 macrophages were simultaneously collected to estimate the gene expression levels of
113 *CD163*, *IL10*, and *MMP12*, a group of classic markers for M2 in the co-culture
114 system.

115 # MATERIALS AND METHODS

116 ***Ethical approval***

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

119 ***Patients and tissue samples***

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

134 ***Immunohistochemical (IHC) staining***

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

140 Cambridge, UK, 1:400), E-cadherin (ab40772, Abcam, Cambridge, UK, 1:400),
141 N-cadherin (ab18203, Abcam, Cambridge, UK, 1:400), vimentin (ab92547, Abcam,
142 Cambridge, UK, 1:700), TGFB1 monoclonal antibody (ab92486, Abcam, Cambridge,
143 UK, 1:200), SMAD3 monoclonal antibody (ab40854, Abcam, Cambridge, UK,1:200),
144 and pSMAD3 monoclonal antibody (ab52903, Abcam, Cambridge, UK,1:100). The
145 secondary antibody kit (CWBIO, Beijing, China) was utilized to link the primary
146 antibody. The sections were counterstained with hematoxylin, dehydrated in ethanol
147 and xylene, and mounted in PermountTM mounting medium. The immunostaining
148 results were evaluated using a previously reported method (Shen et al. 2015). The
149 images were obtained using a microscope (Olympus BX53, Olympus, Tokyo, Japan),
150 which was fitted with a digital camera (Olympus cellSens Standard, Olympus). A
151 series of five images was randomly selected from several sections per tissue sample.
152 Each image was taken for each immunostained marker to yield a mean optional
153 density value by Image Pro-Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA).
154 Staining was defined via color intensity, and a color mask was made. The mask was
155 then applied equally to all images, and subsequent measurement readings were
156 obtained. Immunohistochemical parameters assessed in the area detected included (1)
157 integrated optical density (IOD), (2) total stained area (S) and (3) the mean optical
158 density (MOD), equivalent to the mean intensity of staining across all glands.

159 ***Primary endometrial cell culture***

160 The isolation and culture of adenomyosis-derived primary eutopic endometrial cells

161 and normal endometrial cells were conducted using a previously reported method
162 (Chan *et al.* 2004). The endometrial tissues were minced into small pieces (1 mm^3).
163 After the minced tissues were subjected to enzymatic digestion with 0.25% (w/v)
164 collagenase II mixed with 0.125% (w/v) collagenase IV (Worthington, Lakewood, NJ,
165 USA) for 75 min at 37 °C, the tissues were filtered initially through a 100 μm (pore
166 size) nylon mesh to remove debris and then through a 40 μm (pore size) nylon mesh
167 (Falcon cell strainers; Fisher Scientific, Waltham, MA, USA). The epithelial cells
168 remaining in the cell strainer were collected, resuspended in Dulbecco's modified
169 Eagle's medium/F12 (Gibco, Beijing, China), and plated onto six-well plates. Then,
170 the culture medium was changed to RPMI-1640 medium (Gibco, Beijing, China) to
171 prepare for co-culturing with macrophages. The endometrial cells were observed
172 through an inverted microscope, and the cellular morphology of epithelial cells is
173 shown in Fig.1A. The endometrial cells were verified through immunofluorescent
174 staining by using an antibody against CK7 (ab68459, Abcam, Cambridge, UK, 1:200),
175 which is a specific marker for epithelial cells (Fig.1B). The purity of epithelial cells
176 isolated from endometria was greater than 95% which was in conformity with a
177 previous study (Kao et al. 2011).

178 ***Epithelial cells co-cultured with macrophages***

179 THP-1 cells (acute monocytic leukemia) were provided by Dr. Chengjiang Gao
180 (Shandong University, School of Medicine, Department of Immunology, Jinan,
181 Shandong, China). Macrophage differentiation of the THP-1 cells was conducted

182 using a previously reported method (Dehai *et al.* 2014). The cells were triggered by
183 adding 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis,
184 MO, USA) for two days and washed thrice with phosphate-buffered saline (PBS) to
185 eliminate the effect of PMA. Macrophages were observed through an inverted
186 microscope, and the cellular morphology is shown in Fig. 1A. The macrophages were
187 also verified through immunofluorescent staining using an antibody against CD68
188 (ab955, 1:200; Abcam, Cambridge, UK), which is a marker for macrophages (Fig.
189 1C). The primary endometrial epithelial cells were co-cultured with THP-1-derived
190 macrophages using a standard Transwell insert (0.4 μm; Corning, Corning, NY, USA)
191 (Fig. 1D). Each well was plated with approximately 10,000 primary endometrial cells.
192 The cells were washed after incubation for 24 h with 10% fetal bovine serum medium,
193 and the inserts with induced macrophages (7.5×10^5cells) were added to the wells.
194 The control group had an empty insert. Epithelial cells were harvested at different
195 time points, that is, on days 1, 2, 4, and 6.

196 ***Immunocytochemistry***

197 We performed Immunocytochemistry staining to show morphological changes of
198 epithelial cells with EMT related proteins as previously described (Xue et al. 2013).
199 The cultured epithelial cells were washed twice with PBS and fixed in 4%
200 paraformaldehyde (pH 7.0) 30 minutes. The cells were then washed in PBS, blocked
201 with 10% normal goat serum for 1 hour, and incubated overnight with rabbit
202 antihuman CK7 monoclonal antibody (ab68459, 1:400; Abcam, Cambridge, UK) and

203 rabbit antihuman vimentin monoclonal antibody (ab92547, 1:600; Abcam, Cambridge,
204 UK) at 4°C. The secondary antibody kit (CWBIO, Beijing, China) was utilized to link
205 the primary antibody. Cells were counterstained with hematoxylin before mounting.

206 ***Western blot***

207 The EMT-like process-related proteins were examined using Western blot analysis for
208 primary endometrial cells at four different time points after co-culturing the cells with
209 THP-1-derived macrophages. The cells were scraped and extracted in a commercial
210 kit (BestBio, Shanghai, China) for total protein extraction. All proteins that were
211 mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)
212 loading buffer (P0015; Beyotime, Shanghai, China) were heated for 5 min at 100 °C.
213 Protein samples were loaded onto 10% SDS-PAGE and electroplated onto
214 polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes
215 were blocked in 5% nonfat milk (BD, Franklin Lakes, NJ, USA) reconstituted in
216 TBST (0.15 M NaCl, 0.05% Tween20, and 10 mM Tris–HCl [pH 8.0]) for 70 min at
217 room temperature and subsequently incubated overnight at 4 °C with the following
218 primary antibodies: rabbit antihuman CK7 monoclonal antibody (ab68459, 1:5,000;
219 Abcam, Cambridge, UK), rabbit anti-human E-cadherin monoclonal antibody
220 (ab40772, 1:10,000; Abcam, Cambridge, UK), rabbit anti-human vimentin
221 monoclonal antibody (ab92547, 1:5,000; Abcam, Cambridge, UK), rabbit anti-human
222 N-cadherin monoclonal antibody (ab18203, 1:1,000; Abcam, Cambridge, UK), rabbit
223 anti-human TGFB1 monoclonal antibody (ab92486, 1:1,000; Abcam, Cambridge,

224 UK), rabbit anti-human SMAD3 monoclonal antibody (ab40854, 1:1,000; Abcam,
225 Cambridge, UK), and rabbit anti-human GAPDH polyclonal antibody (10494-1-AP,
226 1:20,000; Proteintech, Wuhan, China). After incubating the membranes with
227 horseradish-peroxidase-labeled secondary antibodies for 70 min at room temperature,
228 the signal was detected by Image Studio Digits Ver 4.0. Three independent
229 experiments were performed.

230 ***Real-time polymerase chain reaction (RT-PCR)***

231 The co-cultured cells (macrophages) were washed with cold PBS, and the total RNA
232 was extracted with TRIzol Reagent (Invitrogen Life Technologies, Waltham, MA,
233 USA) in accordance with the manual of the product owner. RNA (1 μg) was
234 reverse-transcribed into cDNA with ReverTra Ace quantitative PCR (qPCR) RT
235 Master Mix with gDNA Remover (Code No. FSQ-301; Toyobo, Osaka, Japan). Each
236 20 μL of PCR product contained 1×SYBR Green PCR Master Mix (Toyobo, Osaka,
237 Japan), 30 ng of cDNA, and 300 nM of each specific primer. The primers used for
238 each gene were listed in Table 2. Subsequently, qPCR was performed on an Applied
239 Biosystems 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA).
240 Three separate experiments were performed on different cultures, and each sample
241 was assayed in triplicate. The mean was obtained to determine the mRNA levels by
242 quantitative RT-PCR analysis, which was performed using the ABI 7500 RT-PCR
243 system (Applied Biosystems, Foster City, CA, USA). The gene expression levels for
244 each group were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

245 The mean relative gene expression level was determined, and the differences were
246 calculated using the $2^{-\Delta\Delta Ct}$ method.

247 ***Statistical analysis***

248 Graphical and statistical analyses were performed using the GraphPad prism software
249 (San Diego, CA, USA). Comparative statistical analyses were performed using the
250 Mann Whitney test of immunohistochemical staining. And Wilcoxon matched pairs
251 test was performed of the antigen expression in western blot between the study
252 (co-cultured with macrophage) and control groups; between the normal endometrium
253 and eutopic endometrium groups, the Mann Whitney test was used. The gene
254 expression levels of macrophages among the control, normal endometrium, and
255 eutopic endometrium groups were calculated using the one-way analysis of variance
256 (Newman-Keuls Multiple Comparison Test). P values ≤ 0.05 were considered
257 significant.

258 # RESULTS

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

261 To investigate whether EMT process exist in eutopic endometrium of adenomyosis,
262 we compared the expression level of CK7, vimentin, E-cadherin and N-cadherin in
263 the endometrium with or without adenomyosis. As depicted in Fig 2 and Fig 3, we
264 found that the expressions of CK7, E-cadherin, N-cadherin, TGFB1, SMAD3 and

265 pSMAD3 have no significant difference between normal and eutopic endometrial
266 epithelial cells in proliferative phase except vimentin which was upregulated. While
267 in the secretory phase, the expressions of CK7 and E-cadherin were both
268 downregulated in eutopic endometrial epithelial cells compared with the normal; as
269 for vimentin, N-cadherin, TGFB1 and SMAD3, the levels of expressions were both
270 high in secretory phase; the expression of pSMAD3 did not differ between eutopic
271 and normal epithelial cells. We also compared the expression level of CK7, vimentin,
272 E-cadherin, N-cadherin, TGFB1, SMAD3 and pSMAD3 between proliferative and
273 secretory phase. As shown in Fig 2 and Fig 3, in eutopic endometrium, the
274 expressions of TGFB1, SMAD3 and pSMAD3 in secretory phase were higher than
275 proliferative phase; while in normal endometrium, only pSMAD3 was upregulated;
276 however, the expression of E-cadherin downregulated in secretory phase.

277 ***Mesenchymal-like morphological changes of epithelial cells in***
278 ***Immunocytochemistry staining after co-culture***

279 Immunocytochemical analyses of CK7 and vimentin expression were performed to
280 investigate the morphological changes of epithelial cells. As shown in Fig 4, analysis
281 using an ordinary light microscope revealed a mesenchymal-like changes in epithelial
282 cells after cocultured with macrophages for 2 days. The expressions level of CK7 was
283 down-regulated and vimentin was up-regulated in epithelial cells. Although the
284 changes of cell morphology was less obvious than protein expression, some epithelial
285 cells have typical mesenchymal-like morphology.

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

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

308 significance of the fold changes for CK7, E-cadherin, vimentin and N-cadherin
309 expressions between the eutopic endometrium group and the normal endometrium
310 group (Fig. 5E).

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

314 The protein expression levels of TGFB1 and SMAD3 in the eutopic endometrium and
315 normal endometrium groups were insignificantly upregulated after co-culturing
316 endometrial epithelial cells and macrophages (Figs. 6A, 6B, and 6C). The protein
317 expression level of control group epithelial cells isolated from adenomyosis eutopic
318 and normal endometria was also estimated. Statistical analysis indicated no difference
319 in the protein expression levels of TGFB1and SMAD3 between adenomyosis eutopic
320 and normal endometrial epithelial cells without co-culture (Fig. 6D). However, the
321 upregulation of pSMAD3 after co-culturing was higher on day 6 in the epithelial cells
322 both derived from normal and eutopic endometria after co-culture (Figs. 6A, 6B and
323 6C); statistical analysis indicated that the protein expression levels of pSMAD3 in
324 eutopic epithelial cells was higher than normal (Fig. 6D). After co-culturing with
325 macrophages, the fold changes of TGFB1, SMAD3 and pSMAD3 have no difference
326 between the eutopic endometrium group and the normal endometrium group (Fig.
327 6E).

328 ***Co-cultured with endometrial cells, THP-1-derived macrophages polarized to the***

329 ***M2 type; compared with the eutopic endometrium group, further polarization to M2***
330 ***was detected in the normal endometrium group***

331 The interaction of macrophages and endometrial epithelial cells induced the
332 endometrial epithelial cells to undergo mesenchymal transition-like process.
333 Meanwhile, endometrial epithelial cells can induce macrophages to polarize. The PCR
334 results proved that primary endometrial epithelial cells can cause macrophages to
335 polarize to the M2 type. The genetic level of *IL10* in the eutopic endometrium and
336 normal endometrium groups significantly increased when compared with the control
337 group. The expression of *IL10* was higher in the normal endometrium group on day 1
338 and day 4 (Fig. 7B). However, the genetic level of *CD163* increased only in the
339 normal endometrium group on day 1 and day 6. The eutopic endometrium group did
340 not exhibit *CD163* expression in macrophages compared with the control group.
341 Therefore, in terms of induced *CD163*, the normal endometrium group exhibited
342 higher levels than the eutopic endometrium group on day 1 and day 6 (Fig. 7B). The
343 genetic level of *MMP12* increased in the normal endometrium group at all the time
344 points. In contrast to the control group, the *MMP12* levels increased significantly in
345 the eutopic endometrium group only on day 1 and day 2. The induction of *MMP12*
346 expression was higher in the normal endometrium group than in the eutopic
347 endometrium group, except on day 2 (Fig. 7B). Throughout the entire experiment, the
348 polarization of macrophages in the eutopic endometrium group was tender, whereas
349 that in the normal endometrium group was fluctuant, the expression levels of *CD163*,
350 *IL10*, and *MMP12* initially increased, then decreased, and increased again. Although

351 the polarization of macrophages was definite, the morphological change of
352 macrophages was not evident (Fig. 7A).

353 DISCUSSION

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

361 Eutopic endometrium has always been considered the origin of ectopic endometrium
362 in endometriosis or adenomyosis (Benagiano *et al.* 2013). Research on the
363 adenomyosis mechanism gained more attention after the following definition was
364 provided (Bird *et al.* 1972): "Adenomyosis may be defined as the benign invasion of
365 endometrium into the myometrium, producing a diffusely enlarged uterus which
366 microscopically exhibits ectopic non-neoplastic, endometrial glands and stroma
367 surrounded by the hypertrophic and hyperplastic myometrium." However, the specific
368 molecular mechanism is still unclear. In recent years, EMT has emerged in
369 adenomyosis mechanism research. Although these studies had far-reaching
370 significance, the EMT process in adenomyosis research should be supplemented. The
371 analysis of IHC did not find the definite evidence of EMT in the eutopic endometrium

372 of adenomyosis in proliferative phage; however, we found an EMT phenomenon in
373 eutopic endometrium at secretory phase, and the expressions levels of TGFB1 and
374 SMAD3 that indicate signal pathway of EMT were higher in secretory phase, which
375 meant that adenomyosis eutopic endometrium epithelial cells had been abnormally
376 activated and dysfunctional. Through the comparison during the menstrual cycle, we
377 found that the pathway of TGFB1/SMAD3 (pSMAD3) was activated in the secretory
378 phage, and the expression of E-cadherin was downregulated. These phenomena were
379 consistent with previous research (Kim *et al.* 2005, Chen *et al.* 2010). We drew two
380 reasons for the upregulated TGFB1/SMAD3 (pSMAD3) pathway in secretory phase
381 of eutopic endometrium: one is progesterone, as a previous study described that the
382 progesterone induces stromal decidualization indirectly by enhancing the expression
383 and secretion of TGFB1 from epithelial cells (Kim *et al.* 2005); the other is the
384 microenvironment, which stimulated the pathway to lead to EMT.

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

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

407 After comparing the eutopic and normal epithelial cells without co-culturing, changes
408 in the expression of E-cadherin, CK7, N-cadherin, and vimentin in epithelial cells
409 were compared between the eutopic and normal groups after co-culturing with
410 macrophages. Consistent with a recent study (Xiong *et al.* 2016), no significant
411 difference in the expression levels of E-cadherin and N-cadherin was observed
412 between normal and eutopic epithelial cells. Moreover, no significant difference in the
413 expression levels of CK7 and vimentin was observed between the two groups. In
414 addition, after co-culturing with macrophages, the fold changes of downregulated
415 E-cadherin and CK7, and upregulated N-cadherin and vimentin have no difference

416 between the eutopic endometrium group and the normal endometrium group. So, we
417 speculate that macrophages can equally induce EMT process in eutopic and the
418 normal epithelial cells.

419 The TGFB1/SMAD3 signaling pathway is essential for the EMT process. Although,
420 the upregulation of TGFB1 and SMAD3 was statistically insignificant, the protein
421 expression levels of pSMAD3 were higher in the co-cultured epithelial cells than in
422 the control. The expression levels of TGFB1, SMAD3 and pSMAD3 in epithelial
423 cells were compared between eutopic and normal epithelial cells without co-culturing
424 with macrophages. Then, the expression changes of TGFB1, SMAD3 and pSMAD3
425 in epithelial cells were compared between eutopic and normal groups after
426 co-culturing with macrophages. The protein expression levels of TGFB1 and SMAD3
427 between eutopic and normal endometrium groups differed insignificantly, which is
428 inconsistent with previous studies (Johnson *et al.* 2005, Cruz *et al.* 2015). Another
429 research reported that only weak upregulation of TGFB1 in endometriosis eutopic
430 endometrium occurred compared with the controls (Goteri *et al.* 2015). The present
431 study is the first to compare the protein expression levels of TGFB1 and SMAD3
432 between adenomyosis eutopic and normal endometria, and the authors hope that this
433 study could gain more attention from researchers involved in mechanism studies of
434 adenomyosis. No significant difference in protein expression was observed between
435 eutopic and normal endometrium groups without co-culturing; it was the same with
436 the fold changes of TGFB1 and SMAD3 after co-culturing with macrophages in the
437 eutopic endometrium group than in the normal endometrium group. This result was

438 not consistent with our IHC analyses, and we speculated that the lack of steroids in
439 the intro culture medium influenced it. However, it is worth noting that the
440 expressions of pSMAD3 were upregulated both in normal and eutopic epithelial cells
441 after co-culture. But the fold changes of pSMAD3 after co-culturing have no
442 significance. Thus, it proved that the stimulation and induction of EMT process in the
443 epithelial cells of the adenomyosis eutopic endometrium and normal endometrium is
444 parallel.

445 Abnormal levels of immune cells, such as macrophages, have been largely reported in
446 adenomyosis (Ota et al. 1998, Zhihong *et al.* 2016). Increased knowledge on the
447 immune aspects of the pathogenesis of adenomyosis may allow the development of
448 novel medical therapies for this debilitating condition. To our knowledge,
449 macrophages, which play important roles in innate and acquired immunity, together
450 with natural killer cells and cytotoxic T-lymphocytes in healthy women, can destroy
451 misplaced endometrial cells (Dmowski *et al.* 1998). However, during the past decades,
452 several reports identified a decrease or impairment in the cell-mediated immunity of
453 women with adenomyosis or endometriosis (Matarese *et al.* 2003, Guo *et al.* 2016). In
454 this study, epithelial cells from normal and eutopic endometria can induce
455 macrophages to polarize to the M2 type. However, the inducing capability of the
456 normal endometrium group was more intense than that of the eutopic endometrium
457 group. *CD163* was a hallmark of M2 macrophage. In our study, it did not express
458 higher in THP-1 derived macrophages after cocultured with eutopic epithelial cells.
459 That signifies that the ratios of M2 to pan-macrophages were significantly lower in

460 adenomyosis patients than normal group. It can be consistent with a previous study
461 performed in endometriosis (Takebayashi *et al.* 2015). Khan et al. demonstrated that,
462 compared to control women without endometriosis, women with endometriosis had
463 Escherichia coli contamination of menstrual blood with increased levels of bacterial
464 endotoxin in the menstrual fluid and peritoneal fluid (Berbic *et al.* 2009), this will
465 affect the polarization of macrophages. Thus, we suspect that this phenomenon exists
466 in adenomyosis as well. On the other hand, adenomyosis itself might be the cause of
467 the incapable polarization of M2 macrophages in the eutopic endometrium. Given the
468 failure of polarization to M2 in the eutopic endometrium group, the misplaced
469 endometrial cells might escape macrophage's engulfment and elimination. This
470 incapacity can result from the eutopic endometrium itself or pelvic microenvironment.
471 As the uterine cavity is connected to the pelvic cavity through the oviducts, some
472 substances like immune cells in the pelvic cavity can flow into the uterine cavity,
473 especially during the process of ovulation. Moreover, fluctuations in the genetic levels
474 of *CD163*, *IL10*, and *MMP12* were observed in the normal endometrium group
475 because of the sensitive characteristics and homeostasis of macrophages. The weak
476 activation of macrophages in the eutopic endometrium group demonstrated that the
477 eutopic endometrium was immunotolerant.

478 In conclusion, endometrial cells and microenvironment are two important factors in
479 the pathogenesis of adenomyosis. This study showed that both epithelial cells isolated
480 from eutopic and normal endometria can be equally induced by macrophages to
481 undergo EMT. The polarization of macrophages to M2 was less intense in the eutopic

482 endometrium group than in the normal endometrium group. Further study is required
483 to examine the immunity mechanism of the incapability, even failure, of polarization
484 to M2 of inducing macrophages in eutopic endometrium of adenomyosis. In eutopic
485 epithelial cells, the expressions of E-cadherin and pSMAD3 were higher than normal.
486 Thus, we can attribute the incapable polarization to M2 to it. Our current findings
487 suggest that adenomyosis may be promoted by the ability of epithelial cells derived
488 from the eutopic endometrium to undergo EMT and the incapability of inducing
489 macrophages to polarize to M2. Thus, immune regulation and inflammation reaction
490 reduction may help relieve adenomyosis.

491 DECLARATION OF INTEREST

492 None declared.

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500 the patients for agreeing to participate in our study.

501 # AUTHORS' ROLES

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

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1 # Figure legends

2 FIGURE 1

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

10 FIGURE 2

11 The expression of CK7, vimentin, E-cadherin, N-cadherin and several signal pathway proteins
12 (TGFB1, SMAD3 and pSMAD3) in the endometrium of women without adenomyosis and
13 endometriosis (n = 11), eutopic endometrium (n = 16) from women with adenomyosis were
14 analyzed by immunohistochemistry. Original magnification: ×400. NP: endometrium of women
15 without adenomyosis at proliferative phase; EuP: endometrium of patient with adenomyosis at
16 proliferative phase; NS: endometrium of women without adenomyosis at secretory phase; EuS:
17 endometrium of patient with adenomyosis at secretory phase.

18 FIGURE 3

19 Results of MOD analysis (Mann Whitney test) using all immunostaining data. All patients were
20 represented by points. * normal group vs eutopic group; # proliferative phase (eutopic) vs
21 secretory phase (eutopic); & proliferative phase (normal) vs secretory phase (normal). */#/&p
22 <0.05; **/##/&&p <0.01; ***/###/&&&p <0.001. The results are expressed as the mean± SEM.

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 co-culture, the cytoplasm of cells was positive for vimentin (**D**) and less for CK7 (**B**). The nuclei were stained with hematoxylin. Magnification, ×400.

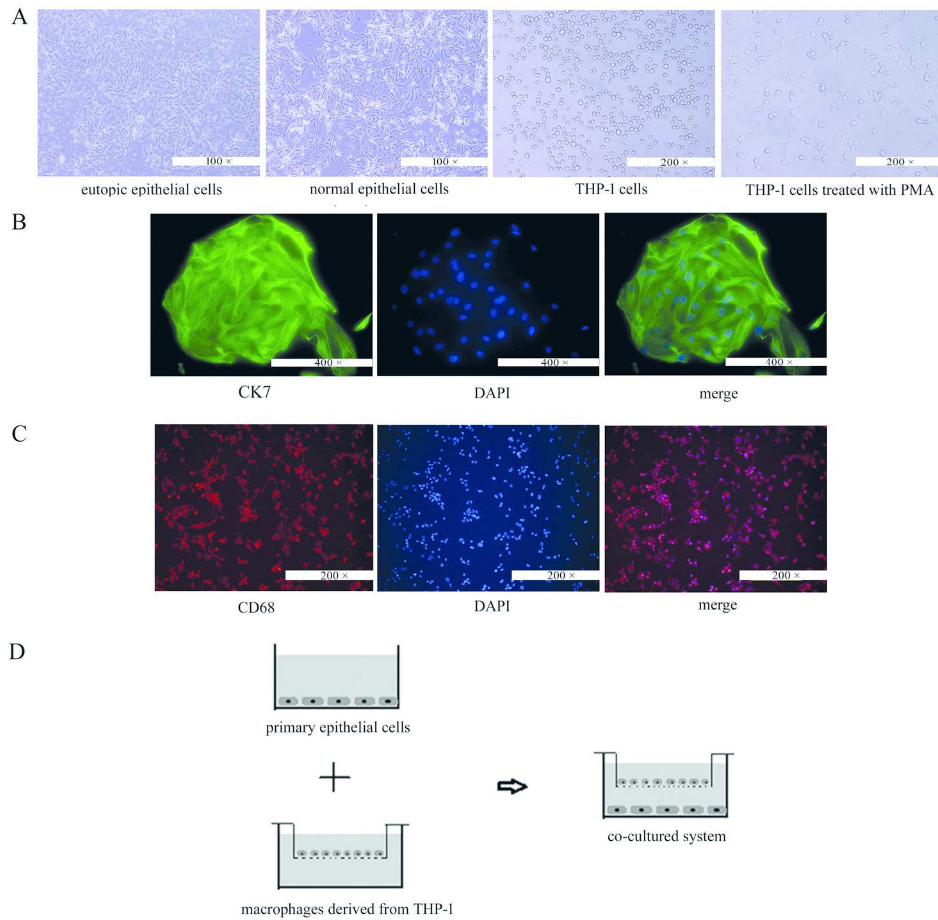
FIGURE 5

(**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± SEM.

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/ pSMAD3in 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

45 co-culturing groups. (**D**) Statistical analysis of the protein expression levels of TGFB1 and
46 SMAD3/ pSMAD3 between normal and eutopic epithelial cells without co-culturing. (**E**)
47 Statistical analysis of the fold changes of the protein expression levels between normal and
48 eutopic epithelial cells after co-culturing. *p <0.05; **p <0.01; ***p <0.001. The results are
49 expressed as the mean± SEM.
50 FIGURE 7
51 (**A**) Representative photomicrographs of macrophages with and without co-culturing (original
52 magnification, ×400). (**B**) Gene expression of THP-1-derived macrophages co-cultured with or
53 without normal or eutopic epithelial cells. * normal group vs control group; # adenomyosis group
54 vs control group; & normal group vs adenomyosis group. */#/&p <0.05; **/##/&&p <0.01; ***/###/&&&p
55 <0.001. The results are expressed as the mean± SEM.



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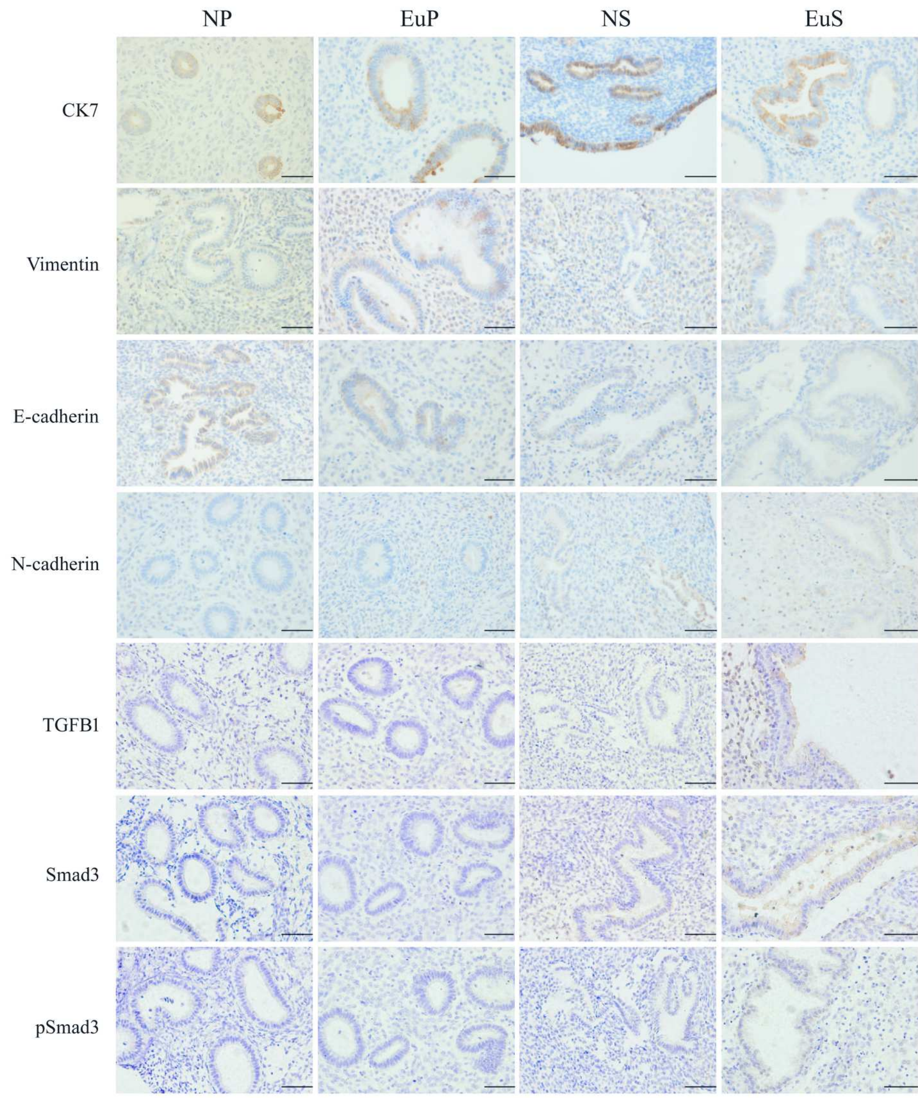

FIGURE 2 The expression of CK7, vimentin, E-cadherin, N-cadherin and several signal pathway proteins (TGFB1, SMAD3 and pSMAD3) in the endometrium of women without adenomyosis and endometriosis (n = 11), eutopic endometrium (n = 16) from women with adenomyosis were analyzed by immunohistochemistry. Original magnification: ×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.

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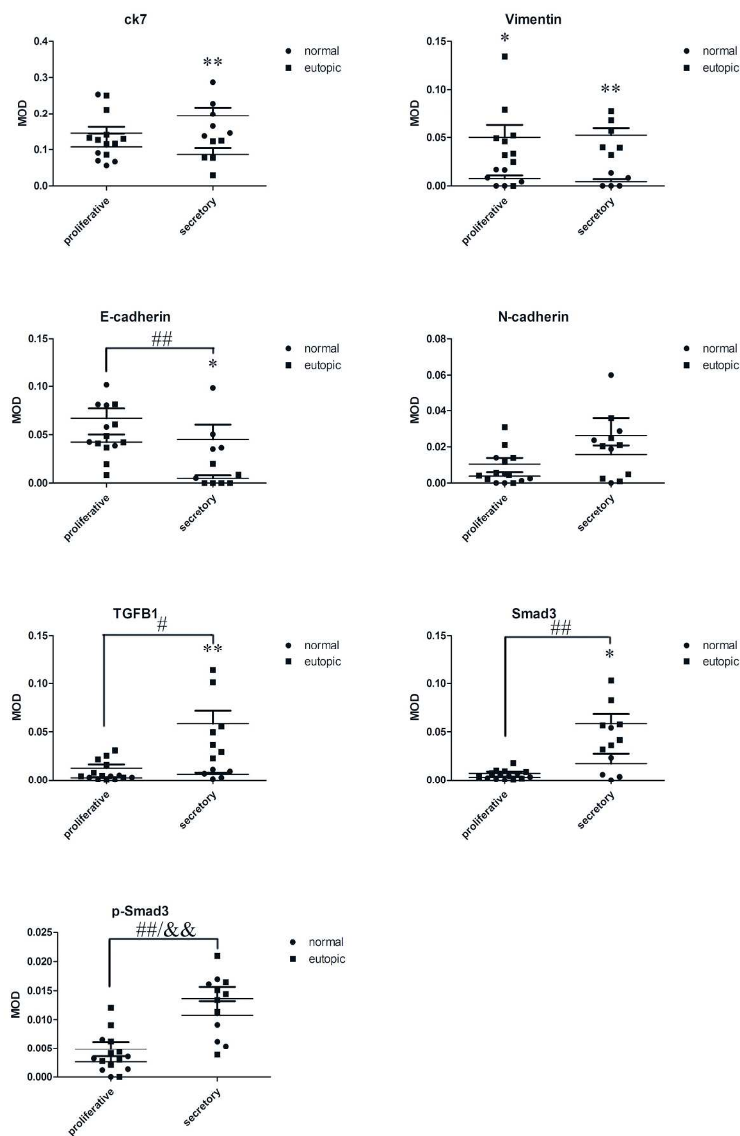

FIGURE 3 Results of MOD analysis (Mann Whitney test) using all immunostaining data. All patients were represented by points. * normal group vs eutopic group; # proliferative phase (eutopic) vs secretory phase (eutopic); & proliferative phase (normal) vs secretory phase (normal). */#/&p <0.05; **/##/&&p <0.01; ***/###/&&&p <0.001. The results are expressed as the mean± SEM.

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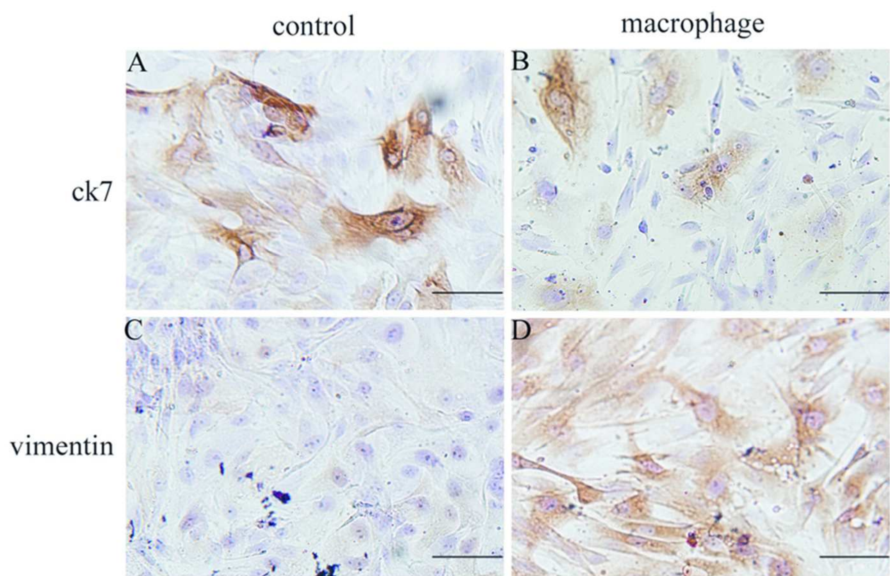
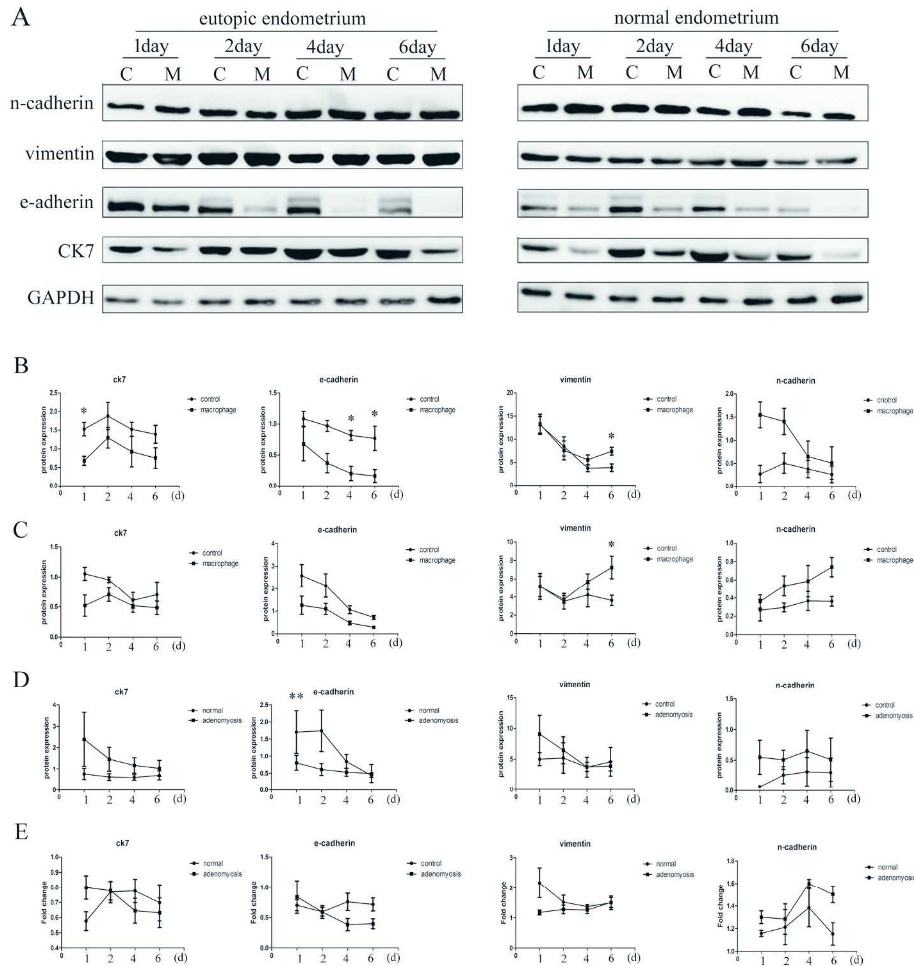


FIGURE 4 Immunocytochemical staining for vimentin and CK7 expression. The cytoplasm of cells was negative for vimentin (C) and positive for CK7 (A) before co-culture; after 2 days co-culture, the cytoplasm of cells was positive for vimentin (D) and less for CK7 (B). The nuclei were stained with hematoxylin.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

80x53mm (300 x 300 DPI)



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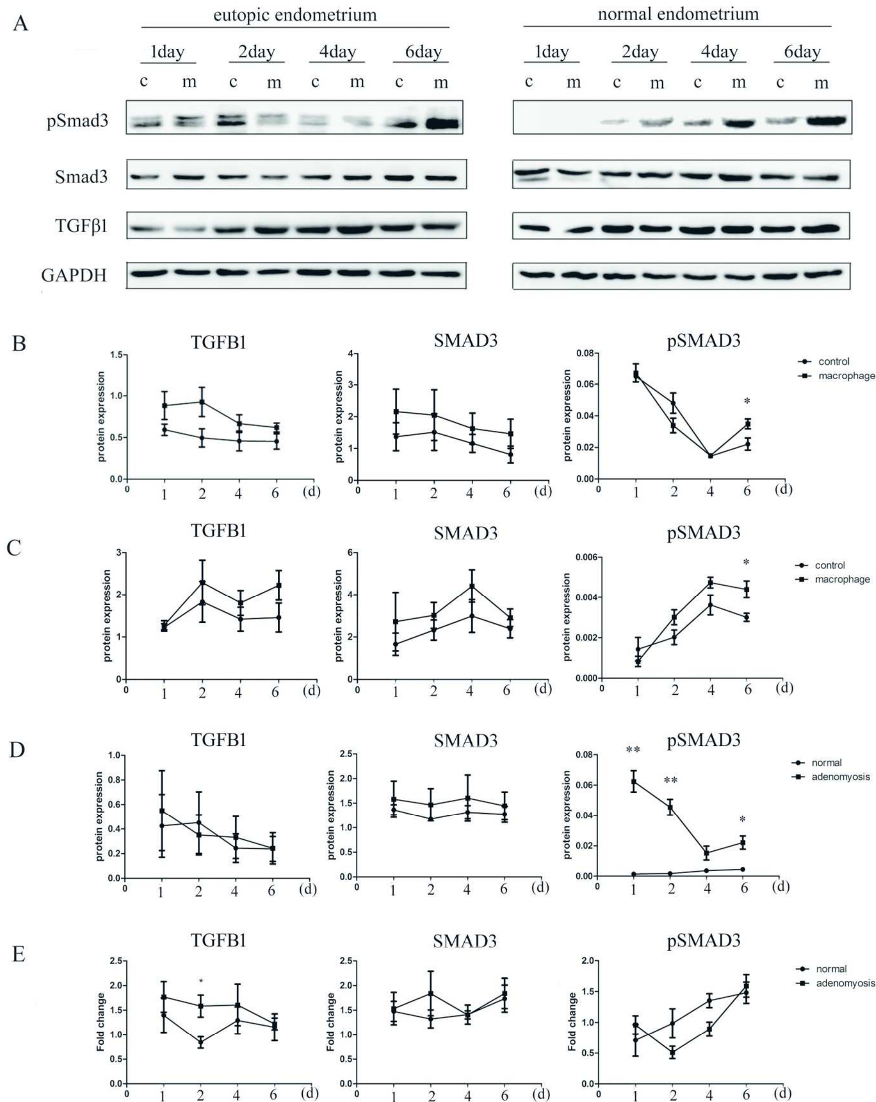

FIGURE 6 (A) Western blot of epithelial cells isolated from normal endometrium and eutopic endometrium of adenomyosis. C: control group; M: macrophages (co-culturing) group. (B) Statistical analysis of the protein expression levels of TGFB1 and SMAD3/ pSMAD3in eutopic epithelial cells between the control 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± SEM.

180x219mm (300 x 300 DPI)

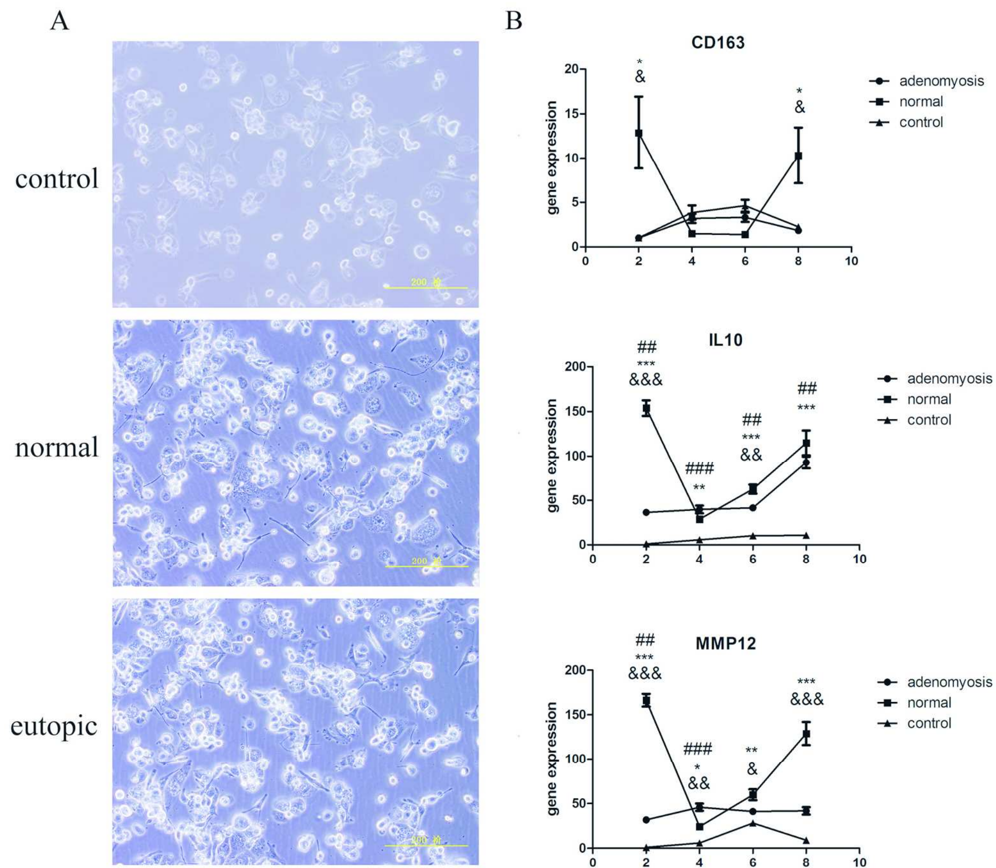

FIGURE 7 (A) Representative photomicrographs of macrophages with and without co-culturing (original magnification, ×400). (B) Gene expression of THP-1-derived macrophages co-cultured with or without normal or eutopic epithelial cells. * normal group vs control group; # adenomyosis group vs control group; & normal group vs adenomyosis group. */#/&p <0.05; **/##/&&p <0.01; ***/###/&&&p <0.001. The results are expressed as the mean± SEM.

180x180mm (300 x 300 DPI)

Table 1 Characteristics of patients recruited with adenomyosis and controls.

Item	Controls (n=11)	Adenomyosis (n=20)	Statistical significance
	Mean=30.0 (SD=8.7)	Mean=40.8 (SD=5.1)	
Age (year)	Median=30	Median=40.5	**
	Range=20-41	Range=31-49	
Menstrual phase			
Proliferative	7(63.6%)	9 (45.0%)	NS
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=0.5(SD=1.1)	Mean=5.9 (SD=2.3)	***
	Median=0	Median=6	
	Range=0-5	Range=0-8	
Uterus size (cm3)	Mean=72.5	Mean=344.3	
	Median=70.1	Median=285.5	***
	Range=52.3-80.1	Range=110.1-628.2	
Co-occurrence of endometriosis			
No	11(100.0%)	18 (90.0%)	NS
Yes	0(0.0%)	2 (10.0%)	
Co-occurrence of endometrial polyps			
No	11(100.0%)	16 (80.0%)	NS
Yes	0(0.0%)	4 (20.0%)	

uterus size calculated as πD1D2D3/6, 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, visual analog scale and uterus size while for other data Fisher's exact test was used.

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

CD163	Forward	GGCTTGCAGTTTCCTCAAGA
CD163	Reverse	AGCTGACTCATGGGAATTTTCTG
MMP12	Forward	ACTACACATTCAGGAGGCACA
MMP12	Reverse	GTCATCAGCAGAGAGGCGAA
IL10	Forward	AGGACTTTAAGGGTTACCTGGG
IL10	Reverse	TTCTCAGCTTGGGGCATCAC
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
GAPDH	Reverse	TGGTGAAGACGCCAGTGGA