Am J Transl Res 2019;11(2):572-585
www.ajtr.org /ISSN:1943-8141/AJTR0086003

Original Article

Telocytes enhanced the proliferation, adhesion and motility of endometrial stromal cells as mediated by the ERK pathway in vitro

Xue-Ling Tang, Fei-Lei Zhang, Xiao-Juan Jiang, Xiao-Jun Yang

Department of Obstetrics and Gynecology, The First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, PR China

Received September 26, 2018; Accepted December 22, 2018; Epub February 15, 2019; Published February 28, 2019

Abstract: Telocytes (TCs) is special interstitial cell that have recently been identified in the female reproductive system. Endometriosis (EMs) is a benign gynecological disease whose etiology is still not fully clear. Implantation and proliferation of endometrial stromal cells (ESCs) out of the uterus are essential processes in the development of EMs. Herein, we investigate the *in vitro* changes of ESCs when cocultured with TCs, and the potential mechanisms involved. The current results demonstrated that, vimentin-positive/pan cytokeratin-negative ESCs, and TCs with a characteristic structure and immunophenotype (CD34/vimentin double-positive) were successfully isolated and harvested. Morphologically, direct cell-to-cell contacts were observed between TCs and ESCs. Quantitatively, TCs treatment clearly promotes the viability of ESCs, enhances cell cycle progression at G2/M phase and upregulates p-ERK1/2 and cyclin-D3 (all $P < 0.05$). Functionally, ESCs educated by TCs displayed significantly enhanced adhesion ability and accelerated invasion and migration capacity (all $P < 0.05$). However, no significant changes were found in the rate of apoptosis and in the expression of AKT signaling pathway proteins in TCs-educated ESCs (both $P > 0.05$). Therefore, TCs treatment obviously enhanced the *in vitro* motile and invasive capacity of ESCs, which were mediated by the ERK-cyclin-D3 signaling pathway, likely through direct intercellular contacts and/or juxta-paracrine effects; signaling through this axis therefore increased the likelihood of EMs. The enhanced functions of TCs-educated ESCs not only contribute to a deeper understanding of TCs, but also highlight a new concept regarding the physiopathology and therapy of EMs and associated impaired reproductive function.

Keywords: Telocytes (TCs), interstitial cells, endometriosis (EMs), endometrial stromal cells (ESCs), EMs-related diseases, extracellular-regulated kinase (ERK) signaling pathway

Introduction

Telocytes (TCs) is a novel type of stromal cell of mesenchymal origin first identified and reported by Popescu and colleagues [1]. Until now, TCs has been reported in a variety of tissues [2-12]. Based on their extremely long telopode (Tp), TCs form wide connections with adjacent surrounding cells and generate a special 3D network within interstitial tissues [13, 14]. Such structural features are the basis of the proposed multiple functions of TCs, which include tissue regeneration and repair processes [14-18], signal transduction [19, 20], pacing of muscular contraction coordination [21], secretory functions [22] and immunomodulation/immunosurveillance in the local microenvironment [23]. Currently, TCs are believed to be essential for intestinal stem cell activation by influencing Wnt proteins to provide niche signals [24-26]. TCs has also been found in the female reproductive system [27, 28]: including in the vagina [29], endometrium [30], myometrium [31-33], fallopian tube [34], and chorionic villi of the placenta [11, 12]. In normal myometrium and salpinx, TCs express estrogen and progesterone receptors, potentially function as steroid sensors and participate in the coordination of human myometrial contractions and fallopian tube motility [31, 34]. In oviduct tissues affected by disease, TCs and its Tps were obviously decreased or lost, and widespread severe ultrastructural damages was observed, which was further linked with tissue fibrosis and

reduced tubal fertility function [35-37]. Based on current available evidence, TCs is a key component in maintaining the normal tissue structure of the female reproductive tract, and it has distinct roles in the physiopathology of various obstetrical and gynecological conditions.

Endometriosis (EMs), which is a common gynecological condition that involves the migration of viable endometrial tissue fragments beyond the uterine cavity, affects 6-10% of women of childbearing age [38]. Currently, EMs-related pelvic pain and reproductive disorders cumulatively affects many individuals [39, 40]. Several classical theories have been proposed for the pathogenesis of EMs, including Sampson's theory of retrograde menstruation [41], immune origin, and stem cell origin, among others [42]. Nevertheless, none of the existing theories alone can explain the development of EMs in all patients, and thus a more the accurate pathogenesis remains to be elucidated. Prevailing points support that the establishment and development of EMs require retrograde menstruation or endometrial fragments, which contain aberrantly activated of eutopic endometrial stromal cells (ESCs). This is followed by the migration, invasion, implantation and proliferation of these ESCs on the surface or inner part of pelvic tissues or organs; this occurs, simultaneously with a controlled local inflammatory response and angiogenesis [41]. Multiple studies have suggested that ESCs in patients with EMs possess different properties from ESCs in normal controls [43-45]. Eutopic ESCs demonstrate with increased motile and invasive capacity compared with that of their ectopic counterparts from patients with EMs and those from nonendometriotic controls [44, 46, 47]. Identification of the underlying mechanisms that change the activity of ESCs might be a major step toward an understanding of the pathogenesis of EMs.

The presences of TCs in the endometrium and intercellular connections between Tp and adjacent cells, including ESCs, have been previously observed. This finding has revealed potential cell-to-cell communication and the maintenance of homeostasis over short and long distances within the endometrium [30]. However, further evidence as to the exact roles of TCs in ESCs is lacking. Based on our previous observation regarding the immunomodulatory effects of TCs on peritoneal macrophages and the potential roles of TC-educated macrophage in the onset of EMs [23, 48], further assumptions were made: TCs might also change the behavior of ESCs, which is mediated by intercellular contacts and/or by juxta-paracrine effects, and this finally contribute to the development of EMs. The purpose of this study was to investigate the *in vitro* changes in phenotype and the metergasis of ESCs when cocultured with TCs, and to analyze the underlying mechanisms. This study will be helpful to reveal new functions of TCs and the implications of TCs-educated ESCs in the pathogenesis and therapy of EMs.

Materials and methods

Animals

For this study, 8-week-old BALB/c (20-25 g) adult mice were used and purchased from the Laboratory Animal Center of Soochow University (Laboratory animal certificate: SCXK 2013-0006). All mice were bred in a specific pathogen-free environment with ad libitum access to food and water before the experiments. Animal experiments, including animal care, surgery and handling procedures were approved and conducted under the guidelines published by the University Health Network Animal Care Committee.

Isolation and primary culture of normal eutopic ESCs

Primary ESCs were prepared as previously described [49]. To obtain primary ESCs, a polyculture ratio of male to female mice (1:2) was designed. The estrous cycle was verified through daily vaginal smear examinations. Three days after mating, pregnant mice were sacrificed with an overdose of sodium pentobarbital (50 mg/kg; Fuyang Pharmaceutical Factory, Fuyang, China), and uterine tissues were removed and rinsed three times with phosphate buffered saline (PBS) containing 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA). Uterine samples were then placed in a sterile dish and subjected to cutting and gentle, repeated washes with PBS. Then, ophthalmic tweezers were used to softly scrape the endometrium. Endometrial tissues were collected in a sterile tube (Corning, NY, USA) and centri-

fuged at 335 g for 5 mins. After the supernatant was removed, the final sediment was resuspended in DMEM/F12 containing 0.1% type-II collagenase (Sigma-Aldrich, St. Louis, MO, USA). Digestion was performed at 37°C with vigorous shaking at 9 g for 60 mins and gentle agitation using a Pasteur pipette every 15 mins. After the cells were passed through 100 μm and 40 μm nylon mesh (Becton Dickinson, USA), they were harvested by centrifugation at 400 g for 5 mins, cultured in 25 cm^2 cell culture flasks (Corning, New York, USA), and maintained in a humidified atmosphere containing 5% CO_2 at 37°C for 24 hrs. After the culture medium was removed, the cells were rinsed three times and fresh complete medium was added; the culture medium was changed every other day. Finally, the ESCs were observed by light microscope.

Immunodiagnosis of ESCs

Fresh ESCs were harvested and plated at a low density on coverslips, which was followed by fixation in 4% paraformaldehyde for 20 mins and permeabilization with 0.5% Triton X-100 for 10 mins. Next, ESCs were blocked in 3% bovine serum albumin for 60 mins after another wash in PBS. The primary antibodies were as follows: rabbit anti-vimentin (1:100; cat. no. 5741S, Cell Signaling, USA) and mouse anti-pan Cytokeratin (PCK) (1:200; cat. no. 4545S Cell Signaling, USA). Fixed cells were incubated with the primary antibodies overnight at 4°C and then with Alexa Fluor 594 Donkey anti-rabbit (1:400; cat. no. abs20021, absin, China) and FITC Goat anti-Mouse (1:100; cat.no. abs20012, absin, China) for 30 mins at 37°C. Finally, DAPI counterstaining solution (1:50; cat. no.C1002, Beyotime, Shanghai, China) and mounting medium were added (1:1000; cat. no. p0126; Beyotime, Shanghai, China). The stained cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan).

Isolation, primary culture and immunodiagnosis of uterine TCs

Uterine tissue sampling, isolation, primary culture and immunodiagnosis of uterine TCs were performed according to our previously successfully developed procedures [23, 48]. Complete medium was changed every 48 hrs after monolayer attachment of TCs to the plate surface. In 3 or 4 days, once the typical morphology and special immunophenotype of CD-34-positive/vimentin-positive/c-kit-negative TCs could be observed by fluorescence microscopy, TCs was used for the following experiments. TCs-conditioned media (TCM) were also collected by using serum-free DMEM/F12 (Gibco, New York, USA) after 24 hrs of primary culture.

Direct cocultured system

The ESCs (5×10^4 cells/well) were inoculated into 6-well plates for direct coculture with TCs (5×10^4 dissociated cells/well) at a ratio of approximately 1:1. Then morphological alterations of the coculture system were observed by crystal violet (0.01 mg/ml, Beyotime, Shanghai, China) staining at the 0, 24 and 48 hr time points.

Indirect cocultured system

Transwell chambers (Corning Costar, 6.5 mm diameter, 0.4 μm pore size, 24-wells) were used for indirect cocultured of TCs and ESCs. The upper chambers were seeded with TCs (1×10^5 cells/well), and the lower chambers were seeded with ESCs (2×10^5 cells/well). Transwell chambers were maintained for 1 or 2 days, at 37°C with 5% CO_2. TCs-educated ESCs were then harvested and digested by trypsin for further determination of the phenotype and metergasis alterations at the 0, 24 and 48 hr time points, respectively. Moreover, Non-educated ESCs, which were cocultured with DMEM/F12 instead of TCs in the upper chambers of the Transwell system, served as the negative control.

Cell viability assessment

After 48 hrs of coculture with TCM, TCM-educated ESCs (1×10^4 cells/well) was seeded in a 96-well microplate. Then 10 μl of CCK-8 reagent solution (Dojindo Laboratories, Tokyo, Japan) was added to each well in the dark after which the plate was incubated for another 3 hrs. The same number of Non-educated ESCs served as the negative control. Then, the absorbance of both groups of ESCs was determined at 450 nm using a microplate reader (Multiscan MK3; Thermo Labsystems, Waltham, MA, USA). Data were obtained from at least three separate experiments with three identical wells in each group.

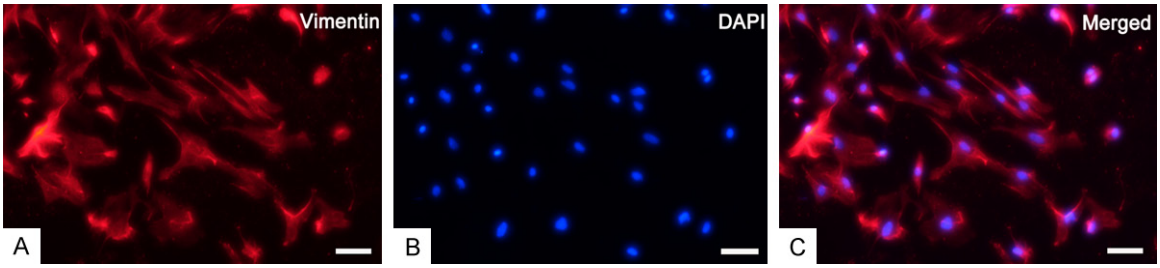

Figure 1. Representative immunofluorescence-based identification of ESCs, murine uterus. Images of negative staining for PCK are not shown. Scale bar =50 μm. A. Positive Alexa Fluor 594 labeling for vimentin (red). B. Nuclei were counterstained with DAPI (blue). C. In the merged figure, the purity of murine ESCs was than 95%.

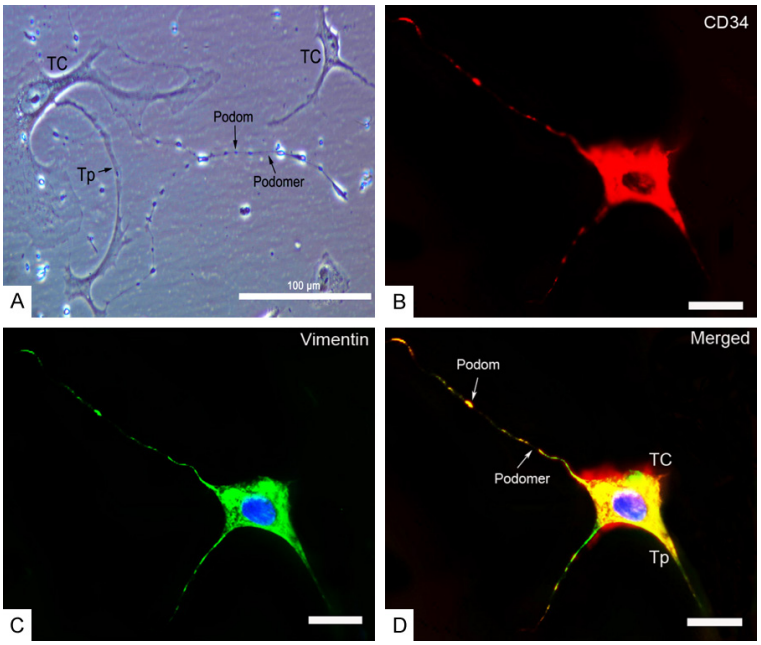

Figure 2. Representative identification of TCs, murine uterus. Images of c-kit negative staining are not shown. A. Typical structures of TCs under phase-contrast microscopy. TCs have small oval cell bodies and one or more extremely long, characteristic Tps, which composed of podomers (thin segments) and podoms (thick segments) arrayed alternatively along Tp. B. Positive CY3 labeling for CD34 (red) within the cell body and the whole length of Tps under fluorescence microscopy. Scale bar =50 μm. C. Positive FITC labeling for Vimentin (green) within cellular body and the whole length of Tps. Nuclei were counterstained with DAPI (blue). Scale bar =50 μm. D. In the merged figure, CD34 and Vimentin expression was colocalized, as both types of immunofluorescence overlapped each other in the TCs, including in the cell body and Tp, which contains podomers and podoms; staining is manifested as an intensity of colorful lights of yellow color and intact DAPI-stained nuclei (blue). This confirmed the special immunophenotype of TCs as CD-34-positive/vimentin-positive/c-kit-negative. Scale bar =50 μm.

Adhesion assay

After 48 hrs of indirect coculture with TCs, TCs-educated ESCs (1×10^4 cells/well) were seeded into a 96-well plate precoated with extracellular matrix (ECM; 1:300 dilution) for 1 h at 37°C with 5% CO_2. The same number of Non-educated ESCs served as the negative control. Then, unattached ESCs were removed and adhesive cells were fixed in 4% paraformaldehyde, stained with crystal violet and destained in acetic acid. Then the absorbances of both groups of ESCs were measured at 540 nm using the aforementioned microplate reader. Data were obtained from at least three separate experiments with three identical wells in each group.

Migration assay

Transwell chambers (Corning Costar, 6.5 mm diameter, 8 μm pore size, 24-wells) were used for the migration assay. Non-educated ESCs (5×10^4 cells/well), which were maintained in serum-free medium for 24 hrs, were plated in the upper chamber, with DMEM/F12 plus 10% FBS (served as the blank control; group A, C) or TCM (potential chemoattractant, TCM-educated ESCs) (group B) in the lower chamber. In addition, after 24 hrs of indirect coculture in a Transwell chamber, the same amount of TC-educated ESCs in DMEM/F12 plus 1% FBS (Gibco) were plated in the upper chamber, with DMEM/F12 plus 10% FBS at the lower chamber (TCs-educated ESCs, group D). This system was maintained for 24 hrs at 37°C in 5% CO_2/95% air, and afterwards,

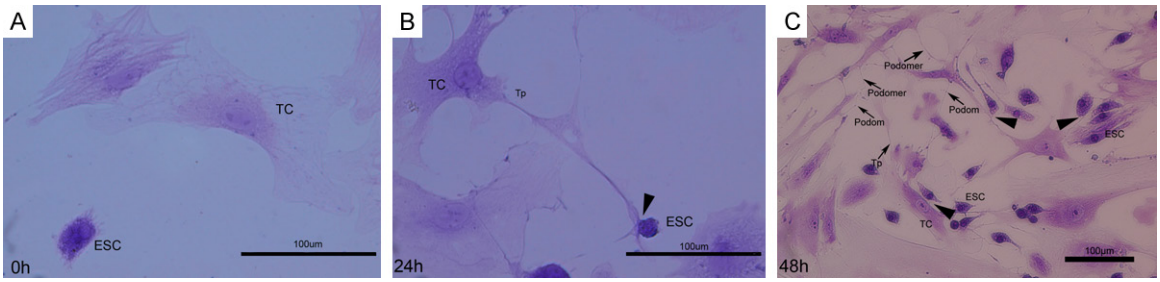

Figure 3. Real-time dynamic alterations of the direct coculture system; crystal violet vital staining at 0, 24, 48 hr intervals; phase-contrast microscopy; murine uterus. A. At 0 hr, TCs and ESCs were scattered in the coculture medium without any intercellular contacts between them. ESCs were observed with their original round shape, which indicated no activation. B. At 24 hrs, direct intercellular contacts between Tps and ESCs were observed (black arrowhead), which indicated that chemokinesis or chemotaxis occurred within the coculture system; this induced the formation of intercellular contacts between Tps and ESCs. C. At 48 hrs, a rich number of direct intercellular contacts (black arrowhead) developed between Tps (black arrow) and extended pseudopodia of ESCs, accompanied by the morphological changes of ESCs from round to long strip/spindle shapes, which indicated enhanced activity after coculture with TCs.

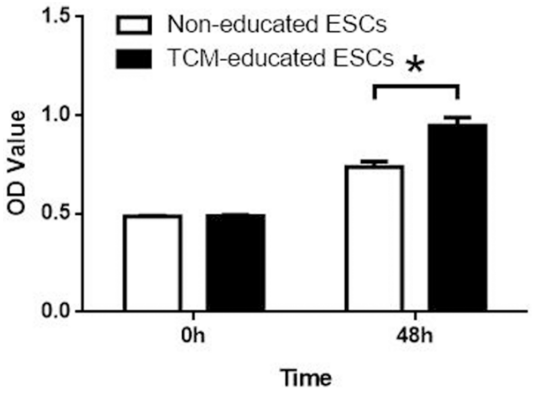

Figure 4. Cell viability of ESCs after 48 hrs of coculture. As demonstrated by OD values, no significant differences were observed between TCM-educated and Non-educated ESCs at 0 hr ($P > 0.05$). However, TCs significantly activated ESCs at 48 hrs ($*P < 0.05$ *versus* Non-educated ESCs); error bars = SD. This indicated enhanced viability after TCM treatment. The data are representative of at least 9 values from three separate experiments.

the nonemigrated cells on the top side of the filter were removed using a wet cotton swab. Then, the migrated ESCs remaining on the bottom surface were fixed in methanol and stained with crystal violet. The results were observed by light microscopy, where five random fields for each experiment were captured. The number of stained ESCs on the images was quantified by ImageJ software (V1.8.0, NIH, USA). Data were obtained from at least three separate experiments with three identical wells in each group.

Matrigel invasion assay

Transwell chambers (Corning Costar, 6.5 mm diameter, 8 μm pore size, 24-wells) were used to observe the invasion ability of ESCs. The upper insert was precoated with Matrigel (1:8 dilutions). After 48 hrs of coculture, TCs-educated or Non-educated ESCs (1×10^5 cells/well) in serum-free medium were seeded in the upper chamber. DMEM/F12 containing 10% FBS was added to the lower chamber. After 48 hrs of incubation, the ESCs on the upper surface of the membrane that had not invaded the lower chamber were removed. The next steps were the same as those in the aforementioned "migration assay". Data were obtained from at least three separate experiments with three identical wells in each group.

Cell cycle evaluation

After 48 hrs of coculture, TCs-educated or Non-educated ESCs (1×10^6 cells/well) were suspended in a precooled 70% ethanol solution and kept at 4°C overnight. Then, cells were collected by centrifugation and stained with 0.5 ml working solution (including 25 μl 20X propidium iodide (PI), 10 μl 50X RNase-A and Dye buffer; Beyotime, Shanghai, China) for 30 mins at room temperature. Cell cycle distribution was determined by flow cytometry (Novocyte™; ACEA Bioscience, San Diego, CA, USA) and analyzed by ModFit LT software (v.3.1. Topsham, ME, USA).

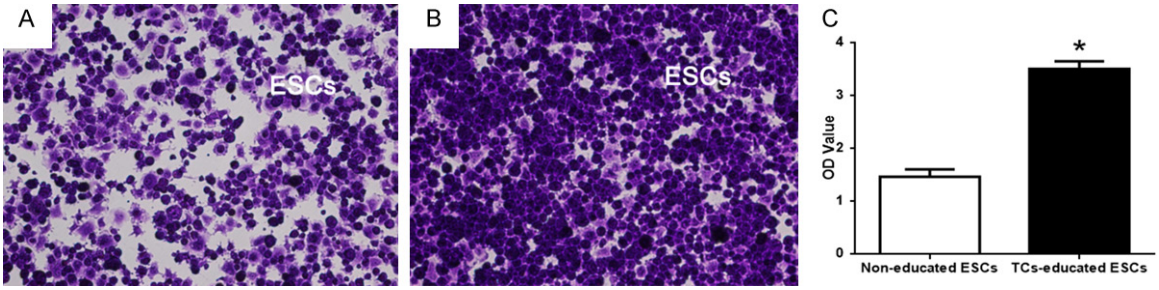

Figure 5. One hour adhesion assay of ESCs after 48 hrs of coculture. Scale bar =100 μm. A. When cultured alone, adhesion of Non-educated ESCs was relatively sparse. B. Adhesion of TCs-educated ESCs was obviously higher than that of the Non-educated group. C. The average OD value of TCs-educated ESCs adhesion was significantly increased (*P < 0.05 *versus* Non-educated ESCs). Error bars = SD. This indicated enhanced adhesion ability after coculture with TCs. The mean and SD were calculated from at least 9 values from three separate experiments.

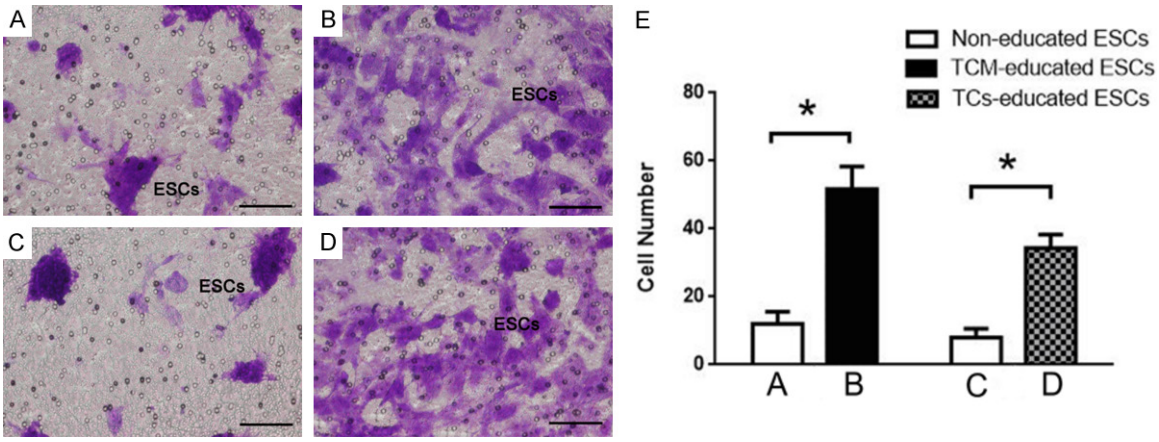

Figure 6. Migration assay of ESCs after 24 hrs of coculture. Scale bar =100 μm. A, C. Non-educated ESCs in the upper chamber; DMEM/F12 in the lower chamber served as a control. B. Non-educated ESCs in the upper chamber, with TCM in the lower chamber. The migration density of so-called TCM-educated ESCs was obviously higher than in the control. D. TCs-educated ESCs (pretreatment for 24 hrs) in the upper chamber with DMEM/F12 in the lower chamber. The migration density of TCs-educated ESCs was also obviously higher than in the control. E. The average number of ESCs that migrated significantly increased after exposure to TCM or upon pretreatment with TCs. (*P < 0.05 *versus* Non-educated ESCs). This indicated enhanced migration ability after coculture with TCs. Error bars = SD. The mean and SD were calculated from at least 9 values from three separate experiments.

Protein expression analysis

After 48 hrs of indirect coculture with TCs, thirty micrograms of total proteins was extracted from TCs-educated or Non-educated ESCs, separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes. Primary antibodies, including anti-p42/44 MAPK (extracellular-regulated kinase 1/2, ERK1/2) (1:1000, #4695, Cell Signaling Technology), anti-Phospho-p44/42 MAPK (P-ERK1/2) (1:1000, #4377, Cell Signaling Technology), anti-cyclin-D3 (1:2000, #2936, Cell Signaling Technology) and anti-β-Actin (1:3000, ab133626; Abcam) were each incubated with the membranes at 4°C overnight with gentle shaking. All subsequent steps were performed at room temperature. After washing thoroughly, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 hr. Bound antibodies were visualized with an enhanced chemiluminescence system. Original western images for all relevant western blots as shown in Figure S1.

Statistical analysis

The data are presented as the mean values ± standard deviation (Mean ± SD), and the results were analyzed by two independent samples Student's t-test or separate variance estimation t-test using SPSS (version 22; SPSS Inc., Chicago, IL, USA). A level of P < 0.05 was con-

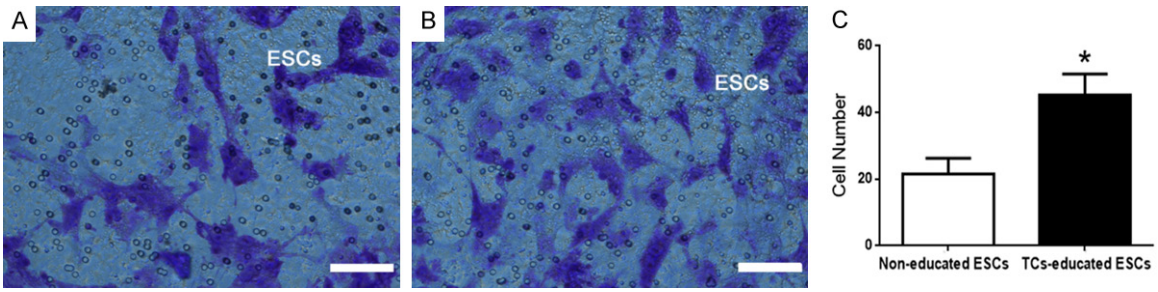

Figure 7. Invasion assay of ESCs after 48 hrs of coculture. Scale bar=100 μm. A. Non-educated ESCs group served as the control. B. TCs-educated ESCs group; invasiveness of ESCs was obviously higher than in the control cells. C. The average number of ESCs that invaded significantly increased in the TCs-educated ESCs group. (*P < 0.05 *versus* Non-educated ESCs). Error bars = SD. This indicated enhanced invasion ability after coculture with TCs. The mean and SD were calculated from at least 9 values from three separate experiments.

sidered statistically significant, while P < 0.01 was considered highly significant.

Results

ESCs immunodiagnostics

The immunophenotype of normal eutopic ESCs in the murnine uterus was vimentin-positive (red) and PCK-negative (**Figure 1**), which was in consistent with in agreement with previous studies [49]. The merged images show that the cell purity of ESCs was over 95%.

Identification of TCs

Representative uterine TCs were successfully isolated and confirmed by their special morphology and immunophenotype. TCs were observed to have one or more extremely long and thin Tp, and alterations in characteristic thick (podoms) and thin segments (podomers) arrayed alternatively within the whole length of the Tp (**Figure 2A**). Moreover, uterine TCs demonstrated a special CD-34-positive/vimentin-positive/c-kit-negative immunophenotype (**Figure 2B-D**), which is in accordance with our previous findings [23, 48].

Direct cocultured system

With the aid of crystal violet staining, the dynamic changes in TCs and ESCs in the coculture system were observed at different time intervals (0, 24 and 48 hrs) under phase contrast microscopy. In the beginning, ESCs and TCs were randomly scattered in the coculture system, without any intercellular contacts between them (**Figure 3A**). Then, at 24 hrs, chemokinesis or chemotaxis seemed to have occurred, and spatial focal contacts between Tps and the ESCs emerged (**Figure 3B**). At 48 hrs, dense and rich intercellular contacts were observed between Tps and the newly formed cellular protrusions of ESCs (**Figure 3C**). Thus, these data indicated that direct cell-to-cell interaction can promote intercellular connections between these cells.

Viability of ESCs

As shown in **Figure 4**, at 48 hrs, the viability of TCM-educated ESCs was significantly higher than that of Non-educated ESCs (0.92 ± 0.04 *versus* 0.72 ± 0.02, P < 0.05), but no significant difference was seen at 0 hr. Thus, this indicated that the viability of TCM-educated ESCs was clearly enhanced.

Cell adhesion

As shown in **Figure 5A**, **5B**, by fluorescence microscopy, the adhesive cell density in the TCs-educated group was higher than that of the control. The OD values also confirmed the significant differences (3.501 ± 0.08 *versus* 1.460 ± 0.08, P < 0.01; **Figure 5C**). This finding indicated that indirect coculture with TCs can enhance cell adhesion of ESCs.

Migration analysis

As shown in **Figure 6A**, **6B**, **6E**, the average number of TCM-educated ESCs that migrated was significantly higher compared to with that of Non-educated control cells (51.667 ± 6.658 *versus* 12 ± 3.606, P < 0.05). Moreover, in **Figure 6C-E**, the average number of TCs-

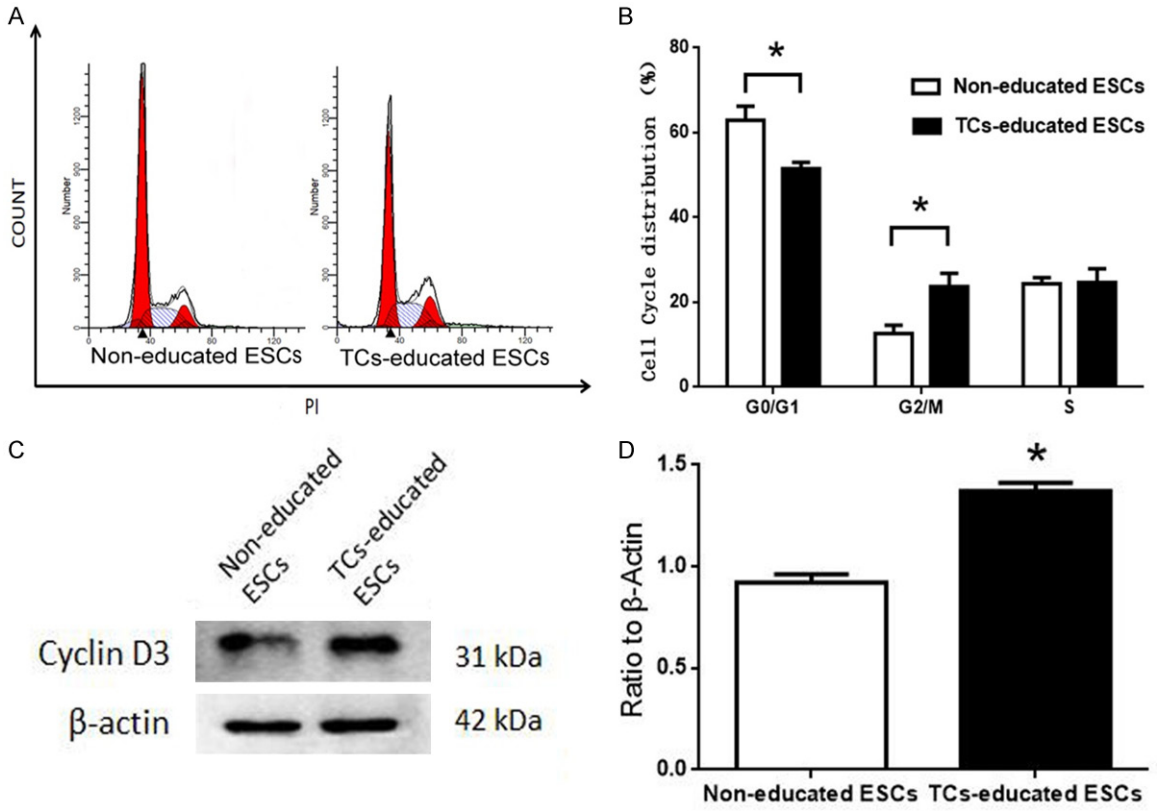

Figure 8. Cell cycle distribution and cyclin D3 expression in both groups of ESCs after 48 hrs of coculture. A. Cell cycle assay by flow cytometry. B. Proportion of cell cycle. TCs treatment reduced the percentage of ESCs in G0/G1 phase (*$P < 0.05$) and upregulated the percentage of cells inG2/M phase (*$P < 0.05$) but with no difference was observed in the percentage of cells in S phase ($P > 0.05$). This indicated that TCs treatment enhanced cell proliferation during G2/M. C. Representative Western blot results of cyclin D3 protein. D. The ratio of cyclin D3 to β-actin was significantly increased in TCs-educated ESCs (*$P < 0.05$ *versus* Non-educated ESCs). This indicated that cyclin-D3 participated in the enhanced cell proliferation during G2/M in TCs-educated ESCs. Data are expressed as the mean ± SD. The mean and SD were calculated from at least 9 values from three separate experiments.

educated ESCs that migrated was higher than that of the Non-educated control (34.333 ± 4.041 *versus* 8 ± 2.646, $P < 0.05$). This suggested that TCM or indirect coculture with TCs can accelerate the migration of ESCs.

Matrigel invasion assay

As shown in **Figure 7**, the average number of TCs-educated ESCs that penetrated through the Matrigel was significantly higher than that of Non-educated ESCs (45.25 ± 6.315 *versus* 21.50 ± 4.758, $P < 0.05$). This finding suggested that indirect coculture with TCs can reinforce the invasion ability of ESCs.

Cell cycle and cyclin-D3 expression of ESCs

Cell cycle changes in both groups of ESCs (TCs-educated and Non-educated) were examined by PI staining and flow cytometry. Compared with Non-educated ESCs, TCs treatment significantly reduced the percentage of ESCs in G0/G1 phase (51.55 ± 1.53 *versus* 62.98 ± 3.32, $P < 0.05$) but upregulated the percentage of ESCs in G2/M phase (23.74 ± 3.13 *versus* 12.65 ± 1.97, $P < 0.05$). However, no significant difference was observed in the percentage of ESCs in S phase (24.71 ± 3.20 *versus* 24.36 ± 1.48, $P > 0.05$; **Figure 8A**, **8B**). Such results indicated that TCs treatment can enhance cell cycle proliferation at G2/M. Furthermore, cyclin-D3 is a type of D-type cyclin that is associated with cell cycle progression; TCs treatment also upregulated cyclin-D3 expression (0.920 ± 0.0428 *versus* 1.372 ± 0.039, $P < 0.05$; **Figure 8C**, **8D**), which indicated that cyclin-D3 participates in the enhanced cell proliferation at G2/M in TCs-educated ESCs.

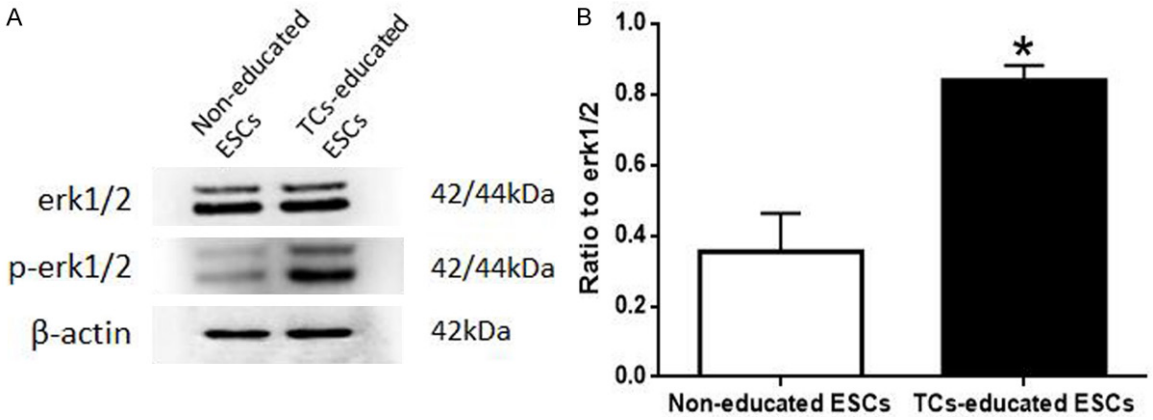

Figure 9. Representative Western blot results of erk1/2 and p-erk1/2 after 48 hrs in TCs-educated and Non-educated ESCs. A. Representative Western blot results of erk1/2 and p-erk1/2 proteins in both groups. B. The ratio of p-erk1/2 to erk1/2 was significantly increased in TCs-educated ESCs (*$P < 0.05$ *versus* Non-educated ESCs). This indicated that the ERK signaling pathway was involved in the activation of TCs-educated ESCs. Data are expressed as the mean ± SD. The mean and SD were calculated from at least 9 values from three separate experiment.

Protein expression of ERK1/2 and P-ERK1/2

To elucidate the underlying mechanisms involved in the phenotype and metergasis alterations in TCs-educated ESCs, the protein expression levels were measured in both groups of ESCs. As shown in **Figure 9**, the protein levels of P-ERK1/2 were significantly higher in TCs-educated ESCs than in Non-educated control cells (0.810 ± 0.086 *versus* 0.374 ± 0.138, $P < 0.05$), while no significant difference was observed in the levels of total ERK1/2 between both groups of ESCs. Western blot analysis indicated that the ERK signaling pathway is involved in the activation of TCs-educated ESCs.

Discussion

Since the first report of the newly found interstitial stromal cells by Prof Popescu's team, many papers on TCs have demonstrated its existence, characteristic structure and immune markers expression in various normal organs and in different species [1]. Multiple physiologic functions have been proposed for these cells, although the majority of potential functions remain to be fully confirmed. Then, pathological alterations in TCs in disease-affected tissues were reported in myocardial infarction [50], the dermal cellular network of skin systemic sclerosis [51, 52], Crohn's disease [53], gallbladder disorders [54] as well as liver fibrosis [55], together with assumed specific pathophysiologic roles for TCs in the pathogenesis, progression and recovery process. Specially, in disease-affected oviduct tissue, in vivo ultrastructure damage of TCs was observed by our team along with intercellular contacts between the damaged TCs and immunocytes, which can lead to tissue fibrosis and attenuated fertility [35, 36]. Subsequently, an *in vitro* coculture study confirmed that TCs can develop intercellular contacts with macrophages and that TCs-educated macrophages were activated with increased cytokine secretion, enhanced invasion ability and inhibition of apoptosis, which in turn were considered to be associated with the onset of EMs [23, 48].

Numerous reports have indicated that EMs is associated with poor fertility capacity and adverse pregnancy outcomes, such as implantation failure and recurrent miscarriage [56, 57]. Additionally, ESCs are increasingly recognized as an essential component in the development of EMs. ESCs from EMs patients demonstrate stronger adhesion [44], migration and invasion ability [46], increased proliferation [58, 59] and are less likely to undergo apoptosis [58, 60]. The underlying multiple signaling pathways include activated Ras/Raf/MEK/ERK [61, 62] and PI3K/AKT pathways [62], among others. Based on the intercellular connections between TCs and ESCs, the functional roles of TCs remain unclear [30]. The purpose of the current study is to explore the exact modulatory function of TCs on normal eutopic ESCs

and the underlying mechanisms. Herein, we established a direct and indirect coculture system for TCs and ESCs. In the direct coculture system, similar intercellular contacts gradually developed between Tps and extended pseudopodia of ESCs. This was consistent with previous *in vitro* observations by Kota Hatta [30]. Such direct cell-to-cell contacts presumably contribute, at least partially, to functional alterations in ESCs.

Furthermore, the indirect coculture system was used to detect the *in vitro* behavioral changes of TCs-educated ESCs. The results indicated that TCs treatment obviously promotes viability, proliferation, invasion, adhesion and migration ability of normal eutopic ESCs, all of which are mediated by the ERK pathway. Moreover, the apoptosis of ESCs was analyzed by Annexin V-FITC/PI staining and flow cytometry, but no difference in apoptosis was found between TCs-educated and Non-educated ESCs (data not shown). Such behavioral changes were similar to characteristics of eutopic ESCs in patients with EMs [44, 63, 64]. In those patients, both eutopic and ectopic ESCs from EMs show more invasive characteristics than normal control ESCs, and the aberrant metergasis of eutopic or ectopic ESCs is a source of EMs occurrence and EMs-related pregnancy failure or pregnancy loss, among other adverse consequences [63, 65-67]. Thus, after the observation of intercellular contacts between TCs and ESCs in the current study and in a previous report [30], we provided the first evidence for the modulatory function of TCs on ESCs and showed that the phenotype and metergasis of TCs-educated ESCs changed markedly from that of normal ESCs. The current results support the hypothesis that TCs-educated ESCs have the potential to facilitate or contribute positively to the onset and progression of EMs.

Further studies confirmed involvement of the ERK pathway in regulating the behavioral changes of TCs-educated ESCs. However, no differences were found in the protein expression of AKT and p-AKT between TCs-educated and Non-educated ESCs (data not shown). The ERK/MAPK and PI3K/AKT signaling pathways are known to be involved in the regulation of cell proliferation, motor function (migration, invasion), autophagy, senescence and apoptosis, especially in ESCs [62, 68-71]. ERK1/2 signaling during ESC migration was found to be activated through the binding of various growth factors and receptors [62]. In addition, increased expression of matrix metalloproteinase 2 (MMP2), which degrades the extracellular matrix and facilitates migration and invasion of ESCs, was observed [72, 73]. Furthermore, cyclin-D3 was reported to function as a downstream target of the ERK signaling pathway [74]. ERK1/2 phosphorylation induces c-Myc, which promotes the transcriptional activation of D-type cyclins [75]. The observed upregulation of cyclin-D3 further points to a central role of ERK1/2-cyclin-D3 activation in the migratory and invasive capacities of TCs-educated ESCs motile, invasive capacit and in their proliferation. In addition to direct cell-to-cell contacts, indirect coculture results suggest an important juxta-paracrine effect of TCs in regulating ESC activity. However, we only analyzed the abovementioned two signaling proteins and obtained one positive result. Therefore, whether ERK signaling is the only pathway involved in one or all of these observed behavioral changes in TCs-educated ESCs, is worthy of further study.

Nevertheless, the current study only investigates normal eutopic ESCs from normal mice and discusses the potential implications of TCs-educated ESCs in EMs. ESCs from patients with EMs demonstrated a different migratory capability than normal controls [76]. Therefore, future studies are needed to test these results in an EMs model. On the contrary, this novel concept of TCs-educated ESCs is worthy of additional investigation. A deeper understanding of the enhanced migratory and invasive capacity of TCs-educated ESCs can also support EMs-related reproductive disorders and endometrial tissue repair or regeneration [47]. In the current study, enhanced motor function of ESCs mediated by ERK1/2 signaling upon exposure to direct cell-to-cell connections or growth factors, chemokines, or cytokines, which might be present in the juxta-paracrine secretome of TCs, is essential not only for the successful implantation of embryos and maintenance of normal pregnancy [77-79] but also for endometrial regeneration following parturition, endometrial resection, or intrauterine adhesions. Therefore, targeting the regulatory effects of TCs and pathway proteins in TCs-educated ESCs holds promise for developing new strategies and tools for the treatment of

EMs and related common reproductive disorders.

Conclusions

In conclusion, although the nature of the paracrine substances of TCs and their downstream molecular pathways in eutopic ESCs should be further investigated, the current results demonstrated that TCs treatment obviously promotes vitality, motility, invasive capacity and proliferation of ESCs. Our study also demonstrated that these characteristics are the mediated by ERK pathway, likely through direct intercellular contacts and/or juxta-paracrine effects. The enhanced functions of TCs-educated ESCs not only contribute to a deeper understanding of TCs, but they also highlight a new concept regarding the physiopathology and more effective treatment strategies for EMs.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81571415 and 81270679).

Disclosure of conflict of interest

None.

Abbreviations

CCK-8, Cell counting kit-8; ECM, extracellular matrix; Ems, Endometriosis; ERK, extracellular-regulated kinase; ESCs, Endometriosis; FCM, Flow cytometry; PBS, phosphate buffered saline; PCK, Pan Cytokeratin; TCs, Telocytes; TCM, TC- conditioned media; Tp, telopode.

Address correspondence to: Xiao-Jun Yang, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Soochow University, 188 Shizi Road, Suzhou 215006, Jiangsu Province, PR China. Tel: +86 18626292163; Fax: +86 512 66919883; E-mail: yang.xiaojun@hotmail.com

References

[1] Popescu LM, Faussone-Pellegrini MS. TELOCYTES - a case of serendipity: the winding way from Interstitial Cells of Cajal (ICC), via Interstitial Cajal-Like Cells (ICLC) to TELOCYTES. J Cell Mol Med 2010; 14: 729-740.

[2] Hashitani H and Lang RJ. Functions of ICC-like cells in the urinary tract and male genital organs. J Cell Mol Med 2010; 14: 1199-1211.

[3] Corradi LS, Jesus MM, Fochi RA, Vilamaior PS, Justulin LA, Goes RM, Felisbino SL and Taboga SR. Structural and ultrastructural evidence for telocytes in prostate stroma. J Cell Mol Med 2013; 17: 398-406.

[4] Luesma MJ, Gherghiceanu M and Popescu LM. Telocytes and stem cells in limbus and uvea of mouse eye. J Cell Mol Med 2013; 17: 1016-1024.

[5] Li H, Zhang H, Yang L, Lu S and Ge J. Telocytes in mice bone marrow: electron microscope evidence. J Cell Mol Med 2014; 18: 975-978.

[6] Yang P, Ahmad N, Hunag Y, Ullah S, Zhang Q, Waqas Y, Liu Y, Li Q, Hu L and Chen Q. Telocytes: novel interstitial cells present in the testis parenchyma of the Chinese soft-shelled turtle Pelodiscus sinensis. J Cell Mol Med 2015; 19: 2888-2899.

[7] Xu T, Lu S and Zhang H. Transmission electron microscope evidence of telocytes in canine dura mater. J Cell Mol Med 2016; 20: 188-192.

[8] Zhang H, Yu P, Zhong S, Ge T, Peng S, Guo X and Zhou Z. Telocytes in pancreas of the Chinese giant salamander (Andrias davidianus). J Cell Mol Med 2016; 20: 2215-2219.

[9] Zhang H, Zhong S, Ge T, Peng S, Yu P, Zhou Z and Guo X. Telocytes in ileum of the Chinese giant salamander: ultrastructural evidence. J Cell Mol Med 2016; 20: 568-574.

[10] Ullah S, Yang P, Zhang L, Zhang Q, Liu Y, Chen W, Waqas Y, Le Y, Chen B and Chen Q. Identification and characterization of telocytes in the uterus of the oviduct in the Chinese soft-shelled turtle, Pelodiscus sinensis: TEM evidence. J Cell Mol Med 2014; 18: 2385-2392.

[11] Suciu L, Popescu LM, Gherghiceanu M, Regalia T, Nicolescu MI, Hinescu ME and Faussone-Pellegrini MS. Telocytes in human term placenta: morphology and phenotype. Cells Tissues Organs 2010; 192: 325-339.

[12] Suciu L, Popescu LM and Gherghiceanu M. Human placenta: de visu demonstration of interstitial Cajal-like cells. J Cell Mol Med 2007; 11: 590-597.

[13] Gherghiceanu M and Popescu LM. Cardiac telocytes - their junctions and functional implications. Cell Tissue Res 2012; 348: 265-279.

[14] Bei Y, Zhou Q, Sun Q and Xiao J. Telocytes in cardiac regeneration and repair. Semin Cell Dev Biol 2016; 55: 14-21.

[15] Wang F, Song Y, Bei Y, Zhao Y, Xiao J and Yang C. Telocytes in liver regeneration: possible roles. J Cell Mol Med 2014; 18: 1720-1726.

[16] Bei Y, Wang F, Yang C and Xiao J. Telocytes in regenerative medicine. J Cell Mol Med 2015; 19: 1441-1454.

[17] Ceafalan L, Gherghiceanu M, Popescu LM and Simionescu O. Telocytes in human skin--are

they involved in skin regeneration? J Cell Mol Med 2012; 16: 1405-1420.
[18] Chaitow L. Telocytes: connective tissue repair and communication cells. J Bodyw Mov Ther 2017; 21: 231-233.
[19] Edelstein L and Smythies J. The role of telocytes in morphogenetic bioelectrical signaling: once more unto the breach. Front Mol Neurosci 2014; 7: 41.
[20] Radu BM, Banciu A, Banciu DD, Radu M, Cretoiu D and Cretoiu SM. Calcium signaling in interstitial cells: focus on telocytes. Int J Mol Sci 2017; 18: 397.
[21] Di Benedetto A, Arena S, Nicotina PA, Mucciardi G, Gali A and Magno C. Pacemakers in the upper urinary tract. Neurourol Urodyn 2013; 32: 349-353.
[22] Albulescu R, Tanase C, Codrici E, Popescu DI, Cretoiu SM and Popescu LM. The secretome of myocardial telocytes modulates the activity of cardiac stem cells. J Cell Mol Med 2015; 19: 1783-1794.
[23] Chi C, Jiang XJ, Su L, Shen ZJ and Yang XJ. In vitro morphology, viability and cytokine secretion of uterine telocyte-activated mouse peritoneal macrophages. J Cell Mol Med 2015; 19: 2741-2750.
[24] Shoshkes-Carmel M, Wang YJ, Wangensteen KJ, Toth B, Kondo A, Massasa EE, Itzkovitz S and Kaestner KH. Subepithelial telocytes are an important source of Wnts that supports intestinal crypts. Nature 2018; 557: 242-246.
[25] Degirmenci B, Valenta T, Dimitrieva S, Hausmann G and Basler K. GLI1-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells. Nature 2018; 558: 449-453.
[26] Samuelson LC. Debate over the identity of an intestinal niche-cell population settled. Nature 2018; 558: 380-381.
[27] Varga I, Urban L, Kajanova M and Polak S. Functional histology and possible clinical significance of recently discovered telocytes inside the female reproductive system. Arch Gynecol Obstet 2016; 294: 417-422.
[28] Aleksandrovych V, Walocha JA and Gil K. Telocytes in female reproductive system (human and animal). J Cell Mol Med 2016; 20: 994-1000.
[29] Shafik A, El-Sibai O, Shafik I and Shafik AA. Immunohistochemical identification of the pacemaker cajal cells in the normal human vagina. Arch Gynecol Obstet 2005; 272: 13-16.
[30] Hatta K, Huang ML, Weisel RD and Li RK. Culture of rat endometrial telocytes. J Cell Mol Med 2012; 16: 1392-1396.
[31] Cretoiu D, Ciontea SM, Popescu LM, Ceafalan L and Ardeleanu C. Interstitial Cajal-like cells (ICLC) as steroid hormone sensors in human myometrium: immunocytochemical approach. J Cell Mol Med 2010; 10: 789-795.
[32] Cretoiu SM, Cretoiu D and Popescu LM. Human myometrium - the ultrastructural 3D network of telocytes. J Cell Mol Med 2012; 16: 2844-2849.
[33] Hutchings G, Williams O, Cretoiu D and Ciontea SM. Myometrial interstitial cells and the coordination of myometrial contractility. J Cell Mol Med 2009; 13: 4268-4282.
[34] Cretoiu SM, Cretoiu D, Suciu L and Popescu LM. Interstitial Cajal-like cells of human Fallopian tube express estrogen and progesterone receptors. J Mol Histol 2009; 40: 387-394.
[35] Yang J, Chi C, Liu Z, Yang G, Shen ZJ and Yang XJ. Ultrastructure damage of oviduct telocytes in rat model of acute salpingitis. J Cell Mol Med 2015; 19: 1720-1728.
[36] Yang XJ, Yang J, Liu Z, Yang G and Shen ZJ. Telocytes damage in endometriosis-affected rat oviduct and potential impact on fertility. J Cell Mol Med 2015; 19: 452-462.
[37] Aleksandrovych V, Sajewicz M, Walocha JA and Gil K. Tubal telocytes: factor infertility reason? Folia Medica Cracoviensia 2016; 56: 17.
[38] Khan KN, Kitajima M, Fujishita A, Hiraki K, Matsumoto A, Nakashima M and Masuzaki H. Pelvic pain in women with ovarian endometrioma is mostly associated with coexisting peritoneal lesions. Hum Reprod 2013; 28: 109-118.
[39] Facchin F, Barbara G, Saita E, Mosconi P, Roberto A, Fedele L and Vercellini P. Impact of endometriosis on quality of life and mental health: pelvic pain makes the difference. J Psychosom Obst Gyn 2015; 36: 135.
[40] Ozkan S, Murk W and Arici A. Endometriosis and infertility: epidemiology and evidence-based treatments. Ann N Y Acad Sci 2008; 1127: 92-100.
[41] Sampson and John A. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol 1927; 14: 93-94.
[42] Riccio LGC, Baracat EC, Chapron C, Batteux F and Abrao MS. The role of the B lymphocytes in endometriosis: A systematic review. J Reprod Immunol 2017; 123: 29-34.
[43] Klemmt PA, Carver JG, Kennedy SH, Koninckx PR and Mardon HJ. Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. Fertil Steril 2006; 85: 564-572.
[44] Klemmt PA, Carver JG, Koninckx P, McVeigh EJ and Mardon HJ. Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mecha-

nistic model for endometriosis progression. Hum Reprod 2007; 22: 3139-3147.
[45] Sundqvist J, Andersson KL, Scarselli G, Gemzell-Danielsson K and Lalitkumar PG. Expression of adhesion, attachment and invasion markers in eutopic and ectopic endometrium: a link to the aetiology of endometriosis. Hum Reprod 2012; 27: 2737-2746.
[46] Banu S, Lee J, Speights JV, Starzinski-Powitz A and Arosh J. Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms. Endocrinology 2008; 149: 1180-1189.
[47] Weimar CH, Macklon NS, Post Uiterweer ED, Brosens JJ and Gellersen B. The motile and invasive capacity of human endometrial stromal cells: implications for normal and impaired reproductive function. Hum Reprod Update 2013; 19: 542-557.
[48] Jiang XJ, Cretoiu D, Shen ZJ and Yang XJ. An in vitro investigation of telocytes-educated macrophages: morphology, heterocellular junctions, apoptosis and invasion analysis. J Transl Med 2018; 16: 85.
[49] Arnold JT, Kaufman DG, Seppala M and Lessey BA. Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. Hum Reprod 2001; 16: 836-845.
[50] Zhao B, Chen S, Liu J, Yuan Z, Qi X, Qin J, Zheng X, Shen X, Yu Y, Qin TJ, Chan JY and Cai D. Cardiac telocytes were decreased during myocardial infarction and their therapeutic effects for ischaemic heart in rat. J Cell Mol Med 2013; 17: 123-133.
[51] Manetti M, Guiducci S, Ruffo M, Rosa I, Faussone-Pellegrini MS, Matucci-Cerinic M and Ibba-Manneschi L. Evidence for progressive reduction and loss of telocytes in the dermal cellular network of systemic sclerosis. J Cell Mol Med 2013; 17: 482-496.
[52] Manetti M, Rosa I, Messerini L, Guiducci S, Matucci-Cerinic M and Ibba-Manneschi L. A loss of telocytes accompanies fibrosis of multiple organs in systemic sclerosis. J Cell Mol Med 2014; 18: 253-262.
[53] Milia AF, Ruffo M, Manetti M, Rosa I, Conte D, Fazi M, Messerini L and Ibba-Manneschi L. Telocytes in Crohn's disease. J Cell Mol Med 2013; 17: 1525-1536.
[54] Matyja A, Gil K, Pasternak A, Sztefko K, Gajda M, Tomaszewski KA, Matyja M, Walocha JA, Kulig J and Thor P. Telocytes: new insight into the pathogenesis of gallstone disease. J Cell Mol Med 2013; 17: 734-742.
[55] Fu S, Wang F, Cao Y, Huang Q, Xiao J, Yang C and Popescu LM. Telocytes in human liver fibrosis. J Cell Mol Med 2015; 19: 676-683.
[56] Simon C, Gutierrez A, Vidal A, de los Santos MJ, Tarin JJ, Remohi J and Pellicer A. Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum Reprod 1994; 9: 725-729.
[57] Stephansson O, Kieler H, Granath F and Falconer H. Endometriosis, assisted reproduction technology, and risk of adverse pregnancy outcome. Hum Reprod 2009; 24: 2341-2347.
[58] Delbandi AA, Mahmoudi M, Shervin A, Akbari E, Jeddi-Tehrani M, Sankian M, Kazemnejad S and Zarnani AH. Eutopic and ectopic stromal cells from patients with endometriosis exhibit differential invasive, adhesive, and proliferative behavior. Fertil Steril 2013; 100: 761-769.
[59] Nishida M, Nasu K, Ueda T, Fukuda J, Takai N and Miyakawa I. Endometriotic cells are resistant to interferon-gamma-induced cell growth inhibition and apoptosis: a possible mechanism involved in the pathogenesis of endometriosis. Mol Hum Reprod 2005; 11: 29-34.
[60] Nasu K, Yuge A, Tsuno A, Nishida M and Narahara H. Involvement of resistance to apoptosis in the pathogenesis of endometriosis. Histol Histopathol 2009; 24: 1181-1192.
[61] Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA and McCubrey JA. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. Leukemia 2003; 17: 1263-1293.
[62] Gentilini D, Busacca M, Di Francesco S, Vignali M, Vigano P and Di Blasio AM. PI3K/Akt and ERK1/2 signalling pathways are involved in endometrial cell migration induced by 17beta-estradiol and growth factors. Mol Hum Reprod 2007; 13: 317-322.
[63] Wingfield M, Macpherson A, Healy DL and Rogers PAW. Cell proliferation is increased in the endometrium of women with endometriosis. Fertil Steril 1995; 64: 340-346.
[64] Gaetje R, Kotzian S, Herrmann G, Baumann R and Starzinskipowitz A. Invasiveness of endometriotic cells in vitro. Lancet 1995; 346: 1463.
[65] Yang JH, Wu MY, Chen CD, Chen MJ, Yang YS and Ho HN. Altered apoptosis and proliferation in endometrial stromal cells of women with adenomyosis. Hum Reprod 2007; 22: 945-952.
[66] Weimar CH, Kavelaars A, Brosens JJ, Gellersen B, de Vreeden-Elbertse JM, Heijnen CJ and Macklon NS. Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos. PLoS One 2012; 7: e41424.
[67] Ornek T, Fadiel A, Tan O, Naftolin F and Arici A. Regulation and activation of ezrin protein in endometriosis. Hum Reprod 2008; 23: 2104-2112.

[68] Qu JL, Qu XJ, Zhao MF, Teng YE, Zhang Y, Hou KZ, Jiang YH, Yang XH and Liu YP. Gastric cancer exosomes promote tumour cell proliferation through PI3K/Akt and MAPK/ERK activation. Dig Liver Dis 2009; 41: 875-880.

[69] Kavurma MM and Khachigian LM. ERK, JNK, and p38 MAP kinases differentially regulate proliferation and migration of phenotypically distinct smooth muscle cell subtypes. J Cell Biochem 2003; 89: 289-300.

[70] Saxena NK, Sharma D, Ding X, Lin S, Marra F, Merlin D and Anania FA. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. Cancer Res 2007; 67: 2497-2507.

[71] Cagnol S and Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. FEBS J 2010; 277: 2-21.

[72] Li MQ, Li HP, Meng YH, Wang XQ, Zhu XY, Mei J and Li DJ. Chemokine CCL2 enhances survival and invasiveness of endometrial stromal cells in an autocrine manner by activating Akt and MAPK/Erk1/2 signal pathway. Fertil Steril 2012; 97: 919-929.

[73] Matsuda M, Sasabe H, Adachi Y, Suzuki T and Mori T. Increased invasion activity of endometrial stromal cells and elevated expression of matrix metalloproteinase messenger RNA in the uterine tissues of mice with experimentally induced adenomyosis. Am J Obstet Gynecol 2001; 185: 1374-1380.

[74] Jiang W, Zhu J, Zhuang X, Zhang X, Luo T, Esser KA and Ren H. Lipin1 regulates skeletal muscle differentiation through extracellular signal-regulated kinase (ERK) activation and cyclin D complex-regulated cell cycle withdrawal. J Biol Chem 2015; 290: 23646-23655.

[75] Filigheddu N, Gregnanin I, Porporato PE, Surico D, Perego B, Galli L, Patrignani C, Graziani A and Surico N. Differential expression of MicroRNAs between eutopic and ectopic endometrium in ovarian endometriosis. J Biomed Biotechnol 2010; 2010: 369549.

[76] Gentilini D, Vigano P, Somigliana E, Vicentini LM, Vignali M, Busacca M and Di BA. Endometrial stromal cells from women with endometriosis reveal peculiar migratory behavior in response to ovarian steroids. Fertil Steril 2010; 93: 706-715.

[77] Salker M, Teklenburg G, Molokhia M, Lavery S, Trew G, Aojanepong T, Mardon HJ, Lokugamage AU, Rai R, Landles C, Roelen BA, Quenby S, Kuijk EW, Kavelaars A, Heijnen CJ, Regan L, Macklon NS and Brosens JJ. Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss. PLoS One 2010; 5: e10287.

[78] Chang HJ, Lee JH, Hwang KJ, Kim MR, Chang KH, Park DW and Min CK. Transforming growth factor (TGF)-beta1-induced human endometrial stromal cell decidualization through extracellular signal-regulated kinase and Smad activation in vitro: peroxisome proliferator-activated receptor gamma acts as a negative regulator of TGF-beta1. Fertil Steril 2008; 90: 1357-1365.

[79] Yoon MS, Koo JB, Hwang JH, Lee KS and Han JS. Activation of phospholipase D by 8-Br-cAMP occurs through novel pathway involving Src, Ras, and ERK in human endometrial stromal cells. FEBS Lett 2005; 579: 5635-5642.

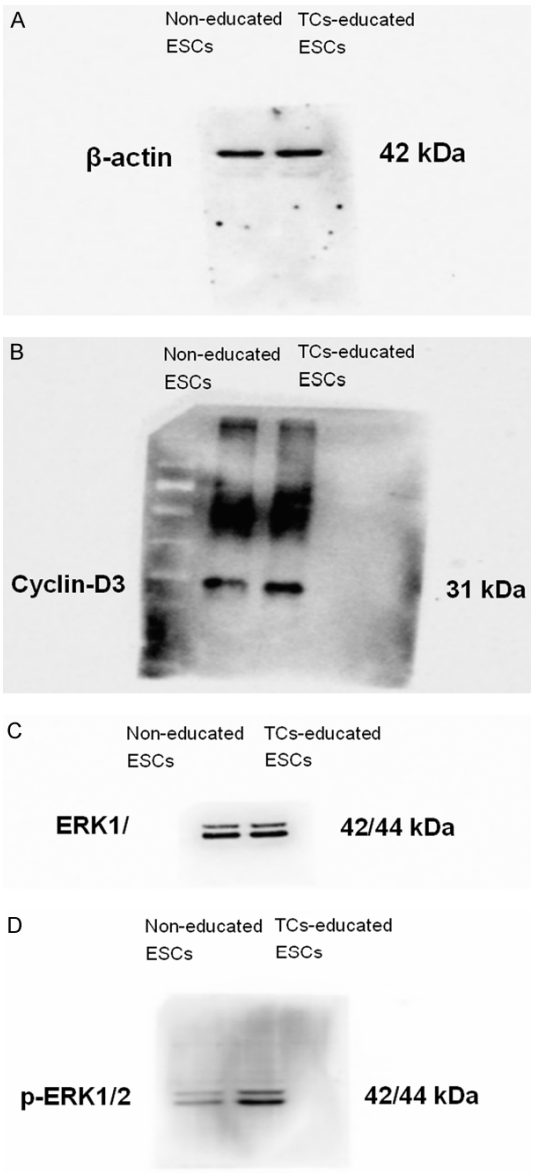

Figure S1. Original Western blots of β-actin (A), cyclinD3 (B), ERK1/2 (C) and p-ERK1/2 (D) after Telocytes treatment endometrial stromal cell. Pathway analysis for non-educated ESCs and TCs-educated ESCs.