



# Endometriosis Cell Proliferation Induced by Bone Marrow Mesenchymal Stem Cells

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## Abstract

Endometriosis is an estrogen-dependent gynecological disorder that affects 10% of reproductive-aged women and causes pelvic pain and infertility. Bone marrow–derived stem cells (BMDCs) are known to engraft endometriosis in association with lesion growth; however, they do not undergo significant clonal expansion. The indirect effects of BMDCs on endometriosis growth and cell proliferation are not well characterized. Here, we demonstrate that BMDCs' co-culture increased endometrial stromal cell proliferation. In vitro studies using endometrial cells showed that BMDCs increased cell proliferation and activation of *CDK1* in both an endometriosis cell line and primary endometrial stromal cells from women with endometriosis, however not in normal endometrial cells. In vivo studies using a mouse model of endometriosis showed increased CDK1+ expression associated with engrafted GFP + BMDCs. These results suggest that endometrial cell proliferation is induced by stem cell–derived trophic factors leading to the growth of endometriotic lesions. Targeting the specific signaling molecules secreted by BMDC may lead to novel therapeutic strategies for controlling cell proliferation in endometriosis.

**Keywords** Endometriosis · Cell proliferation · CDK1 · Bone marrow–derived cells (BMDCs)

## Introduction

Endometriosis is a common, chronic inflammatory disorder in women of reproductive age. This disease has an unknown

Peng Chen and Ramanaiah Mamillapalli contributed equally to this work.

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pathogenesis and is characterized by pain, infertility, and ectopic pelvic lesions resembling endometrium [1, 2]. Current medical therapies treat endometriosis by altering sex steroid hormones. However, side effects that develop during these treatments may be cumbersome and threaten patient compliance.

Bone marrow–derived cells (BMDCs) migrate to multiple organs and differentiate into tissue-specific cells. BMDCs are involved in the pathogenesis of endometriosis and contribute to the development of this disease [3]. They participate in epithelial and stroma regeneration in endometrial tissue [4] and endometriotic lesions [5, 6]; they are also likely the principal source of extra-pelvic endometriosis [3, 6]. However clonal expansion and cell replacement alone do not account for the extensive effects of these stem cells on the engrafted tissues [7]. The effect of BMDCs' engraftment on the endogenous resident endometrial cells is still poorly characterized.

The peritoneal microenvironment is significantly altered in women with endometriosis. Endometrial cells refluxed into the peritoneal cavity secrete chemokines [8], creating a feed-forward loop [9] that stimulates the infiltration of BMDCs including immune cells [10] and bone marrow–derived stem cells. Both endometriotic and immune cells [11–13] produce pro-inflammatory cytokines and prostaglandins [14, 15], and they suppress anti-inflammatory interleukins [16], creating an

inflammatory imbalance. The ability of this altered microenvironment to support endometriotic cells is dependent on kinase signaling pathways [17]. In this study, we focused mainly on three signaling pathways involving nuclear factor (NF)kB, mitogen-activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR) as well as factors related to the cell cycle. We carried out co-culture of endometrial stromal cells and BMSCs to examine the differential expression of molecules involved in these kinase signaling pathways to delineate the effects of BMDCs on endometrial cells.

## Materials and Methods

### Sample Collection

Institutional Review Board (IRB) approval was obtained from Yale School of Medicine (New Haven, Connecticut) allowing the use of human samples (Human Investigations Committee protocol, #1004006657). Recruited subjects were women who underwent surgery for benign disease in the reproductive endocrinology practice of the Yale School of Medicine. Written informed consent was obtained from subjects admitted to the hospital prior to undergoing laparoscopy or laparotomy for suspected benign indications such as pelvic masses, pelvic pain, infertility, or endometriosis. Endometrial biopsies in the secretory phase were obtained from women with moderate-to-severe endometriosis (stages III and IV). Inclusion criteria included women who were aged 18–49 years, had regular menstrual cycles, and used no hormonal therapy for at least 3 months preceding surgery. Exclusion criteria included post-menopausal status, previous hormone use within 3 months of surgery, hyperplasia, polyps, malignancy, autoimmune disease, or use of anti-inflammatory medications. Twenty subjects were included in each of the endometriosis and control study groups. The endometriosis group was surgically diagnosed and had histologically verified endometriosis, and the control group was visually verified to be free of endometriosis during the surgery. The phase of the menstrual cycle was determined based on the subjects' menstrual history and confirmed by histologic evaluation.

### Cell Culture

The immortalized endometrial stromal cell line (HESC) and endometriosis stromal cell line were a gift from Prof. Gil Mor at Yale School of Medicine, New Haven, CT, USA. Eutopic endometrial tissues were processed and stromal cells cultured using a protocol previously described by Ryan et al. [18] with minor modifications [19]. Briefly, endometriotic or endometrial tissue was minced finely and digested in digestion medium containing collagenase B (1 mg/ml, Roche Diagnostics, Indianapolis, IN, USA) and deoxyribonuclease I (0.1 mg/ml,

Sigma-Aldrich, St. Louis, MO, USA) in DMEM medium, at 37 °C for 30 min, and tissue was pipetted gently to disperse the cells every 10 min. Stromal cells were filtrated through a 40- $\mu$ m cell strainer and cultured in DMEM/F-12 containing 10% fetal bovine serum and 1% penicillin-streptomycin. The fibroblast-like appearance of endometriosis-derived stromal cells in culture under phase contrast microscopy appeared identical to that of endometrial stromal cells. Primary eutopic endometrial stromal or endometriosis derived cells were used at 2 to 3 passages.

BMDCs were obtained from the American Type Culture Collection (ATCC, cat. # PCS-500-012, Manassas, VA, USA). Co-culture between BMDCs and endometrial stromal cells was carried out using 4- $\mu$ m pore size polycarbonate membrane (Millipore, Burlington, MA, USA). Endometrial stromal cells were seeded into a 6-well plate at a concentration of  $1 \times 10^5$  cells per well. BMDCs were plated into the trans-well insert at a concentration of  $1 \times 10^4$  cells per insert and the insert was placed into the 6-well plate. Each well contained 3 ml growth media (ATCC CRL-11731) with a final concentration of 15% fetal bovine serum. Total RNA and protein were collected from the cell cultures grown for 24, 48, and 72 h. Total RNA was used for the analysis of gene expression by qRT-PCR while protein was analyzed by western blot.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from endometriotic lesions by TRIzol reagent (Life Technologies), followed by purification using Qiagen cleaning kit (Qiagen, Valencia, CA, USA) to prepare cDNA with 50 ng RNA in a 20  $\mu$ l reaction mixture by iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real-time PCR was performed using SYBR Green (Bio-Rad) and optimized in the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Twenty-six genes which were significantly differentially expressed between normal and endometriosis cells were selected from in the initial screen of more than 150 genes. qRT-PCR was used to quantify gene expression using specific primers for these 26 genes (Table 1). The specificity of the amplified transcript (39 cycles) and absence of primer-dimers were confirmed by a melting curve analysis. Gene expression was normalized to the expression of  $\beta$ -actin for each sample. Relative mRNA expression for each gene was calculated using the comparative cycle threshold (Ct) method, also known as the  $2^{-\Delta\Delta CT}$  method [20]. All experiments were carried out three times and each experiment was performed in duplicate. Nuclease-free water was used as a negative control replacing the cDNA template.

**Table 1** Primer sequences

Gene	Forward Primer Sequence	Reverse Primer Sequence
<i>NFκB1</i>	GTGGTGCCTCACTGCTAACT	GGATGCACTTCAGTTCTGT
<i>NFκB2</i>	TAGCCACAGAGATGGAGGAG	CCGAGTCGCTATCAGAGGTA
<i>PPAR-γ</i>	AGGAAGGGGCCTTAACCTCT	TCTCCGGAAGAAACCCCTTGC
<i>ERK1</i>	CTGGCAAGCACTACCTGGAT	TGGAGGGCAGAGACTGTAGG
<i>ERK2</i>	TACACCAACCTCTCGTACATCG	CATGTCTGAAGCGCAGTAAGATT
<i>p38-α</i>	ATGCATAATGGCCGAGCTGT	TATGGTCAACTTACCCAGGGGAT
<i>JNK</i>	CTGAAGCAGAAGTCCACCA	TGCACCTAAAGGAGAGGGCT
<i>PTEN</i>	CGGCAGCATCAAATGTTTCAG	AACTGGCAGGTAGAAGGCAACTC
<i>AKT1</i>	CAGCCTGGGTCAAAGAAGTCA	TGTACTCCCCTCGTTTGTGC
<i>mTOR</i>	TCGCTGAAGTCACACAGACC	CTTTGGCATATGCTCGGCAC
<i>CDK2</i>	GACACGCTGCTGGATGTCA	GAGGGGAAGAGGAATGCCAG
<i>CDK4</i>	GTGTATGGGGCCGTAGGAA	GATCACGGGCCTTGTACACT
<i>CDK6</i>	TGCAGGGAAAGAAAAGTGCAATG	CCTCGAAGCGAAGTCCCTCAA
<i>CDK1</i>	GGATGTGCTTATGCAGGATCC	CATGTACTGACCAGGAGGGATAG
<i>Ki67</i>	AGCACGTCGTGTCTCAAGAT	AGTCATCTGCGTACTGTCT
<i>Cyclin D1</i>	CAATGACCCCGCACGATTTTC	AAGTTGTTGGGGCTCCTCAG
<i>P15</i>	CGAGGAGAACAAGGGCATGC	CTGTGCGACCTTCTCCACTAG
<i>P16</i>	GCTAAGTGCTCGGAGTTAATAGCA	GGACCGCGGTATCTTTCCA
<i>P18</i>	GGGGACCTAGAGCAACTTACT	GGCAATCTCGGGATTTCACAG
<i>P19</i>	GTGCATCCCAGCCCTCAAC	TGGCACCTTGCTTCAGCAGCTC
<i>P21</i>	TGCCGAAGTCAGTTCCTTGT	CATTAGCGCATCACAGTCGC
<i>P27</i>	CAGCTTGCCCGAGTTCTACT	CGTGTCTCAGAGTTAGCCG
<i>P57</i>	TTTAGAGCCCAAAGAGCCCC	AACAAAACCGAACGCTGCTC
<i>BCL2</i>	CTTTGAGTTCGGTGGGGTCA	GAAATCAAACAGAGGCCGCA
<i>CASPASE 8</i>	CATCCAGTCACTTTGCCAGA	GCATCTGTTCCCCATGTTT
<i>CASPASE 9</i>	GGTCACGGCTTTGATGGAGAT	CCACCTCAAAGCCATGGTCTT

## Western Blotting

Protein was extracted from the cells with RIPA lysis buffer containing protease inhibitors (Bio-Rad Laboratories, Hercules, CA, USA). Total protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (25 µg) from lysates were separated on 4–20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) and transferred on to a polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked in 5% non-fat milk at room temperature for 1 h and incubated overnight at 4 °C with anti-CDK1(1:500) and anti-GAPDH (1:1000) primary antibodies purchased from Abcam (Cambridge, MA, USA) and Cell Signaling Technology, (Beverly, MA, USA), respectively. On the following day, membranes were washed three times each for 5 min in 1% TBST, followed by incubation with a goat anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody (Abcam) in 5% BSA. The protein bands on the membrane were visualized using enhanced chemiluminescence solutions A and B mix for 3 min (PerkinElmer, Inc., Waltham, MA). The density of the bands was

assessed by the ImageJ software, and values were normalized to the densitometric values of GAPDH. Western blots were run twice with duplicate samples.

## Endometriotic Lesions of Mice

C57BL/6J wild-type and ubiquitin-GFP mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. All animal experiments were conducted in accordance under an approved protocol from the Yale University Institutional Animal Care and Use Committee (IACUC). Briefly, wild-type mice (6 weeks) received 125 mg/kg of 5-fluorouracil (5-FU) by i.p. on 6 and 1 days (day 6 and 1) before bone marrow transplantation (BMT, day 0). In addition, stem cell factor (SCF, 50 mg/kg) was injected twice before BMT, as previously described [21], and fresh BM cell transplantation was performed as described previously [5].

Endometriosis in BM transplanted mice ( $N=12$ ) was surgically induced under aseptic conditions and anesthesia using a modified method previously described [6, 22]. Surgery was

performed 30 days following BMT. Uterine horns were removed from wild-type female donor mice at diestrus (low estrogen stage), opened longitudinally, cut into fragments of 3-mm size, and transplanted onto the peritoneal wall of recipient mice. Three fragments were systematically transplanted and sutured to the peritoneal wall of each mouse. Control mice were subjected to sham surgery, where, in place of uterine tissue, peritoneal tissue from the ventral midline was used.

Tissue from endometriotic lesions was fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer tissue sections were mounted on slides, steamed in sodium citrate at pH 6 for 10 min for antigen retrieval, and blocked using 10% donkey serum (Vector Laboratories, Burlingame, CA, USA). Sections on slides were incubated at 4 °C overnight with primary antibodies, rabbit anti-Cdk1 (catalog #PA5–82086; 1:200; Invitrogen, CA, USA) and goat anti-GFP (catalog #ab6673; 1:400, Abcam, Cambridge, MA, USA). Rabbit anti-CDK1 specifically targets human, mouse, and rat species. Secondary antibodies used were Alexa Fluor 568-conjugated donkey anti-goat (catalog #A11057, Invitrogen) and Alexa Fluor 488-conjugated donkey anti-rabbit (catalog #A21206, Invitrogen). Sections were mounted under coverslips using Vectashield fluorescent mounting media with 46-diamidino-2-phenylindole (DAPI) (catalog #H-1200; Vector Laboratories.). Visualization of the slides was performed using a laser scanning confocal microscope (LSM 710; Zeiss) and the ZEN software (Carl Zeiss).

### Statistical Analysis

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA). An unpaired Student's *t* test for percentage of labeled cells (PLC) and RT-PCR data were used to determine statistical significance. Data are expressed as means ± standard error mean (SEM).

## Results

### BMDCs Increase *CDK1* Expression in Endometriosis Cells

In order to understand the effect of BMDCs on endometriosis gene expression, we determined the expression of several genes of interest in immortalized human endometrial cells (HESC) as well as an endometriosis stromal cell line with and without BMDC co-culture. Gene expression was determined in these cells after 24 h (day 1), 72 h (day 3), and 144 h (day 6). As shown in Table 1, we screened 26 genes related to the NFκB, MAPK, and mTOR signaling pathways, the cell cycle, and apoptosis. Among the 26 genes, a single one, *CDK1*, was significantly downregulated in endometrial cells while upregulated in endometriosis cells. As shown in

Fig. 1A, *CDK1* mRNA levels significantly decreased on days 3 and 6 in HESC cells co-cultured with BMDCs compared with the mRNA levels in HESC cells alone. In contrast, we found the opposite in endometriosis stromal cells when co-cultured with BMDCs. In endometriosis cells that were co-cultured with BMDCs, *CDK1* mRNA levels were reduced on days 1 and 3 but interestingly increased significantly on day 6 compared with the mRNA levels in endometriosis cells alone (Fig. 1A). Further, we observed that Ki67 (cell proliferation marker) mRNA levels were increased significantly on day 6 in endometriosis cells co-cultured with BMDCs compared with control endometrial cells (HESCs) as shown in Fig. 1B.

### Effect of BMDCs on *CDK1* Expression in Primary Endometrial Stromal Cells

We confirmed the changes in *CDK1* expression observed in cell lines, in eutopic endometrial primary stromal cells that were cultured from subjects with and without endometriosis. Primary stromal cells from control subjects showed a similar pattern of *CDK1* mRNA levels as observed in HESC controls on day 1, day 3, and day 6 when co-cultured with BMDCs (Fig. 2A). BMDCs have a similar effect on *CDK1* protein levels as shown in Fig. 2C. *CDK1* mRNA and protein levels are not significant on day 1 in primary cells in accordance with the mRNA and protein levels in HESC cells on day 1 (Fig. 2A and C). Primary stromal cells from endometriosis subjects had significantly upregulated *CDK1* mRNA expression as well as protein levels on all other days (3 and 6) when co-cultured with BMDCs, as shown in Fig. 2B and D respectively.

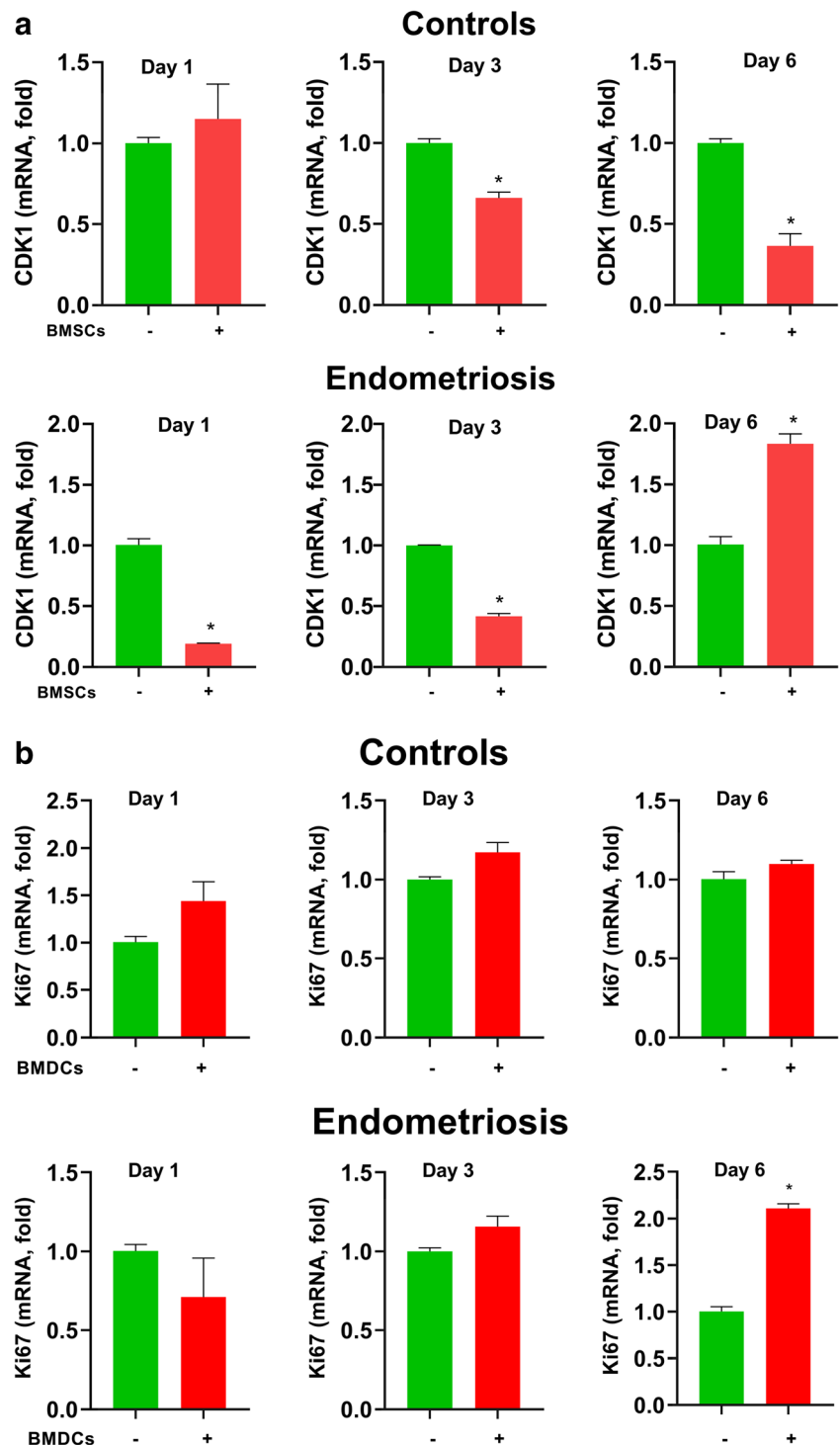
### BMDCs Increased Eutopic Stromal Cell Proliferation

In order to determine the effect of BMDCs on cell proliferation in eutopic endometrial primary cells, we co-cultured primary cells from eutopic endometrial tissue obtained from subjects with and without endometriosis with BMDCs. Cell proliferation was determined by quantifying the number of cells using bromophenol blue staining for live cells. As shown in Fig. 3, when primary cells from control subjects were co-cultured with BMDCs, the number of cells was significantly decreased compared with the primary cells alone, while the number of primary cells from endometriosis subjects was significantly increased by co-culture with BMDCs.

### BMDCs Increase *Cdk1* Expression in Endometriotic Lesions In Vivo

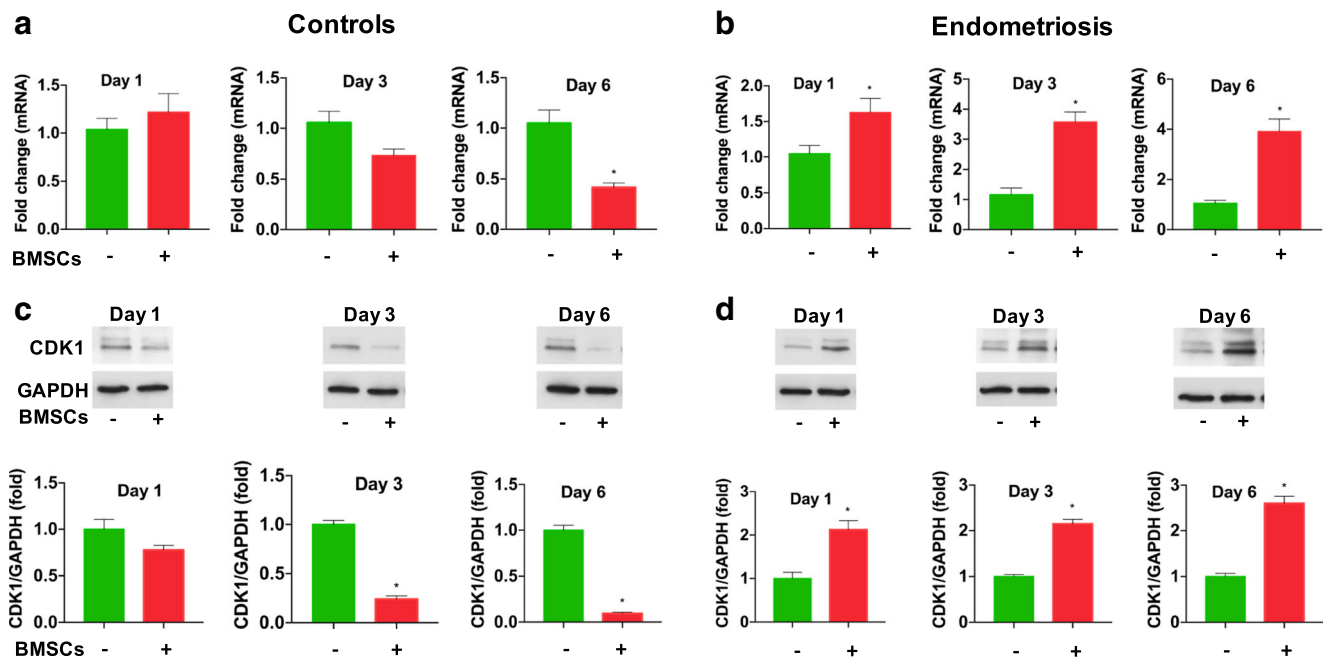
To characterize BMDCs' engraftment in endometriosis in vivo, we used a 5-FU-based submyeloablation mouse bone marrow transplantation model according to our published protocol [21]. Sections from endometriotic lesions from

**Fig. 1** **A** BMDCs increased CDK1 expression in an endometriosis cell line. CDK1 mRNA levels were significantly increased in endometriosis cells on day 6 compared with day 1 and 3 and also compared with the control endometrial stromal cells (HESC). In controls, CDK1 levels were reduced significantly on days 3 and 6 compared with day 1. Each bar represents the mean  $\pm$  SEM for data from three individual experiments and each experiment was performed in duplicate. Asterisk denotes statistical significance ( $p < 0.05$ ) between cells without and with BMDCs. **B** BMDCs increased Ki67 expression in an endometriosis cell line. Ki67 mRNA levels were significantly increased in endometriosis cells on day 6 compared with day 1 and 3 and also compared with control endometrial stromal cells (HESC). Each bar represents the mean  $\pm$  SEM for data from three individual experiments and each experiment was performed in duplicate. Asterisk denotes statistical significance ( $p < 0.05$ ) between cells without and with BMDCs



endometriosis mice and uterus from sham mice that received GFP expressing BM cell transplantation were stained with anti-GFP (green) and anti-Cdk1 (red). Gross morphology of the endometriotic lesions induced in our mouse model is shown in supplemental figure 1. The stained endometriosis sections were visualized for green and red labeled cells under

confocal microscopy. Immunofluorescence results showed that the number of Cdk1 expressing stromal cells (red) nearby engrafted GFP-positive cells (green) were increased (Fig. 4A) and significantly higher ( $p < 0.05$ ) as shown in Fig. 4B in endometriotic lesions from mice with endometriosis compared with the sham group mice.



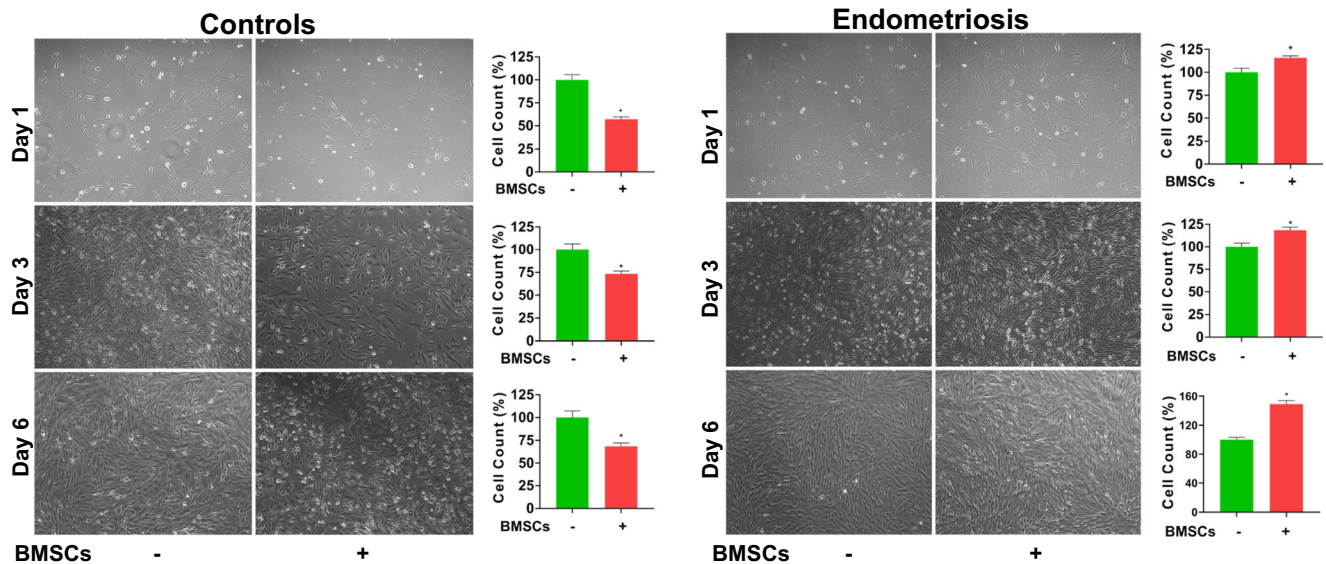
**Fig. 2** BMDCs increased CDK1 expression in primary stromal cells from endometriosis. After co-culture with BMDCs, CDK1 mRNA levels were significantly decreased in primary stromal cells from normal subjects (A) while increased in stromal cells from subjects with endometriosis (B). C and D show the CDK1 protein levels in normal subjects and subjects with

endometriosis, respectively. Quantification of protein bands were measured by densitometry and normalized to respective GAPDH. Bar graphs represent the average values of three individual experiments performed in duplicate. Asterisk denotes statistical significance ( $p < 0.05$ ) between cells without and with BMDCs

**Discussion**

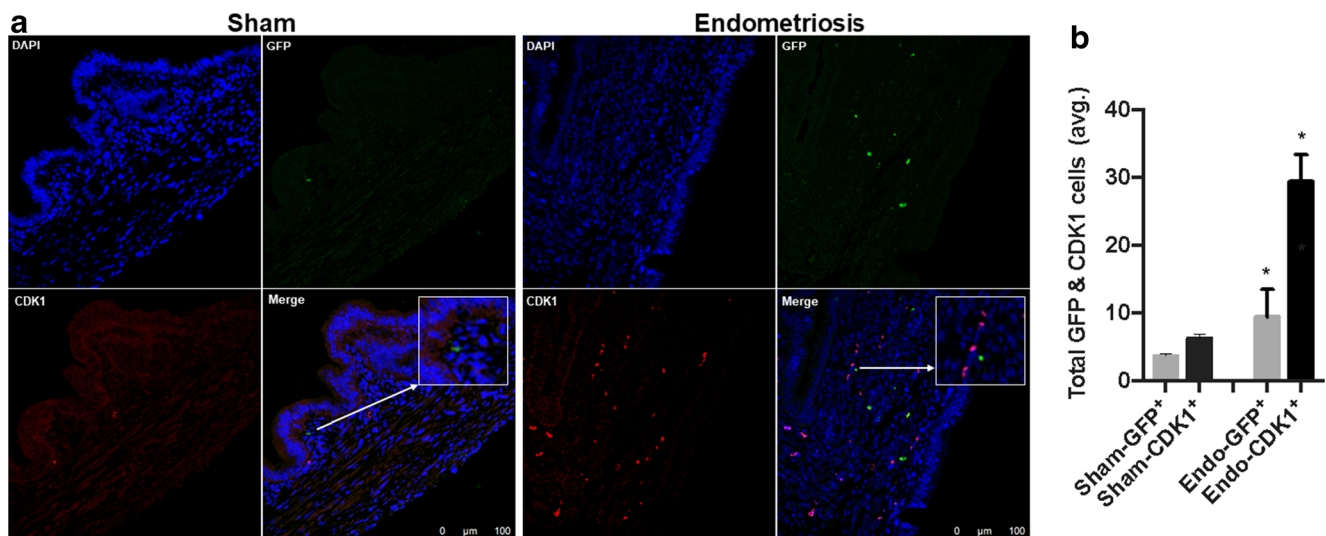
In this study, we demonstrated that BMSCs increased stromal cell proliferation and upregulated *CDK1* expression in endometriosis. In cell lines and primary cells obtained from eutopic endometrial

tissue of subjects with endometriosis, cell proliferation and CDK1 expression were increased by co-culture with BMDCs. We further showed in vivo that engraftment of BMDCs into endometriotic lesions (ectopic) increased the number of endometrial stromal cells that express *CDK1*.



**Fig. 3** Increased cell proliferation in endometriosis induced by BMDCs: Representative microscopic images showing cell growth on days 1, 3, and 6 from primary stromal cell cultures from normal subjects (controls) and subjects with endometriosis (endometriosis). Primary stromal cells from normal endometrium co-cultured with BMSCs showed decreased and cell count significantly on all days, Stromal cells from endometriosis

subjects demonstrated increased numbers of cells on all days. Each bar represents the mean  $\pm$  SEM for data from three individual experiments and each experiment was performed in duplicate. Asterisk denotes statistical significance ( $p < 0.05$ ) primary stromal cells vs those co-cultured with BMSCs



**Fig. 4** **A** Representative images of Cdk1 expressing cells adjacent to BMDCs in endometriosis lesions as demonstrated by immunofluorescence. Tissue sections from uterus of sham mice and lesions from mice with endometriosis were immune-stained by anti-GFP, anti-Cdk1, and DAPI for nuclear stain. Endometriosis lesions

(right) showed more Cdk1 expressing cells (red) adjacent to engrafted BMDCs (green) comparing to tissue from sham (left) surgery treated mice. **B** shows that GFP positive and CDK1 protein expressing cells are significantly higher ( $p < 0.05$ ) in endometriosis compared to sham mice. Scale bar: 100  $\mu$ m

Endometriosis is a gynecological disorder common among reproductive-aged women characterized by the growth of endometriotic lesions in pelvic region, causing pain and infertility. Lesion growth is due to active cell proliferation and is essential to the development of endometriosis. Until now, the most widely accepted theory for pathogenesis of endometriosis is Sampson theory, in which the exfoliated menstrual endometrial cells attach to the peritoneal membrane, and subsequent cell proliferation and invasion into the underlying tissue results in endometriotic lesions [23]. Retrograde menstruation is a physiologic process that takes place almost in all menstruation cycles of reproductive-aged women. Accumulated evidence suggests that endometrial cells from both eutopic and ectopic endometrium in endometriosis exhibit excessive proliferation which is essential for the progression of endometriosis [24–27]. miRNAs, histone deacetylation, and several signaling pathways play a role in the regulation of endometrial cell proliferation and apoptosis in disease [28–31]. Our previous studies [3, 5, 6, 32] show that BMDCs are incorporated into endometriotic lesions and play a role in the development and progression of peritoneal and extra-peritoneal disease. The importance of bone marrow-derived stem cells (BMDCs) in human endometrial biology has been previously established; however, the mechanisms by which they influence endometriosis growth have not previously been characterized [3, 33–35].

Cyclin-dependent kinase 1 also known as *CDK1* or cell division cycle protein 2 homolog is a highly conserved protein that functions as a serine/threonine kinase and is a key mediator of cell cycle regulation [36]. Among all the *CDK* family members, only *CDK1* is critical for cell cycle regulation and

cell division [37]. *CDK1* expression is upregulated in several malignancies; increased *CDK1* expression or its activation has been documented in oral squamous cell carcinoma [38], ovarian cancer [39], colorectal cancer [40], and prostate cancer [41]. *CDK1* participates in G2/M conversion by forming a complex with cyclin B. Silencing of *CDK1* increases G2/M arrest in the cell cycle and can lead to the production of polyploid cells [42]. Tang L. et.al demonstrated that cyclin B1 play important roles in the pathogenesis of endometriosis by mediating ectopic endometrial cell proliferation [43]. Therefore, it is evident that *CDK1*/cyclin B1 pathway plays an important role in the development of endometriosis. Our results from both in vitro and in vivo experiments are in agreement with the above reports that *CDK1* expression is upregulated in endometriosis; further, we show that in stromal cells cultured from endometriotic lesions, *CDK1* is upregulated by BMDCs.

In vivo, we established a mouse model of endometriosis with BMDCs engraftment. Some CDK1+ cells were associated with GFP + BMDCs although there are areas of CDK1+ cells without BMDCs. This implies the existence of additional mechanism regulating stromal cell proliferation. As expected, BMDCs are not the sole regulators of endometriosis growth; however, we have clearly identified BMDCs and a substantial factor in inducing endometriosis cell proliferation.

The experiments in vitro and in vivo both suggested that BMDCs can upregulate CDK1 expression and proliferation of endometriosis stromal cells compared with normal endometrial stromal cells. Future studies will define the signaling molecules and the pathways by which BMDCs regulate the *CDK1* expression and endometriosis cell proliferation while not stimulating the growth of normal endometrial cells. There are

limitations to this study; eutopic ESCs were used in vitro studies. Primary ectopic endometrial stromal cells (ESCs) are limited in number, difficult to grow and passage, and may respond differently. Nevertheless, we have identified important distinctions in the effect of BMDCs in endometriosis. Taken together, we conclude that BMDCs increased the proliferation of primary stromal cells from endometriosis patients as demonstrated by the activation of *CDK1*. The effect of BMDCs on endogenous endometrial cell proliferation may explain the profound effect of BMDCs despite the relatively small number of BMDCs engrafted. This study may help in developing novel therapeutic strategies for controlling cell proliferation in endometriosis.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethics Approval** Ethics involved in experiments carried out using cell lines approved by the Yale University.

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