Spatial and Temporal Characterization of Endometrial Mesenchymal Stem-like Cells Activity during the Menstrual Cycle

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SHORT RUNNING TITLE:

Endometrial mesenchymal stem-like cells (eMSCs) activity across the menstrual cycle.

ABSTRACT

The human endometrium is a highly dynamic tissue with the ability to cyclically regenerate during the reproductive life. Endometrial mesenchymal stem-like cells (eMSCs) located throughout the endometrium have shown to functionally contribute to endometrial regeneration. In this study we examine whether the menstrual cycle stage and the location in the endometrial bilayer (superficial and basalis portions of the endometrium) has an effect on stem cell activities of eMSCs ($CD140b^{+}CD146^{+}$ cells). Here we show the percentage and clonogenic ability of eMSCs were constant in the various stages of the menstrual cycle (menstrual, proliferative and secretory). However, eMSCs from the menstrual endometrium underwent significantly more round of self-renewal and enabled a greater total cell output than those from the secretory phase. Significantly more eMSCs were detected in the deeper portion of the endometrium compared to the superficial layer but their clonogenic and self-renewal activities remain similar. Our findings suggest that eMSCs are activated in the menstrual phase for the cyclical regeneration of the endometrium.

Keywords: Adult stem cells, endometrial repair, menstrual cycle, endometrial stem cells.

INTRODUCTION

In response to the cyclical changes in ovarian sex steroids, estrogen and progesterone, the human endometrium displays cyclic and rapid changes in proliferation and differentiation. The inner mucosal lining of the uterus is composed of the lumen epithelium supported by stroma and mature glands. The endometrium is comprised of two layers: the basalis which persists into the next cycle to give rise to a new functionalis that is shed during menses [1]. Approximately 400 cycles of shedding and renewing take place during the lifespan of a woman's reproductive years. The menstrual cycle is divided into 3 phases: proliferative, secretory and menstrual [2, 3]. Endometrial regeneration commences at the beginning of menstrual phase and the growth continues into the proliferative phase. In the estrogen dominating proliferative phase, 5-7 mm of endometrial tissue is generated within 10 days [3]. The secretory phase is characterized by glandular secretion and stromal maturation in response to progesterone from the corpus luteum [4]. In the late secretory phase, luteolysis of the corupus luteum causes withdrawal of estrogen and progesterone that triggers breakdown of the functionalis and shedding of a substantial amount of tissue [5].

In recent years, a distinct adult stem cell population known as endometrial mesenchymal stem-like cells (eMSCs) have been shown to be responsible for endometrial remodeling [6]. Endometrial stromal cells co-expressing two surface markers: CD140b and CD146 are enriched with eMSCs and are localized to perivascular regions in the functionalis and basalis layers [7]. These cells are clonogenic, have broad differentiation capacity, display properties and phenotype similar to other mesenchymal stem cells [8-10]. Although eMSCs share a core genetic profile with bone-marrow mesenchymal stem cells in stemness, several genes mainly related to endometrial functions such as, vasculogenesis, angiogenesis, inflammation,

immunomodulation and cell communication are specifically upregulated in eMSCs [11].

EMSC expressing CD140b and CD146 have been identified in proliferative and secretory endometria and can be isolated from hysterectomy or endometrial biopsy tissues [7, 8, 11]. However, the characterization of eMSCs at menstruation is limited and no studies have compared the properties of eMSCs in the different layers of the endometrium (superficial vs. deep portion of the endometrium). We hypothesize that 1) more eMSCs reside in the basalis but their stem cells activities will be similar between the endometrial layers. 2) These eMSCs will exhibit unique properties at menstruation for the repair and regeneration of the endometrium. Therefore, this study aims to investigate the changes of eMSCs during the menstrual phases (proliferative, secretory and menstrual) and in the endometrial layers.

MATERIAL AND METHODS

Human tissues

Full thickness endometrial tissue was collected from 30 ovulating women, 35- to 50-years old undergoing total abdominal hysterectomy (TAH) for benign non-endometrial pathologies (supplementary data TableS1). They had not taken hormonal therapy for three months before surgery. The phase of the menstrual cycle was categorized into proliferative (n = 14; range: 40 to 48 years old; median: 45yr; mean: 44 yr) and secretory (n = 16, range: 35 to 50 years old; median: 44yr; mean: 42 yr) by experienced histopathologists based on hematoxylin-eosin-stained endometrial sections. Menstrual endometrial tissues were obtained from 11 ovulating women aged from 31- to 40- years old attending the infertility clinic on day 2-3 of their menstrual cycle (median: 38yr; mean: 36 yr, supplementary data TableS2). Ethic approval was

obtained from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Written consents were signed by recruited subjects after detailed counseling prior to participation of the study. All the samples were processed within 24 hours after collection.

Single cell suspensions of endometrial epithelial and stromal cells

For TAH samples (Fig 1A), the endometrial layer was scraped off from the underlying myometrium, minced and digested with PBS containing collagenase type III (0.3 mg/ml, Worthington Biochemical Corporation, Freehold, NJ, USA) and deoxyribonuclease type I (40 μg/ml, Worthington Biochemical Corporation) for one hour at 37^{o}C, as described previously [12]. To separate the superficial layer, a gentle scrape on top of the endometrial layer most distal from the myometrium was performed. The deeper portion of the endometrium was defined as the remnant tissue 1mm-area from the endo-myometrial junction (Fig 1B) [13]. Menstrual samples were obtained on day 2 of the menstrual cycle by aspiration. They were digested as described above. In brief, red blood cells were removed using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density-gradient centrifugation. Leukocytes were eliminated using anti-CD45 antibody-coated Dynabeads (Invitrogen, Waltham, MA, USA). Epithelial cells were removed from the stromal cells by using anti-CD326 (EpCAM) antibody-coated microbeads (Miltenyi Biotec Inc., San Diego, CA, USA). The freshly purified stromal cells (6000 – 8000 cells/cm^2) were plated onto 100 mm dishes (BD Biosciences, San Jose, CA, USA) coated with fibronectin (1 mg/ml, Invitrogen) and cultured in growth medium containing 10% FBS (Invitrogen), 1% antibiotics (Invitrogen) and 1% L-glutamine (Invitrogen) in DMEM/F-12 (Sigma-

Aldrich, St Louis, MA, USA). Stromal cells were expand in culture for 7-14 days in a humidified carbon dioxide incubator at 37°C. Medium was changed every 7 days until it reach 80% confluence.

Flow cytometry

The expression of eMSC markers (co-expression of CD140b and CD146) on freshly purified endometrial cells were analyzed using multicolour flow cytometry (Supplementary data Figure S1). Endometrial cells were labeled with phycoerythrin (PE)-conjugated antibody against platelet-derived growth factor receptor beta (PDGFRβ) (CD140b, 2.5 µg/ml, PR7212 clone, mouse IgG_1, R&D Systems, Minneapolis, MN, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD146 antibody (1 mg/ml, OJ79c clone, mouse IgG_1, Thermo Fisher Scientific, Waltham, MA, USA) or isotype matched controls. The cells were then labeled with allophycocyanin (APC)-conjugated anti-CD45 antibody (10 µg/ml, Thermo Fisher Scientific) before resuspension in 0.1% BSA/PBS for flow cytometric analysis using BD Fortressa (BD Biosciences, San Jose, CA, USA) in the University of Hong Kong Faculty Core Facility. Flow cytometry data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

Magnetic bead selection for endometrial mesenchymal stem-like cells

EMSCs isolated by sequential beading with magnetic beads coated with anti-CD140b and anti-CD146 antibodies were used for various functional assessments. In brief, cultured stromal cells were trypsinized, re-suspended in 0.5% BSA/PBS and incubated with PE-conjugated anti-CD140b antibody (10 µl/10^6 cells) for 45 min at 4°C. The cells were then incubated with anti-mouse IgG1 coated microbeads

(Miltenyi Biotec Inc.) for 15 min at 4°C and the cell suspensions were applied to Miltenyi MS columns with a magnetic field to collect the $CD140b^+$ cells. The stromal $CD140b^+$ population were seeded in fibronectin-coated 100-mm culture dishes (BD Biosciences) containing growth medium and cultured at 37°C in 5% CO_2 for 7 to 10 days to allow detachment of the microbeads during cell expansion. The $CD140b^+$ cells were then trypsinized and incubated with anti-CD146 antibody coated microbeads (Miltenyi Biotec Inc.) for 15 min at 4°C. The $CD140b^+CD146^+$ cells (eMSCs) were obtained after column separation and the phenotype was confirmed by dual immunofluorescence (Supplementary data Fig S2).

Dual Immunofluorescence

Double immunofluorescent staining was performed to evaluate the phenotypic of $CD140b^+CD146^+$ cells. Some of the eMSCs were plated at clonal density of 10-30 cells/cm^2 on fibronectin coated 12-well plates and culture for 15 days. Cells were fixed with 4% paraformaldehyde for 20 min. Permeabilization was performed using 0.1% Triton-X 100 for 10 min and blocked with 2% BSA for 30 min. Cells were incubated with primary antibodies; anti-human CD140b (R&D Systems) and anti-human CD146 (Abcam, Cambridge, UK) antibodies at 4°C overnight. The following day, cells were incubated with the secondary antibodies; donkey anti-mouse antibodies conjugated with Alexa Fluor 564 (Invitrogen) and goat anti-rabbit antibodies conjugated with Alexa Fluor 488 (Invitrogen). The cell nuclei were detected by DAPI (Thermo Scientific). All washing steps were performed with PBS and conducted at room temperature unless specified. Images were captured using a Carl Zeiss LSM inverted confocal microscope and a Zeiss LSM Zen 2010 software (Carl Zeiss, Munich, Germany) at the University Of Hong Kong Core Facility.

Colony initiating cell assay

For assessment of colony-forming ability, eMSCs were plated in triplicates at a clonal density of 10-30 cells/cm^2 on fibronectin coated 100 mm dishes or 6-well plates and cultured for 15 days as previously described [14]. Cells were cultured in growth medium, incubated at 37^oC in 5% CO_2 and medium changed every seven days. Regular monitoring of the cells was performed under an inverted microscope (Nikon) to ensure colonies derived from single cells. Colony forming units (CFUs) were stained with toluidine blue (Sigma-Aldrich) on day 15. Large CFUs were defined as colonies with ≥4,000 cells and small CFUs were those with ≤4,000 cells. The colony forming ability was determined by the number of CFUs formed divided by the number of cells seeded, multiplied by 100.

In vitro serial cloning

Individual large stromal CFUs from passage 1 (P1) were trypsinized using cloning rings (Sigma-Aldrich) to determine the self-renewal capacity of the cells in different stages of the menstrual cycle. Two individual large CFUs per patient samples obtained from the clonogenic assays were used for serial cloning. The cell number of each CFUs was determined and the cells were re-seeded at a density of 35 cells/cm^2 [14]. The process continued until the cells could no longer form CFUs. Similar procedure was carried out for the difference between large and small CFUs.

Total cell output assay

The proliferative potential of CFUs derived eMSC in different menstrual phases was examined by separately pooling 6 large CFUs, and expanding them in culture with a seeding density of 2,000 cells/cm^2 [14]. The growth of the cells was closely monitored and passaged every 4 to 7 days when the culture was 80% confluent. The process continued until senescence of the cells. The cumulative population is the sum of output cell number at each passage.

Statistical analysis

Data were analyzed using GraphPad PRISM software (version 5.00; GraphPad Software Inc., San Diego, CA, USA). Distribution normality was examined using the D'Agostino and Pearson test. Mann-Whitney test was performed to determine statistical significance between two groups. For comparing three groups, the data were analyzed using Kruskal-Wallis test, follow by Dunn's post-test. Wilcoxon matched pairs test was performed to compare proportion of eMSCs in the superficial and deep portion of the endometrium. Data are presented as mean ± SEM. Results were considered statistically significant when $P < 0.05$.

RESULTS

Proportion of CD140b$^+$CD146$^+$ cells in different menstrual phases and endometrial layers

The expression for the eMSC markers (CD140b$^+$CD146$^+$ cells) in freshly isolated endometrial cells was analyzed by flow cytometry to determine the percentage of eMSCs in the total endometrial population. The proportion of eMSCs was similar

among the three phases of the menstrual cycle (Fig 2A). The percentage of cells co-expressing $CD140b^{+}CD146^{+}$ cells in the menstrual phase was 3.83 ± 1.31% (n = 7), in the proliferative phase was 1.60 ± 0.52% (n = 10) and in the secretory phase endometrium was 2.41 ± 1.71% (n = 6).

Assessment of eMSCs in different endometrial layers by flow cytometry showed a significantly higher percentage of $CD140b^{+}CD146^{+}$ cells in the deep portion of the endometrium than in the superficial layer ($n = 4$, $P < 0.05$, Fig 2B).

We also examine the expression of $CD140b^{+}CD146^{+}$ cells in large and small CFUs by dual immunofluorescence. In general, more co-expressing cells were detected within large CFUs compare to small CFUs (supplementary data S3A, S3B).

The clonogenic and self-renewal activity of eMSCs in different menstrual phases and endometrial layers

To examine the effect of menstrual cycling on the clonogenic activity of eMSCs, cloning efficiencies were compared among samples obtained in the menstrual, proliferative and secretory phases using magnetic bead selection. The cloning efficiencies were determined from the number of large and small CFUs relative to the total number of seeded cells. Fig 3A shows the clonogenic activity of eMSCs in the menstrual phase that formed large CFUs (0.79 ± 0.35%, n = 9) was similar to that in the proliferative phase (0.60 ± 0.21%, n = 9) and the secretory phase (0.44 ± 0.15%, n = 12). There was also no difference in the cloning efficiencies for small CFUs (menstruating, 3.62 ± 1.50%, n = 9; proliferative, 2.25 ± 0.90%, n = 9; secretory, 4.02 ± 0.86%, n = 12) (Fig 3B).

The self-renewal ability of large CFUs in different menstrual phases was assessed using a serial cloning strategy (Fig 3C). Clonally derived eMSCs in menstruating endometrium underwent significantly more rounds of self-renewal (5.25 ± 0.48, n = 4) than those in the secretory phase (3.33 ± 0.24, n = 9, P <0.05) but not those in proliferative phase (3.75 ± 0.25, n = 4, P = 0.06).

Large CFUs from eMSCs in the menstrual phase samples were able to serially clone ≥ 5 rounds, where 0.21% of eMSCs were able to initiate CFUs in the second round, 0.80% of in the third round, 0.23% in the fourth and 0.015% in the fifth round (Fig 3D). For the proliferative phase samples, some of the large CFUs could passage ≥ 3 rounds, where 0.45% of the cells could initiate CFUs in the second round and 0.75% in the third round. Similarly, some of the large CFUs in secretory phase could passage ≥ 4 rounds, where 0.26% of the cells initiated CFUs in the second round, 0.01% in the third round and 0.75% in the fourth round.

The percentage of eMSCs that formed large CFUs was 0.63 ± 0.28% (n = 5) in the superficial layer and 2.28 ± 1.02% (n = 5, Fig 3E, P = 0.25) in the deep portion of the endometrium. There was no statistical difference in the formation of small CFUs in superficial (3.51 ± 1.54%) and deep (3.02 ± 1.70%, Fig 3F) layers. The self-renewal activity of large CFUs was similar between the superficial (3.20 ± 0.58%, n = 5) and the deep layer of the endometrium (2.60 ± 0.40%, n = 5, Fig 3G).

In general, eMSCs that formed large CFUs could under more rounds of self-renewal (3.67 ± 0.67, n = 3) than small CFUs (0.33 ± 0.33, n=3) although this was not statistically different (supplementary data S3E, P = 0.07).

Proliferative potential of eMSCs in different menstrual phases

The proliferative potential was determined by serial passaging clonally derived eMSCs until senescence (Fig 4A). The eMSCs from the menstrual phase ($6.41 \pm 3.82 \times 10^{11}$, $n = 5$) and the proliferative phase ($2.02 \pm 1.75 \times 10^{11}$, $n = 6$) produced larger cumulative cell number than those from the secretory phase ($1.10 \pm 0.61 \times 10^{9}$, $n = 4$, $P < 0.05$). The number of cells yielded at each passage is shown in Fig 4B. The total time required for the large CFUs to reach senescence was 106.40 ± 10.29 days for the menstruation phase, 90.17 ± 7.82 days for proliferative phase and 84.25 ± 5.23 days for the secretory phase.

DISCUSSION

In this study, we identified and characterized the putative population of endometrial stromal stem/progenitor cells in three phases of the menstrual cycle and in superficial and deep portions of the human endometrium. We showed that the percentage and clonogenicity of eMSCs ($CD140b^{+}CD146^{+}$ cells) were relatively consistent across the menstrual cycle. An intriguing finding from this study was that eMSCs in the menstrual phase exhibited the greatest self-renewal ability and yielded a higher output of cells. These findings support the notion that endometrial stem cells are responsible for the repair of the endometrium after menstruation. We also showed that more eMSCs resided in the deep endometrial portion but their stem cell properties were similar across the endometrial layers. Hence, eMSCs can readily be obtained from both layers of the endometrium.

Endometrial regeneration starts at menstruation [15, 16]. Therefore, it is logical that the niche at this stage should activate stem cells for endometrial repair. Consistently, our data showed that eMSCs obtained during menstruation exhibit better proliferative

and self-renewal activity than secretory phase. This was not surprising, since in the secretory phase the growth of the endometrium has ceased and decidualization begins [3, 17]. Also, endometrial regeneration continues into the proliferative phase [18], therefore the eMSCs from menstruation and proliferative samples were similar. In adults, homeostasis between the quiescent and activated states of stem cells are important to protect stem cells from losing their potential to self-renewal whilst support ongoing tissue regeneration. The observed phenomenon displayed by eMSCs at menstruation indicates the resident stem cells are responsive to local and systemic signals provided by the niche cells during the tissue breakdown. Although menstruation endometrium displayed a higher percentage of eMSCs, it was not statistically significant. In this study, two methods were used for the collection of endometrial samples. Endometrial aspirations correspond to the superficial parts of the breakdown tissue and hysterectomy samples are full thickness endometrium. Due to the limitation in obtaining hysterectomy samples during menstruation, our current data do not represent the percentage of eMSCs from deep portion of the endometrium. In addition, more active eMSCs detected from menstruation endometrium may be due to the age differences between the sample groups.

Published report examining samples from proliferative and secretory phase demonstrated eMSCs comprises 1.5% of endometrial stromal cells, similar to our current finding [8]. Large CFUs from menstrual phase eMSCs and stromal cells can passage up to five times and display similar proliferative potentials [19]. In this study, a lower clonogenic activity was observed this is likely to be related to the different technical methods used for isolating eMSCs [8].

The endometrium is functionally comprised of a polarized gradient of cells with different phenotypes. The functionalis undergo a striking progression of histological

changes in the menstrual cycle, while the basalis remains relatively unchanged. The existence of putative stem cells in the lower endometrial layer has been well-documented [17, 20, 21]. Here we also found the relative percentage of eMSCs residing in the deeper portion of the endometrium was higher than the superficial layer. Although eMSCs are well distributed throughout the endometrium, no comparative study has been conducted between the endometrial layers [7, 8, 11]. In this study, we demonstrated that the clonogenic activity and self-renewal characteristic of eMSCs in both endometrial layers were similar. These findings support the stem cell theory that eMSCs shed during menstruation may contribute to the pathogenesis of endometriosis [6, 22]. Although the superficial layer contains relatively lower numbers of eMSCs, it has been proposed that endometriotic lesions initiated by endometrial stem/progenitor cells would be more severe than lesions initiated by more differentiated cells [23]. Several functional studies support the presence of endometrial stem cells in ectopic endometriotic lesions [24-26]. A recent gene expression profiling study provided new important information on the molecular phenotype and relationship between eMSCs vs. its progeny stromal fibroblast and their respective roles in endometriosis [27]. The authors demonstrate the differential expression of eMSC lineage genes from normal and endometriotic samples. In addition, eMSCs from women with endometriosis exhibit progesterone resistant and endometrial stromal fibroblast inherit this inability to decidualize *in vitro*. These findings indicate gynecological disorder such as endometriosis can affect eMSCs.

Given that the unique microenvironment at menstruation can active eMSCs, a better understanding between the communications of niche cells to stem/progenitor cells is necessary for future cell-based therapies in tissue engineering applications. This study also confirms that a higher eMSC subpopulation reside in the deeper portion of the

endometrium. Studies understanding how eMSCs respond to shifting physiological cues during the menstrual cycle are needed. In addition, the molecular events governing the transition between quiescence and activation of eMSCs remain to be explored.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no financial or commercial conflict of interest.

FIGURE LEGEND

Figure 1 – Hematoxylin and eosin staining of the human endometrium. (**A**) Full thickness of human endometrium. (**B**) Depth of the scrape showing the remnant deep endometrial portion after separation from the superficial layer. Superficial layer, deep portion of the endometrium and myometrium are separated by dotted lines. The upper superficial layer contains multiple glands supported by loose stroma, while the lower portion consists of branched glands with dense stroma. Scale bar: 100 μM.

Figure 2 – Proportion of eMSCs during the menstrual cycle and in different layers of the endometrium. (**A**) Percentage of $CD140b^{+}CD146^{+}$ cells in menstruation, proliferation and secretory endometrium. (**B**) The relative percentage of $CD140b^{+}CD146^{+}$ cells in the superficial compared to the deep portion of endometrium. Results shown as mean ± SEM. * $P < 0.05$. Numbers in parentheses indicate sample size. EMSCs, endometrial mesenchymal stem-like cells; SEM, standard error of the mean.

Figure 3 – The clonogenic and self-renewal activity of eMSCs in different cycle stage and endometrial layers. Cloning efficiency of human eMSCs from menstruation, proliferative and secretory endometrium for (**A**) large and (**B**) small CFUs. (**C**) Number of serially cloned passages of the large CFUs at different menstrual stage. (**D**) Percentage of large CFUs at each passage of serial cloning. The clonogenicity of human eMSCs from superficial and deep portion of the endometrium for (**E**) large and (**F**) small CFUs. (**G**) Number of serial passage for large CFUs in different endometrial layers. Results shown as mean ± SEM. * $P < 0.05$. Numbers in parentheses indicate sample size. CFUs, colony-forming units; eMSCs, endometrial mesenchymal stem-like cells; SEM, standard error of the mean.

Figure 4 – Long-term Proliferative potential of eMSCs in different menstrual stage. (**A**) The total cumulative output of eMSCs in different menstrual stage. (**B**) Growth curve of eMSCs in menstrual (circle), proliferative (square) and secretory (triangle) phase. Each point represents the cell yield obtained at each passage. Results shown as mean ± SEM. * $P < 0.05$. Numbers in parentheses indicate sample size. EMSCs, endometrial mesenchymal stem-like cells; SEM, standard error of the mean.

Supplementary Figure S1 – Gating strategy for co-expression of CD140b and CD146 in human endometrial cells. (**A**) Freshly isolated human endometrial cells were analyzed by flow cytometry for expression of cell surface markers. Viable cells were selected by their forward scatter (FSC) and side scatter (SSC) profile. (**B**) Cell properties; SSC area (SSC-A) versus SSC height (SSC-H) to gate out cell doublets and aggregates to ensure the signal arises from single cell. Leukocytes were removed by electronic gating using CD45-APC. Single parameter histograms for individual markers CD146-FITC, CD140b-PE. (**C**) Grey line indicates background fluorescence with isotype matched IgG control. Percentage of $CD140b^{+}CD146^{+}$ cells from (**D**) superficial portion, (**E**) deep portion and (**F**) full thickness endometrium. Representative dot-plots for co-staining of CD140b and CD146.

Supplementary Figure S2 – Immunofluorescent staining of CD140b and CD146 on endometrial stromal cells after microbeads isolation. (**A**) The surface marker CD140b (red) on endometrial stromal cells was confirmed by immunofluorescence after $CD140b^{+}$ microbeads selection. (**B**) All cells co-expressed CD140b (red) and CD146 (green) after the second $CD146^{+}$ microbeads isolation. DAPI nuclear stain (blue). Scale bar: 100 μM.

Supplementary Figure S3 – Phenotypic Expression and Serial Activity of large and small eMSC CFUs. Representative images showing the co-expression of CD140b (red) and CD146 (green) cells on **(A)** large and **(B)** small CFUs. DAPI nuclear stain (blue). Images showing the negative control of **(C)** mouse IgG and **(D)** rabbit IgG. **(E)** Number of passage from large and small CFUs (n=3). Results shown as mean ± SEM. Scale bar: 100 μM.

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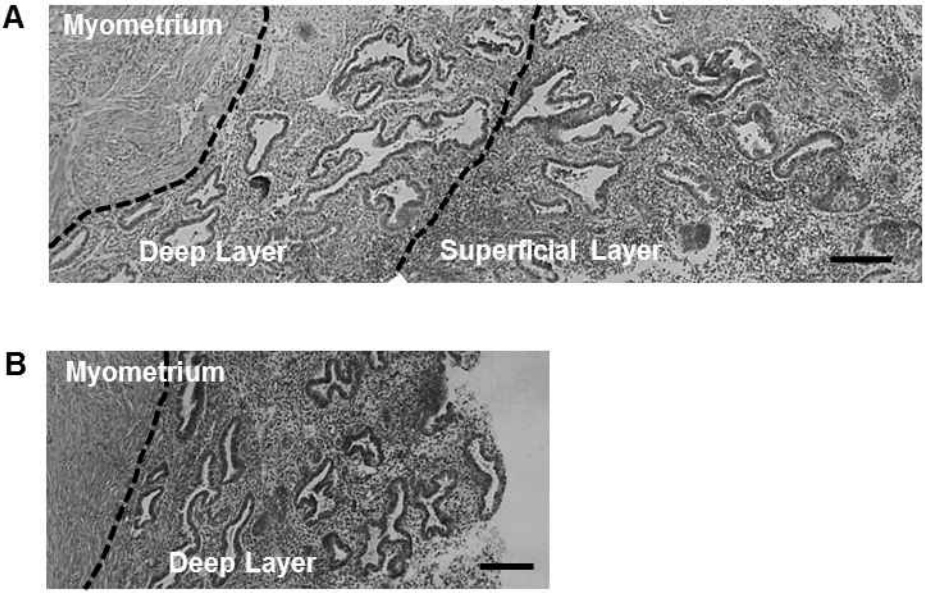
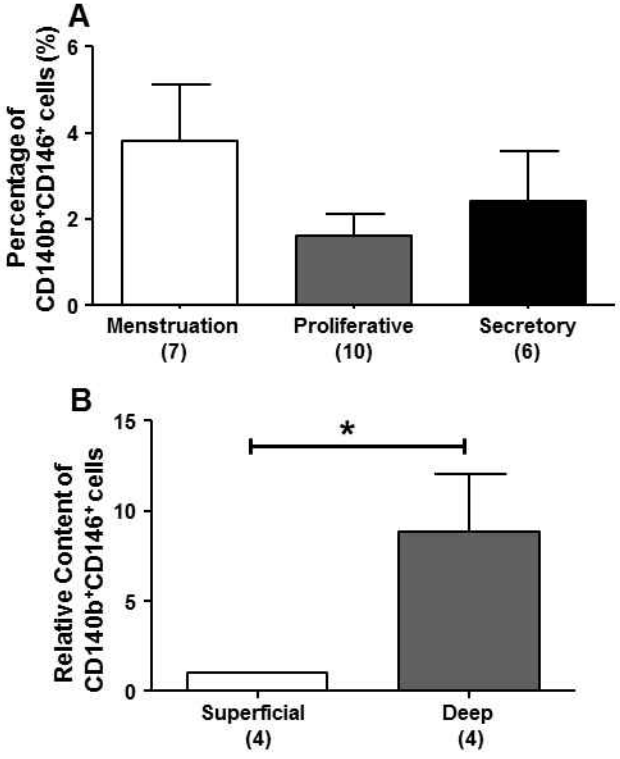
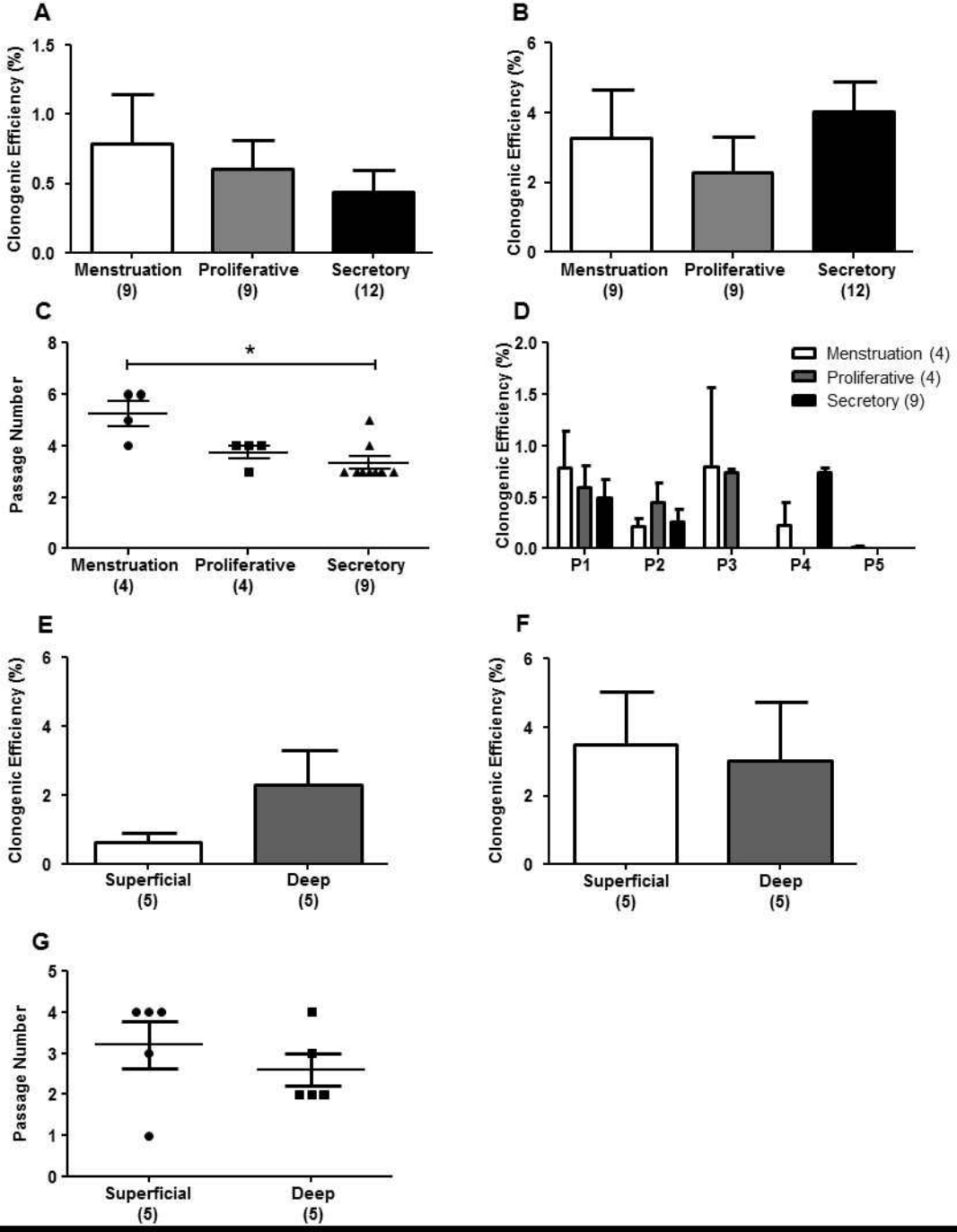
Xu et al –Figure 1



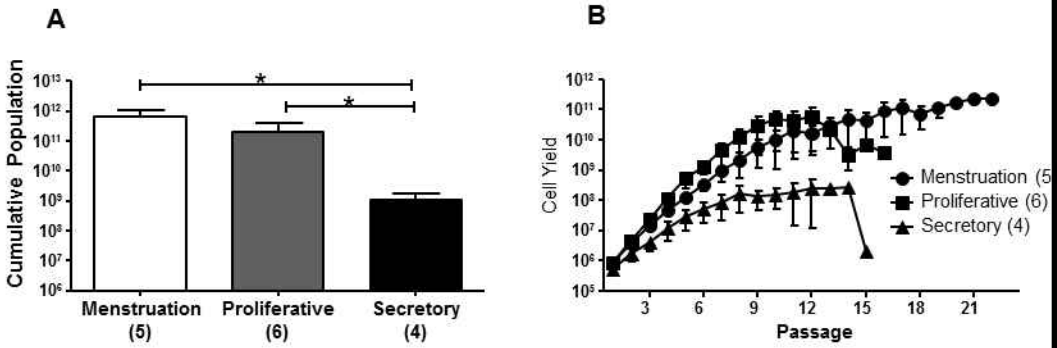
Xu et al – Figure 2



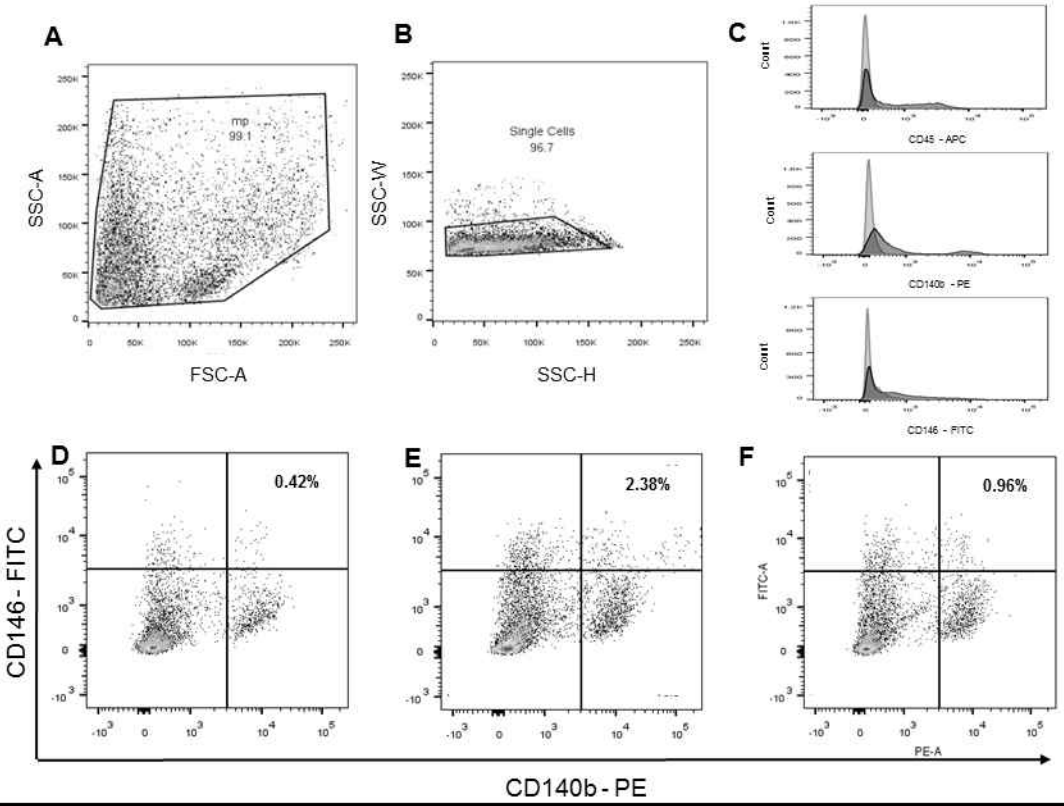
Xu et al – Figure 3



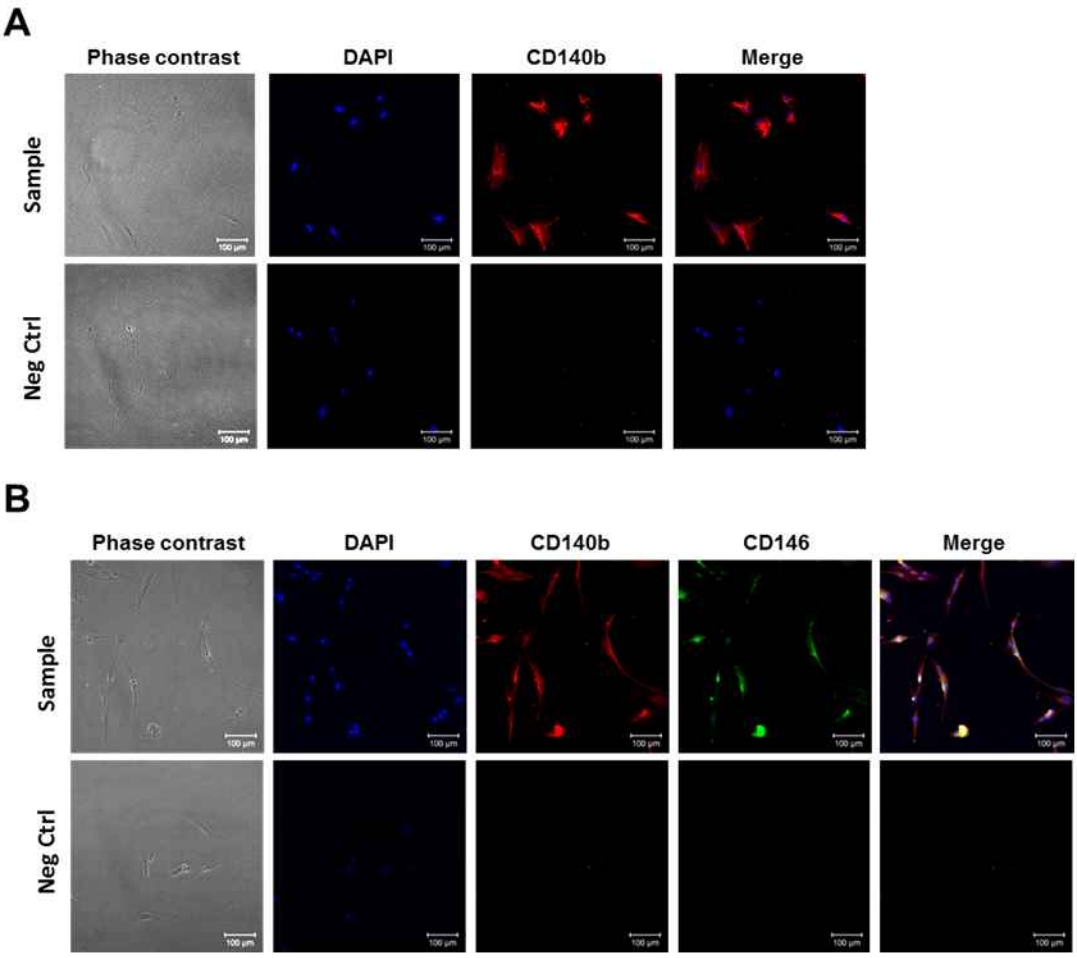
Xu et al – Figure 4



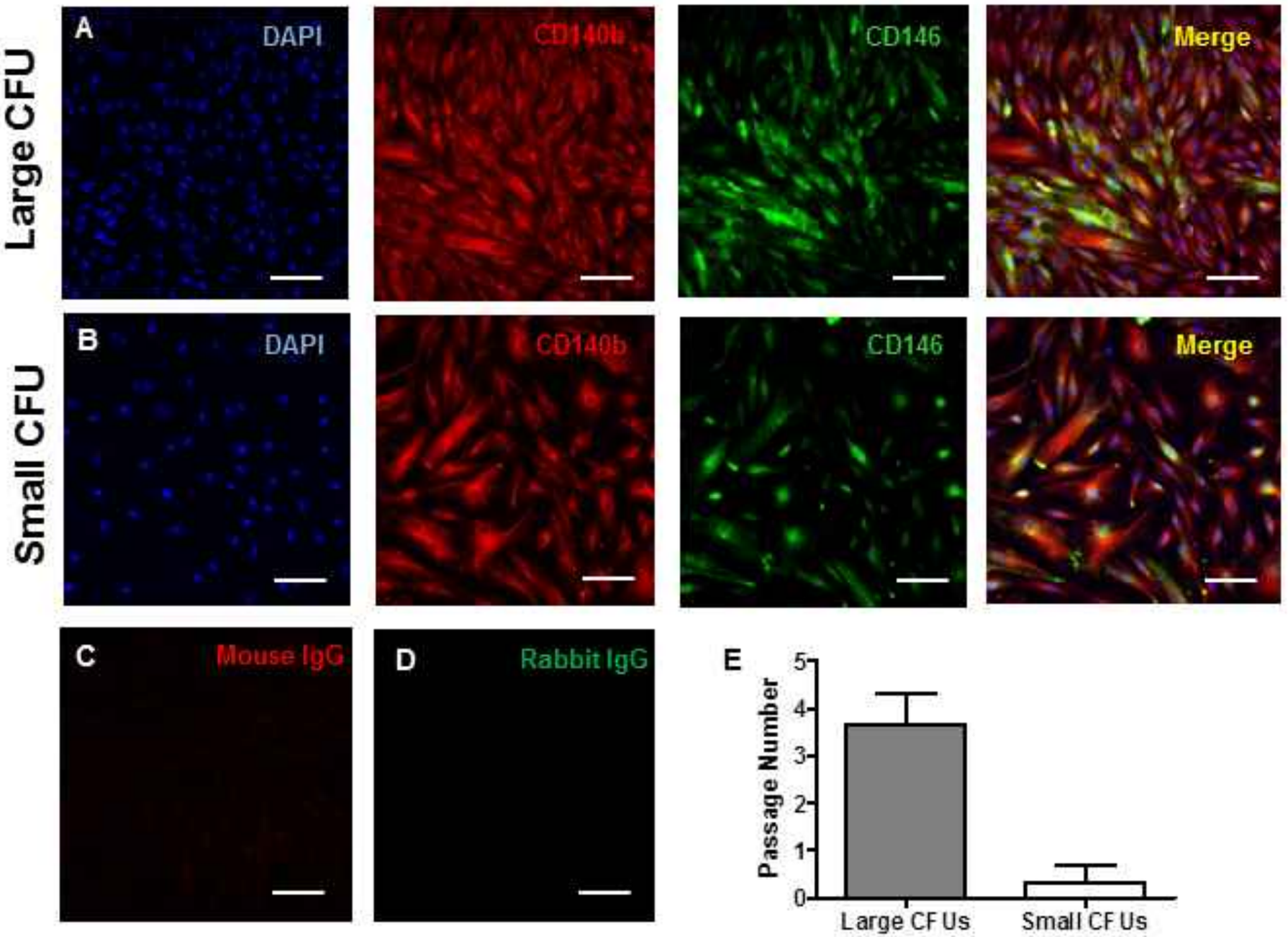
Xu et al – Supplementary Fig S1



Xu et al – Supplementary Fig S2



Xu et al – Supplementary Fig S3



Supplementary Table S1 - Pathological Characteristic of Full Thickness Endometrial Samples

	Age	Menstrual Phase	Pathology
1	47	proliferative	leiomyomas
2	47	proliferative	leiomyomas
3	45	proliferative	leiomyomas
4	44	proliferative	leiomyomas
5	41	proliferative	adenomyosis
6	41	proliferative	adenomyosis + leiomyomas
7	40	proliferative	leiomyomas
8	48	proliferative	leiomyomas
9	43	proliferative	leiomyomas
10	47	proliferative	adenomyosis
11	47	proliferative	leiomyomas
12	45	proliferative	adenomyosis + leiomyomas
13	48	proliferative	leiomyomas
14	41	proliferative	leiomyomas
15	44	secretory	leiomyomas
16	50	secretory	leiomyomas
17	48	secretory	adenomyosis
18	44	secretory	leiomyomas
19	43	secretory	leiomyomas
20	45	secretory	leiomyomas
21	42	secretory	leiomyomas
22	40	secretory	leiomyomas
23	48	secretory	adenomyosis + leiomyomas
24	48	secretory	leiomyomas
25	46	secretory	leiomyomas
26	40	secretory	leiomyomas
27	36	secretory	leiomyomas
28	35	secretory	leiomyomas
29	36	secretory	leiomyomas
30	37	secretory	leiomyomas

Supplementary Table S2 – Age of Menstruation Samples

	Age	Menstrual Phase
1	39	Menstrual
2	31	Menstrual
3	40	Menstrual
4	40	Menstrual
5	38	Menstrual
6	38	Menstrual
7	37	Menstrual
8	32	Menstrual
9	34	Menstrual
10	39	Menstrual
11	33	Menstrual