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Characterization of Endometrial Stem Cells

by

Angela M. Rojas

THESIS

Submitted in partial satisfaction of the requirements for the degree of

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by

Angela M. Rojas

Dedication and Acknowledgments

I owe my deepest gratitude to my advisor, Dr. Linda C. Giudice. This work would not have been possible without her guidance, unconditional support, and admirable expertise. I would also like to thank my committee members Dr. Susan Fisher and Dr. Synthia Mellon for their support and insight.

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Characterization of Endometrial Stem Cells

Angela M. Rojas

Abstract

Human endometrium is a dynamic tissue that responds to the circulating ovarian steroid hormones estradiol (E_2) and progesterone (P_4), and regenerates in the absence of pregnancy on a cyclic basis [1]. The regenerative capacity of the endometrium is remarkable, and this process occurs nearly 400 times during a women's reproductive lifetime. It has been proposed that endometrial stem cells (SC)/progenitor cells mediate endometrial regeneration. Recently, a clonogenic stem cell-like population co-expressing CD146 and platelet-derived growth factor-receptor β (PDGF-R β) has been found in the endometrium [2, 3, 4, 5]. It has been proposed that abnormal functioning of this endometrial stem cell-like population is involved in the initiation and progression of disorders associated with abnormal endometrial proliferation [6]. The concept that a stem cell-like population is responsible for disease progression has been previously postulated in the cancer stem cell model [7]. This model states that tumors contain a subset of cells that both self-renew and give rise to differentiated progeny and are responsible for the tumor's growth [7].

There are several disorders of the endometrium whose etiologies are not well understood and that may have their origins in the endometrial stem/progenitor population, including endometrial hyperplasias, endometrial cancers and endometriosis. Endometriosis is a chronic benign gynecological disorder characterized by the presence of endometrial glands and stroma outside the uterine cavity [8]. It has been established that the eutopic endometrium of women with endometriosis does not appropriately respond to progesterone

(P₄) [8, 9]. We hypothesize that, gynecological disorders such as endometriosis may be driven by a subset of cells, namely SC, that have some unique characteristics that distinguish them from those of SC in normal endometrium, and while not predisposing to uncontrolled growth as in cancers, do predispose to the persistent proliferative phenotype seen in endometriosis.

Herein, we propose experiments to **1)** characterize the SC populations in eutopic endometrium from women with versus without endometriosis, both molecularly and functionally to help elucidate the role of these cells in the development and maintenance of normal endometrium and endometriosis, and **2)** to determine whether the aberrant response to P₄ in endometriosis can be observed in the SC precursor/progenitor cells.

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Introduction

The human endometrium, the lining of the uterus, is a dynamic tissue whose biological role is to provide a site for implantation and development of an embryo. During reproductive years the endometrium responds to the circulating ovarian steroid hormones, E₂ and P₄, and undergoes cyclical processes of regeneration, proliferation, differentiation and shedding. The human endometrium is composed of several cell types: the glandular epithelium and the supporting cells of the stromal compartment, which include stromal fibroblasts, the vascular cells, and leukocytes. Functionally, the endometrium is composed of two layers: the outer functionalis layer and the inner basalis layer [8]. During menstruation, endometrial glands and stroma regenerate presumably from their remnants in the basalis to form the new functionalis layer; however, little is known about the precise mechanisms involved in endometrial regeneration, and the specific cell types that participate in this process. The concept that endometrial stem/progenitor cells are responsible for the remarkable regenerative capacity of the endometrium was postulated a few years ago [4, 5, 10]. The first published evidence for the existence of adult stem/progenitor cells in human endometrium identified a small subset of clonogenic epithelial and stromal cells [4, 5].

Stem cells are undifferentiated cells that have the ability to self-renew, as well as to differentiate toward different cell types. Stem cells can be divided into two major groups: embryonic and adult. Embryonic stem cells are fully undifferentiated and are able to generate all embryonic germ layers (totipotent cells). Adult stem cells have a high proliferative potential, substantial self-renewal capacity and ability to differentiate into at least one type of mature functional progeny (multipotent cells) [11]. In several adult tissues, rare populations of adult stem cells with high proliferative potential have been identified, which are

responsible for regeneration and maintenance of the entire tissue during the lifetime of the individual. These adult stem cells occupy specific niches within the tissue they reside and undergo self-renewal and differentiation, eventually producing numerous functional progeny [5]. In the human endometrium, a mesenchymal stem cell (MSC)-like population was recently isolated by selecting cells co-expressing two perivascular cell markers, CD146 and PDGF-R β [6, 12]. This population, obtained from endometrial stromal fibroblasts had an increased colony forming capability compared to stromal cells that do not express such markers, had the potential to differentiate into different mesenchymal cell lineages, and also expressed known MSC surface markers [6].

It is likely that these newly identified stem cell population and their surrounding niche cells may be involved in the initiation and progression of gynecological disorders associated with abnormal endometrial proliferation such as endometriosis [6]. As described above endometriosis is characterized by the presence of endometrial glands and stroma outside the uterine cavity. The distribution of this tissue is most often within the pelvic peritoneum but can include the pelvic viscera, rectovaginal septum, pleura, abdominal wall, and, rarely, the brain. The consequences of endometriosis often include pelvic pain and infertility. The incidence of the disorder is between 6% and 10% of all women and 35%–50% of women with pelvic pain and infertility [13]. It has been shown that eutopic endometrium of women with endometriosis does not respond appropriately to P₄, which may lead to abnormal signaling between stroma and epithelium, and also between endometrial and embryonic cellular components, which may lead to infertility and poor pregnancy outcome. Gene-expression studies in eutopic endometrium of women with endometriosis have demonstrated a reduced response to P₄ in the transition from proliferative to secretory phases [13]. One of

the genes whose expression is reduced in endometriosis, is FOXO1A. The FOXO1A gene encodes a P₄-regulated transcription factor involved in cell cycle control and the induction of apoptosis. It is markedly induced upon decidualization of endometrial stromal cells in both in vivo and in vitro assays in response to P₄ and cAMP [13]. FOXO1A can up-regulate both IGFBP1 and prolactin (PRL) promoter activities in human endometrial stromal cells [14]. Understanding the cause of aberrant response to P₄ can help elucidate targeted therapies for this disorder.

The objective of this proposal is to investigate potential differences and similarities between the stem cell populations in the endometrium from women with versus without endometriosis, both molecularly and functionally. This will help elucidate whether the aberrant endometrial proliferative behavior and the abnormal response to P₄ present in endometriosis arise from stem cell progenitors.

Preliminary Data

a. Isolation of MSC-like endometrial cells. Human endometrium contains a small population of MSC-like cells co-expressing CD146 and PDGF-R β that may be responsible for its cyclical growth [3, 4, 5]. In preliminary studies herein, I have used the experimental approach of Gargett and colleagues [3, 4, 5] to isolate and characterize these cells and have gone beyond what has been published in the literature – namely to provide proof of concept by preparing RNA from these cells for microarray analysis. Specifically, single cells were isolated from endometrium harvested from n=3 hysterectomy specimens (43-49 years old), using enzymatic and mechanical digestions as previously described [15]. Endometrial epithelial cells were isolated by positive selection using anti-EpCAM-coated magnetic beads (MiltenyiBiotec, Bergisch Gladbach, Germany). This antibody shows specificity for both luminal and glandular epithelium in full thickness endometrium. The stromal cells will then be negatively selected using anti-CD45 antibody-coated magnetic beads for the removal of leukocytes [15]. Purified endometrial stromal cells will be conjugated with CD146 antibody (R&D Systems, Minneapolis, MN, USA), PDGF-R β (R&D Systems, Minneapolis, MN), or isotype matched controls, followed by FITC-conjugated (BD Biosciences, Franklin Lakes, NJ, USA) and phycoerythrin-conjugated (R&D Systems, Minneapolis, MN, USA) secondary antibodies respectively, and analyzed by fluorescent activated cell sorting (FACS) as described [3]. We have been able to use these perivascular markers to successfully isolate this endometrial stem cell population by FACS (Figure 1). We confirmed the Gargett data and also used a commercial antibody to CD146 (whereas the Gargett group used a proprietary antibody).

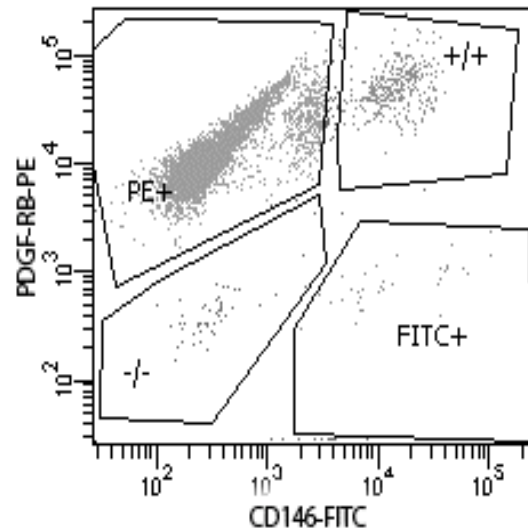


Fig 1. hESF FACS. Fluorescence histogram showing co-staining of CD146 and PDGF-RB in endometrial stromal fibroblast cells. Double positive cells (+/+) indicate the endometrial stem-like cell populations.

b. Transcriptome analysis. Samples from n=2 were used to isolate the four endometrial stromal cell subpopulations: CD146+PDGF-Rβ+, CD146-PDGF-Rβ-, CD146-PDGF-Rβ+ and CD146+PDGF-Rβ-. Freshly isolated cells, and n=1 of cultured CD146+PDGF-Rβ+ cells, were processed for microarray analysis, as described below, to assess the transcriptome of these different populations [3, 15].

c. Characterization of the CD146+PDGFRβ+ cells. Hysterectomy specimens were used to isolate the endometrial stem cell subpopulation: CD146+PDGF-Rβ+. Following isolation, the stem cell-like characteristics of the cells were assessed as described below by *in vitro* colony forming capacity, self-renewal, differentiation capacity *in vitro*, expression of stem cell markers and transcriptome of the cultured clonogenic cells [3, 15].

c.1. Clonogenic potential. A clonogenicity assay was performed to determine the colony-forming potential of each population [15]. Cells were seeded in triplicate at clonal density (50 cells/cm²) in gelatin coated 60-mm Petri dishes (Nunc, Rochester, NY, USA).

Media conditions and fibroblast preparation were conducted as described [3, 9]. Colonies were monitored microscopically on a daily basis to ensure that they were derived from single cells. Cultures were terminated after 3 weeks. The CD146+PDGF-R β + cells had a colony-forming capacity of 2.8%. The number of colonies formed was assessed by the following formula: cloning efficiency (CE (%)) = (number of colonies/number of cells seeded) x 100 [15].

c.2. Expression of stem cell markers. The expression levels for stem cell markers Oct4, Nanog and Sox2 (marker of pluripotency), was analyzed by RT-PCR. A preliminary experiment was performed but due to poor positive controls, and extended culture of the cells prior to RNA isolation the outcome of the experiment was compromised.

c.3. In vitro differentiation. Isolated cells were incubated for 4 weeks with adipogenic differentiation induction media using a commercially available differentiation kit (Chemicon International Inc., USA) following the manufacturer's instructions [3]. We found that these cells are able to differentiate down the adipogenic lineage confirming their MSC-like nature (Figure 2). The stromal phenotype of the MSC-like cells was confirmed by immunohistochemistry for vimentin (Figure 3).

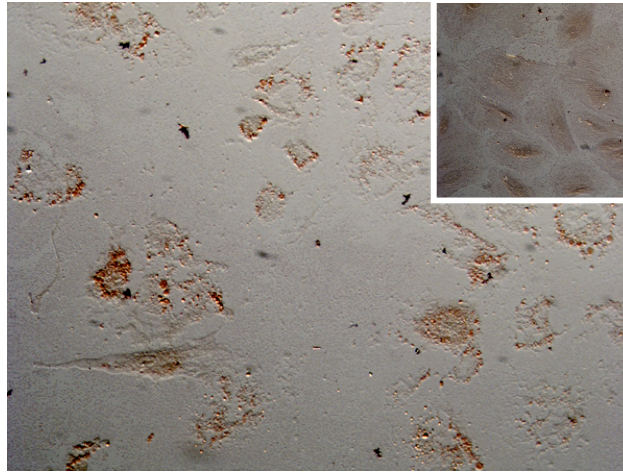


Fig 2. Oil Red-O staining. Adipogenic differentiation of FACS sorted and cultured CD146+PDGF-RB+ human endometrial stromal cells.

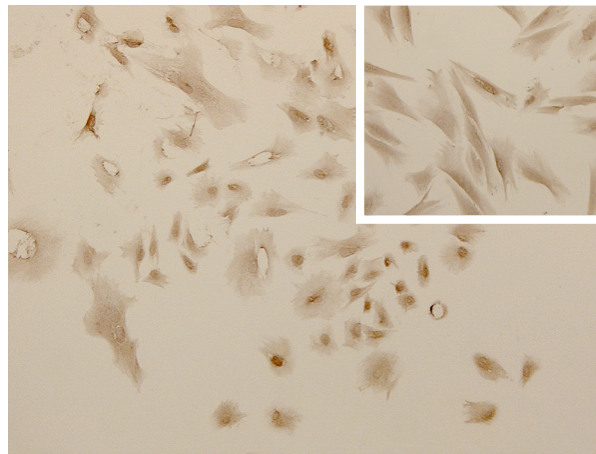


Fig 3. Vimentin immunohistochemistry. FACS sorted stromal cells were positive for vimentin hESF from our tissue bank were used as control (upper right corner).

d. Microarray analysis. To further characterize the various cell populations and compare the CD146+PDGF-R β + cells to the other cell populations identified and isolated by FACS analysis, microarray analysis of the four freshly FACS-isolated stromal cell subpopulations (see above), and also of the cultured CD146+PDGF-R β + [3, 15]. RNA from

5 samples was purified and amplified by reverse transcription, *in vitro* transcription, biotinylation and fragmentation as described [13]. RNA and cDNA quality and integrity were analyzed by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The samples were processed on the Affymetrix Gene ST 1.0 platform and ready to be analyzed by bioinformatic approaches and software (GeneSpring v GX10) for common and differentially expressed genes in the stem cells isolated from normal endometrium and endometriosis [13].

e. Summary. Having isolated a clonogenic population of MSC-like cells with perivascular markers CD146 and PDGF-R β , and demonstrated that the cells have the capability of being differentiated down the adipogenic lineage, and having established feasibility of isolating RNA and performing microarray hybridization, we are now in the position to analyze the transcriptome of this population compared to the other 3 populations (CD146-PDGF-R β -, CD146-PDGF-R β + and CD146+PDGF-R β -) of cells isolated and test the hypothesis of this study—namely if this cells differ in their properties when derived from endometrium from women with versus without endometriosis. This is addressed below, as well as proposed experiments to assess whether these cells have resistance to P₄, as in the mature stromal fibroblast in endometrium from women with endometriosis.

Materials and Methods

AIM 1: To investigate if endometrial stem cells in eutopic endometrium differ molecularly and functionally in endometriosis compared to normal endometrium

We propose to investigate samples from normal endometrium and eutopic endometrium from women with endometriosis. Our lab has an established CHR protocol to obtain and process samples from normal endometrium and endometrium from women with endometriosis. N=10 samples from each of the two experimental groups have been chosen to characterize these cells, as sufficient cells are anticipated to be obtained from this number of samples for replicates and validation studies and for statistical analysis of studies proposed below. N=5 samples from each of the two experimental groups will be used for microarray analysis of freshly isolated cells, and N=5 samples from each group will be used for culture and characterization of the endometrial stem cells.

Single cells from normal endometrium and endometrium from women with endometriosis will be isolated using enzymatic and mechanical digestions as previously described [15]. As described above. Freshly isolated cells will be then be processed for microarray analysis, as described below, to asses the transcriptome of cells isolated from women with versus without endometriosis.

N=5 samples from each of the two experimental groups will be used to isolate the endometrial stem cell subpopulation: CD146+PDGF-R β +. Following isolation, the stem cell-like characteristics of the cells will be assessed as described below by *in vitro* colony forming capacity, self-renewal, differentiation capacity *in vitro*, expression of stem cell markers and transcriptome of the cultured clonogenic cells [3, 15].

a. Clonogenic potential- A clonogenicity assay will be performed as described above.

b. *In vitro* Differentiation- Isolated cells will be incubated with adipogenic, osteogenic and myogenic differentiation induction media for 4 weeks as described [3]. Differentiated cells will be assessed for expression of lineage specific genes using RT-PCR. Amongst the specific genes that will be assessed are CBFA1, LPL, and Calponin, for the osteogenic, adipogenic, and myogenic lineages respectively [3].

c. Expression of stem cell markers- The expression levels of stem cell markers Oct4, Nanog and Sox2 (marker of pluripotency), will be analyzed by RT-PCR [13, 16].

d. Microarray analysis- To further understand if there are differences in gene expression in endometrial stem/progenitor cells isolated from samples obtained from women with versus without endometriosis, we will conduct microarray analysis of the four freshly FACS-isolated stromal cell subpopulations (see above), and also of the cultured CD146+PDGFR β + [3, 15]. RNA will be isolated, purified and amplified by reverse transcription, *in vitro* transcription, biotinylation and fragmentation. The samples will then be processed on the Affymetrix Gene ST 1.0 platform and analyzed by bioinformatic approaches and software (GeneSpring v GX10) for common and differentially expressed genes in the stem cells isolated from normal endometrium and endometriosis [13]. Data will be validated as described [13].

Expected outcomes and alternative methods

We anticipate finding distinct gene expression patterns in the different stromal cell subpopulations. We also expect to find different gene expression patterns in the stem cell subpopulation (CD146+PDGF-R β +) from women with versus without endometriosis, which

will correlate to the high proliferative and invasive profile of the samples being studied. This will indicate that the potential for the establishment and progression of this disorder is determined before terminal differentiation of endometrial cells.

If we have low yield of cells, we will use two alternatives to increase the cell number: 1) we will seek to procure larger endometrial tissue samples as available; or 2) we will pool samples from several patients into one group. Also, if we find no difference between the stem cell populations of normal endometrium and eutopic endometrium in endometriosis, we can speculate that the aberrant proliferative behavior of the cells in endometriosis may be initiated due to unknown modifications in the endometrial stromal microenvironment resulting in persistent changes observed in these cells or that the aberrant proliferative behavior is acquired subsequently.

AIM 2: Determine whether progesterone-resistant endometrial stromal cells in endometriosis arise from an endometrium stem cell precursor population

The goal of this aim is to understand the aberrant response to P₄ by examining the levels of FOXO1A, PRL and IGFBP1 in CD146+PDGF-R β + cells from women with versus without endometriosis to determine whether this abnormal behavior is already present in the progenitor cell population. N=5 will be used from each study group. We will induce decidualization of stromal cells by mimicking the hormonal environment of the cycle using E₂ which induces endometrial cellular growth (proliferative phase) and P₄ which induces differentiation/decidualization (secretory phase).

CD146+PDGF-R β + cells isolated as described in Aim 1 from eutopic endometrium from women with and without endometriosis will be used for the experiments proposed in this section. Cells will be cultured in low-serum DMEM/MCDB-105 medium containing ascorbic acid, transferrin, and gentamicin with 2% charcoal-stripped FBS [18]. Cells will then be treated with E₂ alone (10nM) for 2 weeks, after which E₂+ P₄ (10nM, 1 μ M respectively) will be added to the culture for 2 additional weeks. Each condition will be performed in duplicates with appropriate controls. Supernatants will be collected every 3 days, starting at time 'zero' (before hormone treatment), and RNA will be harvested at time zero, week 2 (after E₂), and week 4 (after E₂+ P₄). The following markers will be assessed by RT-PCR as described: ER α/β , PR A/B, CD146, PDGF-R β , IGFBP1, PRL, vimentin, FOXO1A, Oct4, and Nanog [3].

Expected outcomes and alternative methods

PRL and FOXO1A levels correlate to progesterone action in stromal cells so we expect to see a down-regulation of these genes in endometriosis samples [9, 18]. IGFBP1 levels

correlate the levels of FOXO1A in stromal cells [18]. We also expect to see low levels of both PRA and B in endometriosis samples. We anticipate this phenotype to be present in the progenitor cells as PR mRNA is present in bone-marrow derived MSC [19]. Oct4 and Nanog are pluripotency markers and should not be present in decidualized cells.

If the yield of cells is not sufficient, the same approaches as described in Aim 1 will be pursued. Specifically, we shall seek to procure larger endometrial tissues samples as available, or cells will be pooled from several patients. If we are not able to induce decidualization with E₂/P₄, we will use 8-Br-cAMP treatment, which has been shown to be a more potent and rapid inducer of cell differentiation [17].

Future Studies

Future studies will include the regeneration of endometrial tissue with and without endometriosis *in vivo*. Using immunodeficient mice we will inject human stem/progenitor cells from eutopic endometrium from women with and without endometriosis in the mouse peritoneal cavities. We expect endometrial tissue to arise from both stem/progenitor populations. We would then investigate the proliferation potential and severity of any observed phenotype. Also using siRNA knockdown or over-expression of genes that regulate P₄ action, we expect to rescue the aberrant phenotype *in vivo*.

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