BIOLOGY OF REPRODUCTION (2016) **94**(6):129, 1–4
Published online before print 4 May 2016.
DOI 10.1095/biolreprod.116.141325

Commentary

Endometrial Mesenchymal Stem/Stromal Cells, Their Fibroblast Progeny in Endometriosis, and More[1]

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The human endometrium is a highly regenerative tissue, undergoing monthly cycles of growth, differentiation, and shedding during a woman's reproductive life. Resident mesenchymal stem/stromal cells (MSC) have recently been identified and characterized in human endometrium by numerous investigators (reviewed in [1]) and likely have a key role in its regenerative capacity. In many of these investigations, some or all of the classic properties of bone marrow MSC [2] have been demonstrated: clonogenicity, multipotency (differentiation into mesodermal lineages, adipocytes, osteocytes, and chondrocytes), and a surface phenotype that distinguishes them from leukocytes, hematopoietic, and endothelial cells. Others have shown their self-renewal in vitro [3] and ability to reconstitute endometrial stromal vascular tissue in vivo [4–6].

Specific markers enriching for the most highly clonogenic endometrial MSC (eMSC), $MCAM^+$ ($CD146^+$) $PDGFRB^+$, have revealed their in vivo identity as pericytes [7], and a single marker, SUSD2, as perivascular cells [6] in both the functionalis and basalis layers of the endometrium. Likewise, these and several other markers have identified the in vivo perivascular niche of MSC in many other tissues [8], including bone marrow, placenta, and umbilical cord, and have been used to purify MSC from stromal fibroblast populations also present in these tissues [9]. Similar to bone marrow fibroblasts [10], endometrial stromal fibroblasts (eSF) also exhibit MSC properties [11], except they are weakly clonogenic, generating small, non-self-renewing cell clones, and lack proliferative capacity or the ability to generate neotissue in vivo [10]. Their presence contributes to the heterogeneity of MSC cultures. In response to progesterone, eSF differentiate into decidual cells, which have essential roles in the establishment and maintenance of pregnancy [12]. In contrast to bone marrow MSC, $SUSD2^+$ and $CD146^+PDGFRB^+$ eMSC differentiate into decidual cells in vitro [13–15], indicating a unique property of eMSC.

The perivascular location of eMSC in the endometrial functionalis indicates that they are shed in menstrual blood and may have a role in initiating endometriosis, which is thought to result from retrograde shedding of menstrual debris into the peritoneal cavity [1]. In women with endometriosis, eSF are progesterone resistant, likely contributing to infertility associated with this condition.

MSC are an attractive cell type for regenerative medicine due to their anti-inflammatory, immunosuppressive, and tissue reparative properties, with ~600 clinical trials registered on clinical trials Web sites for a wide range of immune and degenerative disorders [16]. Despite this, the fate and mechanism of action of MSC are poorly understood, confounded by the spontaneous differentiation of MSC to fibroblasts during culture expansion and frequent use of heterogeneous cell preparations of nonpurified fibroblast preparations for clinical application [17]. Allogeneic menstrual blood-derived stromal fibroblasts are being trialed for congestive cardiac failure [18], and autologous eMSC are being developed as a cell-based therapy for urogynecological applications [19].

In a recent issue of *Biology of Reproduction*, an elegant gene profiling study combined with hierarchical clustering and principal component analysis [20] identifies a unique set of lineage genes for eMSC and eSF. Comparison of these in vivo (freshly isolated) phenotypes with their short- and long-term clonal culture counterparts by Barragan et al. confirms the lineage commitment of eMSC to eSF, demonstrating that endometrial pericytes are the lineage precursors of eSF in vitro [14]. The authors also demonstrate that eMSC from women with endometriosis exhibit progesterone resistance that is inherited by their eSF progeny. In contrast, the prominent pro-inflammatory phenotype of endometriosis eSF is not inherited from eMSC but rather induced by unidentified in vivo niche factors. Finally Barragan et al. [20] demonstrate that eMSC rapidly and spontaneously differentiate into eSF in culture, adopting the lineage genes of eSF, and that this process occurs with less fidelity in eMSC derived from endometrium of women with endometriosis. Just as the identification of specific markers for purifying eMSC and eSF has opened the door to investigating their respective roles in endometrial physiology and pathophysiology [1], the discovery of sets of lineage genes for these cells by Barragan et al. opens new avenues of research designed to gain deeper insights into their function at the transcriptional level in both health and disease.

[1]This work was supported by a National Health and Medical Research Council of Australia Senior Research Fellowship (ID 1042298) (C.E.G.), a Monash Graduate Scholarship (S.G.), and the Victorian Government's Operational Infrastructure Support Program.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Barragan et al. [20] provided an in depth study designed to define the transcriptional phenotype of eMSC and eSF in vivo and after short- and long-term culture. The goal was to identify the cellular ontogeny of progesterone resistance characteristic of endometriosis to enable further characterization in in vitro studies. Principle component analysis clearly distinguished the freshly isolated from cultured cells, and hierarchical clustering segregated the gene profiles on cell type followed by disease, which reversed in long-term cultures, suggesting convergence of cell phenotypes and/or magnification of disease phenotype.

The study first determined a set of lineage signature genes for freshly isolated eMSC versus eSF from control samples, identifying 550 upregulated genes in eMSC and 1370 in eSF [20]. The majority of these lineage genes were conserved in the eMSC and eSF of endometriosis samples. These conserved eMSC lineage genes included pericyte markers, hypoxia-related genes, and genes involved in activated Notch and TGFβ signaling pathways, SLIT ligands, WNT inhibition, and various growth factors, chemokines, and cytokines. Conserved eSF lineage genes included phospholipase A2, membrane metallo-endopeptidase (CD10), hormone receptor and metabolism, growth factor, cytokine and receptor genes, ROBO receptors for SLIT ligands, and WNT and Hedgehog signaling pathway genes.

The Barragan et al. study [20] has made a major contribution to the MSC discipline, and the eMSC field in particular, by addressing the issue of spontaneous MSC differentiation into stromal fibroblasts and generation of heterogeneous cultures during expansion. In a comprehensive experimental design, the authors compared gene profiles of pure populations of freshly isolated eMSC ($CD146^+PDGFRB^+$) and eSF ($CD146^-PDGFRB^+$) with early and long-term primary clonal cultures of both cell types. After accounting for the altered expression of culture adaptation genes, 44% of the in vivo eSF lineage genes were still expressed in late passage cultures, providing a robust definition of the eSF molecular phenotype. Importantly, very few eMSC lineage genes were upregulated in eSF cultures, including markers (SUSD2, CD146) used to select eMSC populations. In contrast, highly purified eMSC spontaneously differentiated in culture, downregulating the expression of 81% of their in vivo lineage genes and upregulating 55% of eSF lineage genes until only 19 genes were differentially expressed between late passage eMSC and eSF cultures. This extensive phenotypic analysis confirms previous studies showing loss of SUSD2 expression in purified eMSC cultures [15, 21]. It also recapitulates findings for freshly isolated bone marrow MSC (bmMSC) purified using nerve growth factor receptor (NGFR, CD271) in which 35% of lineage genes were downregulated during subsequent culture in a much smaller study examining only 94 genes [22]. As for eMSC, a WNT receptor (*FRZB*) was downregulated during culture expansion of $CD271^+$ bmMSC. Several genes from the same family were also downregulated in both eMSC (*ANGPT2, BMP8A, CDH2, CDH6, TGFBR2*) and bmMSC (*ANGPTL4, BMP5, CDH5, TGFBR3*). Likewise, several eSF lineage genes were upregulated in cultured $CD271^+$ bmMSC, including *OMD, PDGF-C* and *-D, IGF2, SFRP4,* and *FABP4*. Recently, it was shown that spontaneous differentiation of freshly isolated $SUSD2^+$ eMSC into $SUSD2^-$ eSF during culture expansion was blocked by a small molecule TGFβ receptor inhibitor A83-01 [23] in serum-free medium lacking TGFβ, implying a role for autocrine TGFβ signaling. Under these conditions, inhibition of TGFβ signaling prevented apoptosis and senescence of eMSC. Concurring with these observations, Barragan et al. [20] showed that fresh $CD146^+PDGFRB^+$ eMSC expressed higher levels of the drivers of differentiation *TGFB-1* and *-2* ligands and the *TGFBR2* receptor than fresh $CD146^-PDGFRB^+$ eSF and that these genes were downregulated in eMSC in early and late cultures, implying that the TGFβ signal acts as a switch to initiate eMSC differentiation and subsequent apoptosis. These complementary studies highlight the importance of TGFβ signaling in maintenance of the eMSC phenotype.

Specific markers used for purifying perivascular MSC are frequently downregulated on culture expansion either due to spontaneous differentiation to stromal fibroblasts or culture adaptation. The surface marker *CD146*, *SUSD2*, amino oxidase, copper containing 3 (*AOC3*), and regulator of G-protein signaling 5 (*RGS5*) lineage genes of freshly isolated eMSC are lost during culture expansion [20, 23]. The bmMSC marker CD271 is also expressed in eMSC [6] and downregulated during culture expansion [23]. All are potential markers for monitoring eMSC cultures for the presence of undifferentiated MSC using flow cytometry. However, the percentage of $CD271^+$, $AOC3^+$, or $CD146^+$ cells is drastically and rapidly reduced and does not predict the percentage of clonogenic cells remaining in serum-free eMSC cultures [23] (Gurung and Gargett, unpublished results). In adipose MSC cultures, CD146 is cleaved by matrix metallo-proteinase 3 (MMP3) [24]. This mechanism may also operate during culture of eMSC because *MMP3* expression was markedly upregulated in eMSC cultures in the Barragan et al. study [20] and in A83-01-containing medium [23]. In contrast, the percentage of $SUSD2^+$ cells in eMSC cultures reflects their functionality (clonogenicity) and currently appears to be the most reliable marker for monitoring eMSC cultures [23], an important consideration for cell production for clinical applications. Barragan et al. [20] also found that SUSD2 expression remained low in control eSF cultures but was upregulated in eSF cultures derived from eutopic endometrium of women with endometriosis. The possible inclusion of endometriosis samples in another study may explain the appearance of $SUSD2^+$ cells in $SUSD2^-$ cultures [15].

The main purpose of the Barragan et al. study [20] was to determine whether eSF are the progeny of eMSC precursors and if they inherit progesterone resistance from eMSC or acquire this pathological change in situ as eSF. To answer these questions, the authors compared the molecular phenotypes of freshly isolated and cultured eMSC and eSF obtained from controls and endometriosis samples. In freshly isolated samples, there was considerable similarity in molecular phenotypes, with more than 95% of the top 200 in vivo lineage genes conserved in endometriosis samples, although there were fewer differentially expressed genes in endometriosis eMSC and eSF compared with controls. Of the 320 differentially expressed genes between freshly isolated endometriosis and control eMSC, 22 eMSC lineage genes were downregulated in endometriosis, including thrombospondin-4 (*THBS4*) and *FLT1,* and 61 eSF lineage genes were upregulated, including *PDGFRA, PDGFC, LOX,* and *WNT5A*. Pathway analysis revealed that the upregulated genes were associated with proteolysis and downregulated with colony formation, cell death, and survival, suggesting a role for eMSC in initiating endometriosis lesions when refluxed into the peritoneal cavity during menstruation. Of the 413 differentially expressed genes in freshly isolated eSF from controls and endometriosis samples, 22 eMSC lineage genes were upregulated, including *ANGPT2, FRZB,* and *TGFB2* as well as genes associated with inflammation (*CXCL2, IL8, NFKB1*). There were 62 eSF lineage genes downregulated, including *PLA2G7, IL17RB, DKK1,* and *FGF9*. Pathway analysis showed upregulated genes were associated with numerous pathways,

including inflammation, leukocyte trafficking, angiogenesis and epithelial cell function, indicating a striking pro-inflammatory phenotype of eutopic eSF. This key feature of endometriosis indicates the important contribution of eSF to the pathogenesis of endometriosis and associated infertility [25].

Barragan et al. also demonstrated a progressive change in phenotype of cultured eSF from endometriosis samples with a widening in differential gene expression compared with controls [20]. Only 33% of eSF lineage genes were expressed in eutopic eSF compared with 44% in controls, indicating diminished phenotypic stability in endometriosis eSF cultures. Also more eMSC lineage genes (36%) were retained in endometriosis eMSC cultures compared with control eMSC (23%), resulting in a total of 78 differentially expressed genes in long-term cultures from endometriosis samples compared with 19 in controls. This suggests that eMSC from endometriosis samples have reduced differentiation capacity, which may confer greater ability to initiate lesions in the peritoneal cavity. Despite the increased number of differentially expressed eMSC genes in endometriosis samples in late passage cultures, pathway analysis showed a narrow range of activated genes mainly in lipid metabolism and proteolysis, which may confer invasiveness.

For the first time, Barragan et al. demonstrated that progesterone resistance is a feature of cultured endometriosis eMSC, and that eSF inherit this inability to decidualize in vitro from its precursor, eMSC [20]. Both eMSC and eSF from long-term cultures of endometriosis samples were unable to secrete IGFBP1 in response to progesterone plus estrogen. This suggests that endometriosis is indeed a disorder of the endogenous endometrial stem/progenitor cell population [1], in particular eMSC. There does not appear a direct link between the susceptibility genes identified in genomewide associations studies [26] and the decidualization pathway, suggesting an acquired defect. Epigenetic changes in eutopic and ectopic endometrium are associated with key pathways in steroid hormone production and metabolism [27], but whether altered methylation of eMSC genes have a role is currently unknown. In contrast to endometriosis eSF inheriting a decidualization defect from their eMSC precursors, Barragan et al. showed that the pro-inflammatory phenotype of eSF was not acquired from eMSC in vitro, suggesting that eSF obtained their pro-inflammatory properties in the endometrial in vivo niche, possibly involving systemic mediators [20]. Finally the authors provide a model of the disease phenotype of eMSC and eSF in endometriosis that elegantly summarizes the numerous findings of their study.

In summary, the study by Barragan et al. makes excellent use of gene profiling, bioinformatics, and in vitro studies to generate and interpret important new information on the molecular phenotype and relationship between the pericyte eMSC and eSF, their respective roles in endometriosis and associated infertility, and much more. It shows that the decidualization defect of endometriosis eSF is inherited from eMSC, but not pro-inflammatory characteristics, which are acquired by eSF in vivo. The identification of lineage genes for eMSC and eSF and their comparisons between freshly isolated purified cell populations and their cultured counterparts also informs the MSC field, clearly revealing spontaneous differentiation of MSC to fibroblasts, at a time when the definition of MSC is being questioned. As with all well-designed discovery studies, it also opens new questions for further study. Questions such as what mechanisms lead to the decidualization defect in eMSC of women with endometriosis? Is this due to epigenetic changes known to occur in eutopic endometrium and endometriotic lesions [27], and if so, are the hypo- or hypermethylated genes specific to this stem/progenitor cell type? Or is there a role for micro-RNAs? Are there pharmacological approaches using small molecules or biologicals targeting eutopic eMSC that might promote appropriate differentiation to eSF without the decidualization defect? How is the pro-inflammatory phenotype conferred on eSF in the endometrial niche? Are the lineage gene signatures maintained in eMSC and eSF of ectopic lesions? And could gene profiling ectopic eMSC produce data supporting the concept of retrograde menstruation of endometrial stem/progenitor cells in the pathogenesis of endometriosis?

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