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The Bone Marrow-Derived Human Mesenchymal Stem Cell: Potential Progenitor of the Endometrial Stromal Fibroblast[1]

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ABSTRACT

The cellular sources that contribute to the renewal of human endometrium are largely unknown. It has been suggested that endometrial stem cells originate from bone marrow-derived mesenchymal stem cells (MSC), with subsequent development into endometrial stromal fibroblasts (hESF). We hypothesized that if bone marrow-derived MSC contribute to endometrial regeneration and are progenitors of hESF, their treatment with agents known to regulate hESF differentiation could promote their differentiation down the stromal fibroblast lineage. To this end, we treated bone marrow-derived MSC with estradiol and progesterone, bone morphogenetic protein 2 (BMP2), and activators of the protein kinase A (PKA) pathway and investigated specific markers of hESF differentiation (decidualization). Furthermore, we investigated the transcriptome of these cells in response to cAMP and compared this to the transcriptome of hESF decidualized in response to activation of the PKA pathway. The data support the idea that MSC can be differentiated down the hESF pathway, as evidenced by changes in cell shape and common expression of decidual markers and other genes important in hESF differentiation and function, and that bone marrow-derived MSC may be a source of endometrial stem/progenitor cells. In addition, we identified MSC-specific markers that distinguish them from other fibroblasts and, in particular, from hESF, which is of biologic relevance and practical value to the field of endometrial stem cell research.

cyclic adenosine monophosphate, differentiation, endometrium, fibroblasts, mesenchymal stem cells

INTRODUCTION

Human endometrium is a unique tissue that undergoes regular dynamic processes of growth, differentiation, sloughing, and renewal during a woman's reproductive years, generating 4–14 mm of mucosal tissue each menstrual cycle [1]. To date, the cellular sources that contribute to the complete renewal of endometrium are not known with certainty. The recently identified small population of clonogenic, self-renewing, multipotent mesenchymal stem cells (MSC) with high proliferative potential in human endometrium is a candidate stem/progenitor cell population [2, 3]. Key properties of MSC found in other tissues, such as bone marrow, adipose, dental pulp, amniotic fluid, umbilical cord blood, placenta, skeletal muscle, and pancreas [4, 5], include differentiation in vitro into multiple mesodermal cell types, such as osteoblasts, adipocytes, myoblasts, and chondroblasts [6, 7]. Wolff et al. [8] demonstrated that freshly isolated human endometrial stromal fibroblasts (hESF) can differentiate down the chondrogenic lineage, thus indicating the presence of multipotent stem/progenitor cells in human endometrium [8]. Also, isolated hESF plated at clonal density can differentiate toward adipogenic, osteogenic, myogenic, and chondrogenic lineages [9]. Thus, the stromal fibroblast may derive from a stem/progenitor cell in human endometrium.

Whereas endometrial MSC have properties of progenitors with the capacity to differentiate down multiple lineages, their origin remains unclear. One potential source is bone marrow-derived MSC, which are multipotent cells that, together with hematopoietic stem cells, construct the bone marrow. Osteoblasts, adipocytes, muscle cells, chondrocytes, and neurons originate from MSC in vivo and in vitro [10–12]. Clonogenic endometrial MSC express surface markers typical of bone marrow MSC and fat MSC ($CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD146^+$, $PDGFR\beta^+$, $CD31^-$, $CD34^-$, $CD45^-$, and Stro-1^-) and were found in a perivascular location, suggesting they may be a subset of pericytes involved in regulating both endometrial stromal repair and angiogenesis [9]. Furthermore, Taylor [13] provided evidence that endometrial regeneration may occur as a result of bone marrow-derived stem cells in bone marrow transplant recipients [13]. Donor-derived cells were detected in endometrial samples of bone marrow recipients, and these cells were histologically similar to endometrial epithelial and stromal fibroblasts and expressed appropriate markers of endometrial cell differentiation. It has been proposed that cyclic mobilization of bone marrow-derived stem cells may be a normal physiologic process [13, 14]. Interestingly, bone marrow-derived MSC have been suggested to differentiate not only to stromal fibroblasts but also to endometrial epithelial cells [15, 16].

In the current study, we hypothesized that if bone-marrow derived MSC contribute to endometrial regeneration and are progenitors to hESF, their treatment with agents known to regulate hESF differentiation could promote their differentiation down the stromal fibroblast lineage. To this end, we treated bone marrow-derived MSC with estradiol (E_2) and progesterone

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(P_4), bone morphogenetic protein 2 (BMP2), and activators of the protein kinase A (PKA) pathway and investigated specific markers of hESF differentiation (decidualization). Furthermore, we investigated the transcriptome of these cells in response to cAMP and compared this to the transcriptome of hESF decidualized in response to activation of the PKA pathway. The data support the idea that MSC can be differentiated down the hESF pathway, as evidenced by changes in cell shape and common expression of decidual markers and other genes important in hESF differentiation and function.

MATERIALS AND METHODS

MSC Culture

Bone marrow-derived MSC (Cambrex Biosciences) were expanded in high-glucose Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; HyClone, Thermo Scientific, Inc.), 1% penicillin and 1% streptomycin. At 80% confluency, MSC were trypsinized and plated at 5×10^3 cells/cm^2 in 6-cm plates. Nearly confluent cells were serum-starved overnight and thereafter treated for 0, 3, 7, 14, and 21 days in low-serum medium (2% FBS) with the following treatments: 1) 10 nM E_2 and 1 mM P_4 (E_2+P_4), 2) 0.5 or 1 mM 8-Br-cAMP (Sigma-Aldrich), 3) 50 or 100 nM BMP2 (R&D Systems), 4) 0.5 and 1 mM 8-Br-cAMP plus BMP2, 5) 0.5 and 1 mM 8-BrcAMP plus BMP2 plus E_2+P_4, and 6) 50 or 100 nM human follicle-stimulating hormone (FSH; Sigma-Aldrich). Vehicle controls were present at each time point for all treatments listed. Each experiment was conducted in duplicate and repeated at least three times. Media were changed every third day. Endpoints were prolactin (PRL) and insulin-like growth factor-binding protein-1 (IGFBP1) mRNA, assessed by quantitative real-time RT-PCR, and protein in conditioned media, assessed by ELISA (see below), after 3, 7, 14, and 21 days of incubation. Immunostaining for vimentin, cytokeratin 7, and Oct-4 was performed before and after MSC treatments to assess the stromal lineage of MSC and their differentiation status [17–19]. MSC expressed vimentin and did not express cytokeratin or Oct-4 independent of culture conditions or duration of culture or treatment. The morphological appearance of MSC under different treatment conditions was photographed using a Leica CTR 6500 microscope (Leica Microsystems, Ltd.). Scanned images were processed using the Leica TCS SP5 software package.

Isolation and Culture of hESF

Isolation and culture of hESF from endometrial samples (biopsy and hysterectomy specimens) from subjects without endometrial disease, which were used herein for comparison with MSC, were performed as previously described in detail [20–22]. The University of California, San Francisco (UCSF) Committee on Human Research and the Stanford University Committee on the Use of Human Subjects in Research approved the study. Written informed consent was obtained from subjects. Samples were also obtained through the UCSF National Institutes of Health Human Endometrial Tissue and DNA Bank with appropriate institutional review, approvals, and informed consent from all subjects.

Culture of Human Dermal Neonatal Fibroblasts

To determine specificity of the differentiative response, human dermal neonatal fibroblasts (HDFn; Cascade Biologics, Inc., Invitrogen) were used as a comparator. They were expanded in high-glucose DMEM containing 10% FBS (HyClone), 1% penicillin and 1% streptomycin. At 90% confluency, HDFn were trypsinized and plated at 5×10^3 cells/cm^2 in 6-cm plates. At near confluency, cells were serum-starved overnight and thereafter treated with 1 mM 8-Br-cAMP for 0, 3, 7, 14, and 21 days in triplicate. Endpoint analyses were measurements of *PRL*, *IGFBP1*, and stanniocalcin 1 (*STC1*) mRNA, assessment using quantitative RT-PCR, and PRL and IGFBP1 protein in conditioned media, assessed using specific ELISA assays (see below).

Enzyme-Linked Immunosorbent Assay

Conditioned media from MCS treated under different conditions and for different time periods were subjected to ELISA to determine PRL and IGFBP1 protein concentrations according to the manufacturers' instructions (Alpha Diagnostic International, Inc., and Diagnostic Systems Laboratories, respectively) and as described previously [20, 21]. Inter- and intra-assay coefficients of variation were 5–7.4% and 2.4–3.4%, respectively, for the IGFBP1 ELISA and 6.7–10.4% and 7.8–8.2%, respectively, for PRL. Minimum detection limits for PRL and IGFBP1 assays were 0.14 and 0.4 ng/ml, respectively. Levels of PRL and IGFBP1 were normalized to total RNA.

Total RNA Isolation, Quantitative Real-Time RT-PCR, and Microarray Hybridization

Total RNA from all cell cultures was purified using the Qiagen RNeasy Plus Mini kit according to the manufacturer's instructions. Samples stored in RNase-free H_2O were quantified by spectroscopy, and the purity was analyzed by the 260:280 absorbance ratio. RNA quality was assessed by an A2100 Bioanalyzer (Agilent Technologies). For real-time RT-PCR analysis, 1 μg of RNA was converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories). The mRNA preparations from technical duplicates for each set of treatments were pooled together. The real-time RT-PCR reaction was carried out in duplicates for 40 cycles with the primers listed in Supplemental Table S1 (all supplemental data are available online at www.biolreprod.org).

Microarray analysis was performed with MSC ($n = 3$) at time zero ($t = 0$; untreated, uncultured control), 14-day vehicle control, and 14-day MSC treated with 8-Br-cAMP. Only high-quality samples (RNA integrity number = 9.1–10) were used for microarray analysis. Hybridization was performed with Affymetrix Human Gene 1.0 ST arrays, as described earlier [23]. For each sample, 100 ng of total RNA were reverse transcribed to cDNA using 500 ng of T7-(N6) primers and SuperScript II. A second strand of DNA was generated using DNA polymerase, followed by overnight in vitro transcription to generate cRNA. After processing through cRNA clean-up spin columns, 10 μg of cRNA were reverse transcribed using random primers and SuperScript II. Mixtures were digested with RNase H, and the cDNA was purified by the cDNA clean-up spin columns. Lastly, 5.5 μg of sense cDNA were fragmented and labeled by using the GeneChip WT terminal labeling kit. The quality of cDNA and fragmented cDNA was assessed in the Agilent bioanalyzer. Microarrays were hybridized, washed, stained, and scanned according to the protocol described in WT Sense Target Labeling Assay Manual from Affymetrix (Version 4; FS450_0007) at the UCSF Gladstone Genomics Core Facility.

Statistical Evaluation

Statistical analysis of data generated in the ELISA assays was performed using a two-tailed, type 3 Student *t*-test. For quantitative RT-PCR, the nonparametric Mann-Whitney test was used. Significance was determined at $P \leq 0.05$.

Microarray Gene Expression Data Analysis and Statistical Analysis

As described previously [23], to remove variation of a nonbiological origin, densitometry values among arrays were normalized using the RMA function and further transformed to the logarithmic 2 (log2) scale. Probes with known correspondence to a GenBank accession ID were selected for functional analysis. Statistically significant differences between groups were determined using the Bioconductor (http://www.bioconductor.org/) packages *multtest* (for the parametric MSC intragroup comparison, with a multiple test correction cutoff false discovery rate < 0.01) and *RankProd* (for the nonparametric inter-MSC-hESF group comparison, with a multiple test correction cutoff pfp [*P*-value corrected] < 0.05), both run under R software (http://www.r-project.org/). Functional annotations were carried out using Ingenuity Pathway Analysis (Ingenuity Systems; http://www.ingenuity.com/), where gene symbols and fold-changes of the up- and down-regulated genes were imported. The raw data files of current experiments are uploaded to the National Center for Biotechnology Information Gene Expression Omnibus database (number GSE20631).

Hierarchical Clustering

Herein, we conducted hierarchical cluster analysis of differentially expressed genes from all samples (the combined gene list) using the smooth correlation for distance measure algorithm (GeneSpring 7.3) to identify samples with similar patterns of gene expression (Eisen et al.; see *Note Added in Proof*). The output data are displayed graphically as a Heatmap, based on the measured intensity values of the genes in the gene list, and are represented as a hierarchical tree with branches to indicate the relationships among different groups. Principal component analysis (PCA) was performed to further evaluate similarities in gene expression patterns in our experimental groups. An unsupervised pattern recognition and visualization tool used to analyze large amounts of data derived from array gene expression analysis, PCA displays a multidimensional data set in a reduced dimensionality (three dimensions) to capture as much of the variation in the data as possible [22]. Each dimension represents a component to which a certain percentage of variance in the data is

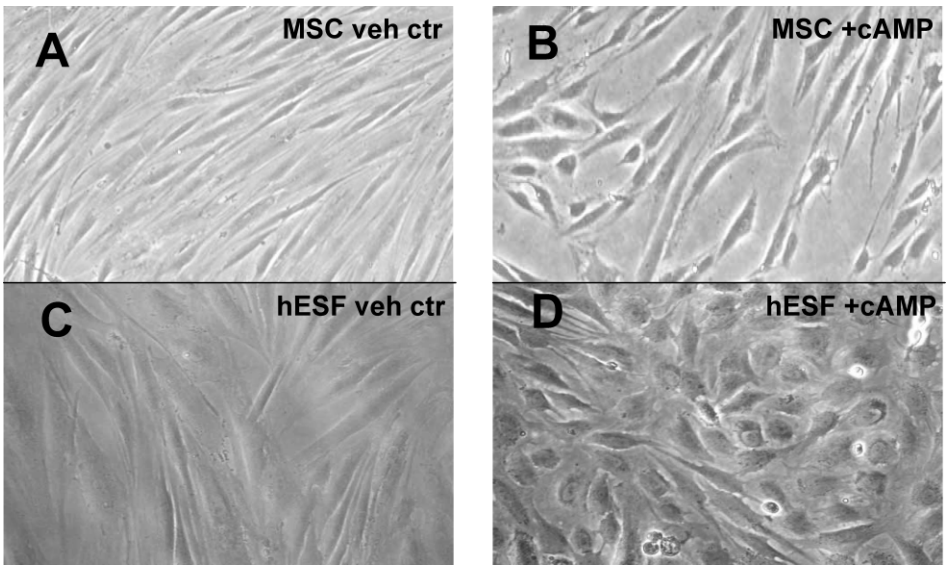


FIG. 1. Morphological transformation of MSC. **A**) MSC cultured for 14 days, vehicle control. **B**) MSC treated with 1 mM 8-Br-cAMP for 14 days. **C**) Human endometrial stromal fibroblasts (hESF) cultured for 4 days, vehicle control. **D**) hESF treated with 8-Br-cAMP for 4 days. Magnification ×40.

attributed. We applied the unbiased PCA algorithm in GeneSpring to all the samples before and after the treatment, using all 19 492 gene probes on the Affymetrix Human Gene 1.0 ST arrays to look for similar expression patterns and underlying cluster structures [24].

The most highly up- or down-regulated genes, as well as some potentially interesting genes, were chosen for validation by quantitative real-time RT-PCR as described above.

RESULTS

Morphological Transformation of MSC

Human endometrial stromal fibroblasts undergo distinct morphologic changes, from a spindle-like to more epithelial appearance with more rounded shape and large nuclei, as they differentiate in response to treatment with cAMP analogues and/or E_2+P_4 [19]. Because MSC have been postulated to be progenitors of hESF, we investigated whether 8-Br-cAMP treatment of MSC elicited similar changes in cell shape and morphology compared to those of similarly treated hESF (Fig. 1). Indeed, the morphological changes of 8-Br-cAMP-treated MSC were very similar to those observed with hESF treated in vitro with 8-Br-cAMP (Fig. 1). No other treatment of the MSC resulted in changes in shape or morphology throughout the culture period (data not shown).

Up-regulation of Decidualization Markers

The classical markers of hESF differentiation (decidualization) in response to 8-Br-cAMP, PRL and IGFBP1, were assessed in MSC treated with 1 mM 8-Br-cAMP. A robust expression of these markers was noted, with maximum mRNA and protein expression observed at 14 days of treatment. *PRL* and *IGFBP1* mRNAs were significantly up-regulated (2600- and 2200-fold, respectively) compared to vehicle controls cultured for the same time period, and corresponding proteins in MSC conditioned media were also increased significantly (Fig. 2A). Treatment with E_2+P_4, BMP2, or FSH did not elicit up-regulation of PRL or IGFBP1 (data not shown). Also, no significant difference in IGFBP1 and PRL mRNA or protein expression was found in MSC treated with cAMP plus E_2+P_4 versus 8-Br-cAMP alone (data not shown), suggesting that the MSC were not responsive to the steroid hormone therapy and that expression of IGFBP1 and PRL was specifically induced by activation of the PKA pathway by the cAMP analogue.

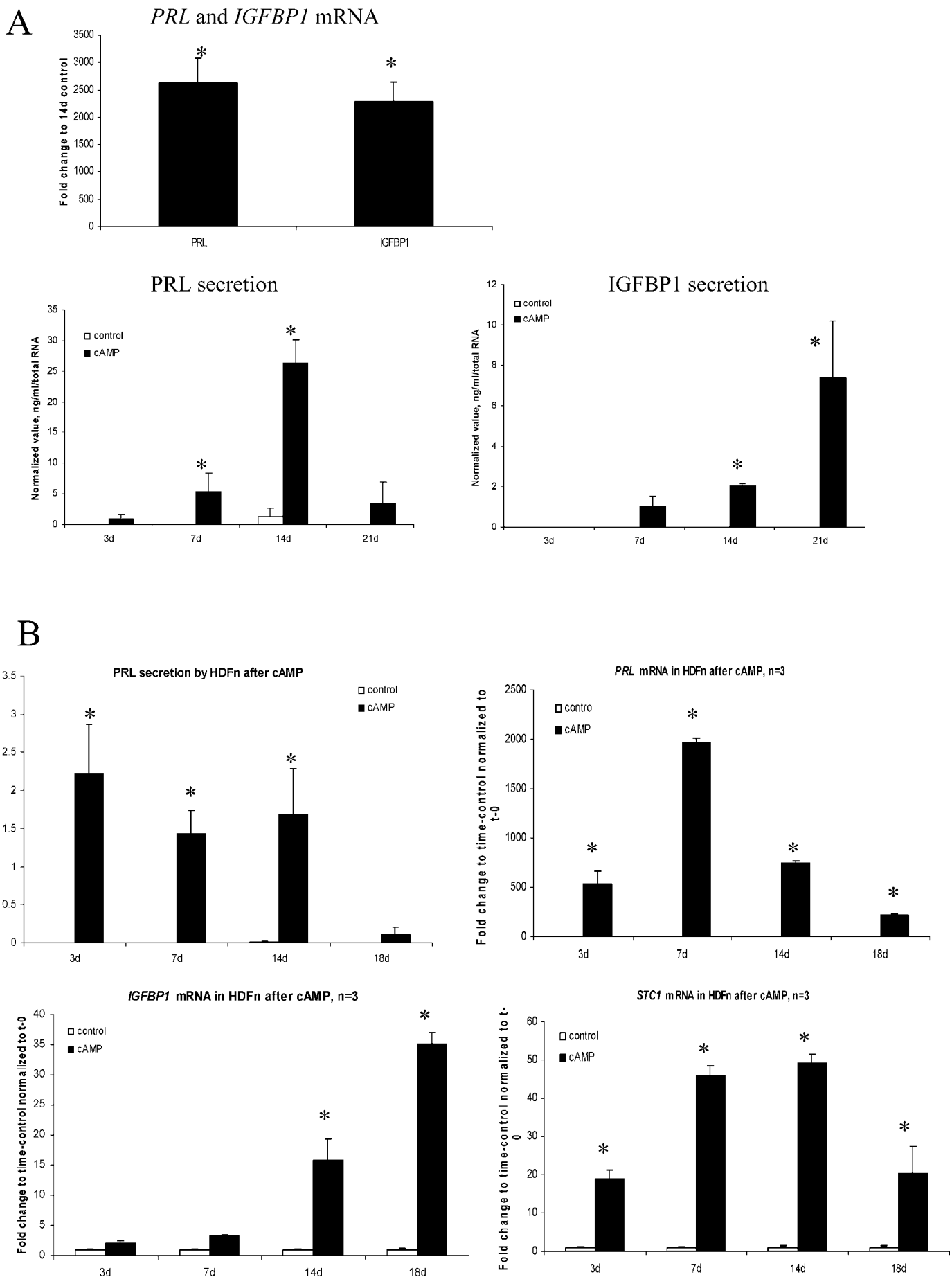
Alternative Fibroblast Cell Line Response to cAMP Treatment

To determine whether the 8-Br-cAMP-induced up-regulation of IGFBP1 and PRL in bone marrow-derived MSC was specific to this cell type (and hESF) or was a general feature of fibroblasts, we treated HDFn with 8-Br-cAMP and assessed expression of *PRL* and *IGFBP1* mRNAs and IGFBP1 and PRL protein secretion. Interestingly, HDFn secreted PRL protein at low levels, confirming a previous report [25], whereas no secretion of IGFBP1 by these cells was observed in response to cAMP treatment (Fig. 2B). However, HDFn expressed high levels of *PRL* mRNA with very low *IGFBP1* mRNA expression upon cAMP treatment (Fig. 2B) compared to levels in cAMP-treated hESF [20, 23] or MSC (Fig. 2A). We also investigated another cAMP-regulated gene, *STC1*, and found that its mRNA was expressed at very low levels in HDFn (Fig. 2B).

Expression of Hormone Receptors and Selected Steroidogenic Enzymes

We and others have previously found that hESF express estrogen receptors (*ESR*), P_4 receptors (*PGR*), androgen

FIG. 2. **A**) *PRL* and *IGFBP1* mRNA levels in MSC treated with 1 mM 8-Br-cAMP for 14 days expressed as fold-change compared to 14 days, vehicle control. Time course of PRL and IGFBP1 protein expression in conditioned media from MSC treated with 1 mM 8-Br-cAMP were normalized to total RNA levels (n = 3). **B**) Expression of PRL, IGFBP1, and STC1 mRNA and/or protein in HDFn (human dermal fibroblasts, neonatal) treated with 1 mM cAMP for 14 days. *PRL*, *IGFBP1*, and *STC1* mRNA levels are expressed as fold-change to time control (vehicle control at 3, 7, 14, and 18 days) normalized to $t = 0$. Note the difference in scale between **A** and **B**. PRL protein secretion at different culture time points was normalized to total RNA. No IGFBP1 secretion by HDFn occurred with or without cAMP treatment. Asterisk indicates statistically significant difference compared to vehicle control at each time point ($P \leq 0.05$). Error bars represent the SEM.



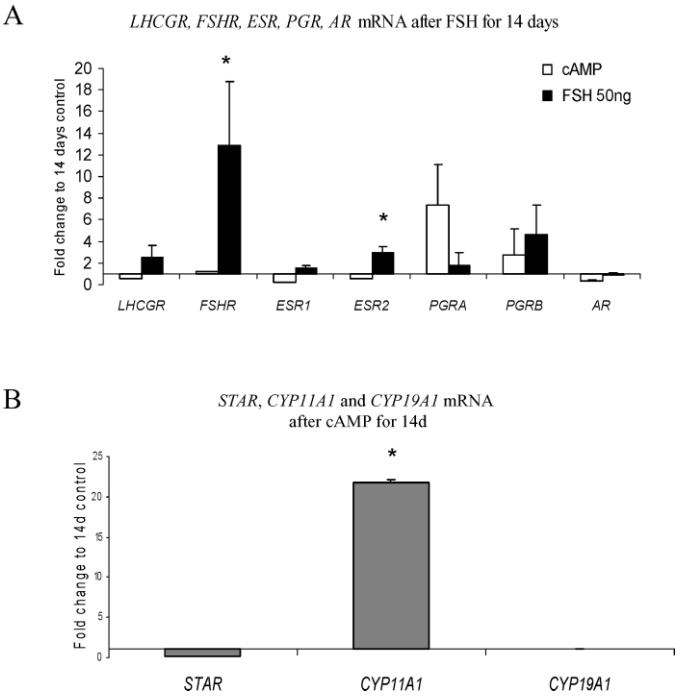


FIG. 3. **A**) LH receptor (*LHCGR*), FSH receptor (*FSHR*), estrogen receptors α and β (*ESR1* and *ESR2*), progesterone receptors A and B (*PGRA* and *PGRB*, respectively), and androgen receptor (*AR*) mRNA expression in MSC treated with 1 mM 8-Br-cAMP for 14 days or 50 nM FSH for 14 days expressed as fold-change compared to 14 days, vehicle control. **B**) Messenger RNA expression for steroidogenic enzymes *STAR, CYP11A1,* and *CYP19A1* in MSC treated with 1 mM 8-Br-cAMP for 14 days expressed as fold-change compared to 14 days, vehicle control. Error bars represent the SEM. $^*P \leq 0.05$.

receptors (*AR*), and gonadotropin receptors, as well as key steroidogenic enzymes [20, 26–29]. We found that MSC expressed *ESR*, *PGR*, and *AR* mRNAs, as well as luteinizing hormone (LH) receptor and FSH receptor (*LHCGR* and *FSHR*) transcripts in $t = 0$, vehicle control, and treatment groups at different time points, without significant influence of cAMP on their expression (Fig. 3A). Treatment with recombinant human FSH at low doses (50 nM) significantly up-regulated mRNA for *FSHR* and *ESR2* (12.5- and 2.5-fold, respectively) compared to vehicle control, whereas changes in *LHR*, *ESR1*, *PGR*, and *AR* were not significant (Fig. 3A). Expression of key steroidogenic enzyme *STAT*, *CYP11A1 (P450scc)*, and *CYP19A1 (aromatase)* transcripts were evaluated. *CYP11A1* mRNA was significantly up-regulated by 1 mM 8-Br-cAMP after 14 days of treatment (Fig. 3B), consistent with our earlier report on *CYP11A1* mRNA in cAMP-treated hESF [20].

Microarray Analysis

To comprehensively assess changes in gene expression in MSC, we performed comparative microarray analysis of MSC untreated and uncultured ($t = 0$; group A), MSC untreated and cultured for 14 days with vehicle control (group B), and MSC treated with 1 mM 8-Br-cAMP for 14 days (group C). Hierarchical clustering analysis of all genes demonstrated that samples clustered according to their assigned group (Fig. 4, A and B). PCA also demonstrated that group A clustered separately from groups B and C, with the latter two groups closer to each other but still distinct, demonstrating a distinctive effect of the culture process in general and 8-Br-cAMP in particular (Fig. 4C).

Network and Pathway Analysis and Gene Regulation

To assess the effect of 8-Br-cAMP on MSC, group C was compared to group B (Table 1). Results of the other comparisons can be found in Supplemental Tables S2 and S3. Ingenuity Pathway Analysis of group C versus group B identified 91 regulated genes, of which 78 were eligible for generating networks. Top networks activated by cAMP in MSC included 1) tissue development, cancer, and cellular growth and proliferation; 2) cell cycle, molecular transport, and gene expression; and 3) organismal functions, skeletal and muscular development and function, and connective tissue development and function, indicating the presence of differentiation signs.

Of note, the microarray data (Table 1) support our findings of cAMP induction in MSC of the decidualization biomarkers *PRL* and *IGFBP1* (Fig. 2A). Furthermore, they demonstrate up-regulation of other cAMP-regulated genes with known functions in endometrial physiology—specifically, solute carrier family 16, member 6 (monocarboxylic acid transporter 7) (*SLC16A6*); *IGF1*; inhibin beta A (*INHBA*); *VEGFA*; pappalysin (*PAPPA*); parathyroid hormone-like hormone (*PTHLH*); dual-specificity phosphatase 4 (*DUSP4*); and others. Moreover, 40 genes, presented in Table 1, were uniquely regulated in group C (cAMP-treated MSC) only and were not found in groups A and B.

Multiple canonical pathways were regulated in all three comparison groups of the present study, with involved genes being both up- and down-regulated. One should note that several molecules can participate in different pathways simultaneously. Treatment with cAMP compared to the vehicle control (group C vs. group B comparison) revealed the following pathways: activated hepatic fibrosis/hepatic stellate cell activation, caveolar-mediated endocytosis, arginine and proline metabolism, glycine, serine and threonine metabolism, and LXR/RXR activation pathways.

Microarray Result Validation with Quantitative Real-Time RT-PCR

Expression of selected genes (*STC1, SMOX, SPON1,* and *IGFBP5*) was validated with quantitative real-time RT-PCR (Fig. 5A) using the same samples that were used for microarray analysis. These data are consistent with and validate the microarray results, even though the scale of the fold-change is different.

Comparison of Response to 8-Br-cAMP in MSC Versus hESF

We compared two independent gene lists, MSC plus 8-Br-cAMP versus control with hESF plus 8-Br-cAMP versus control. Both used the same platform for analysis (Affymetrix Human Gene 1.0 ST arrays) [23] (unpublished data). We found 484 genes were up- and down-regulated in MSC treated with cAMP versus hESF plus cAMP (Supplemental Table S4). Of 484 genes, 333 were unique to the hESF response, 71 were unique to the MSC response, and 20 were in common (Fig. 5B and Table 1). Interestingly, in MSC, cAMP did not induce expression of somatostatin (*SST*), a known cAMP-regulated gene that is highly up-regulated in hESF upon cAMP treatment [19, 30]. The 20 genes in common are known to be involved in endometrial physiology, underscoring that activation of the PKA pathway in MSC directs this cell type down the hESF lineage, with genes in common that have physiologic relevance to the differentiation and function of the hESF (see below).

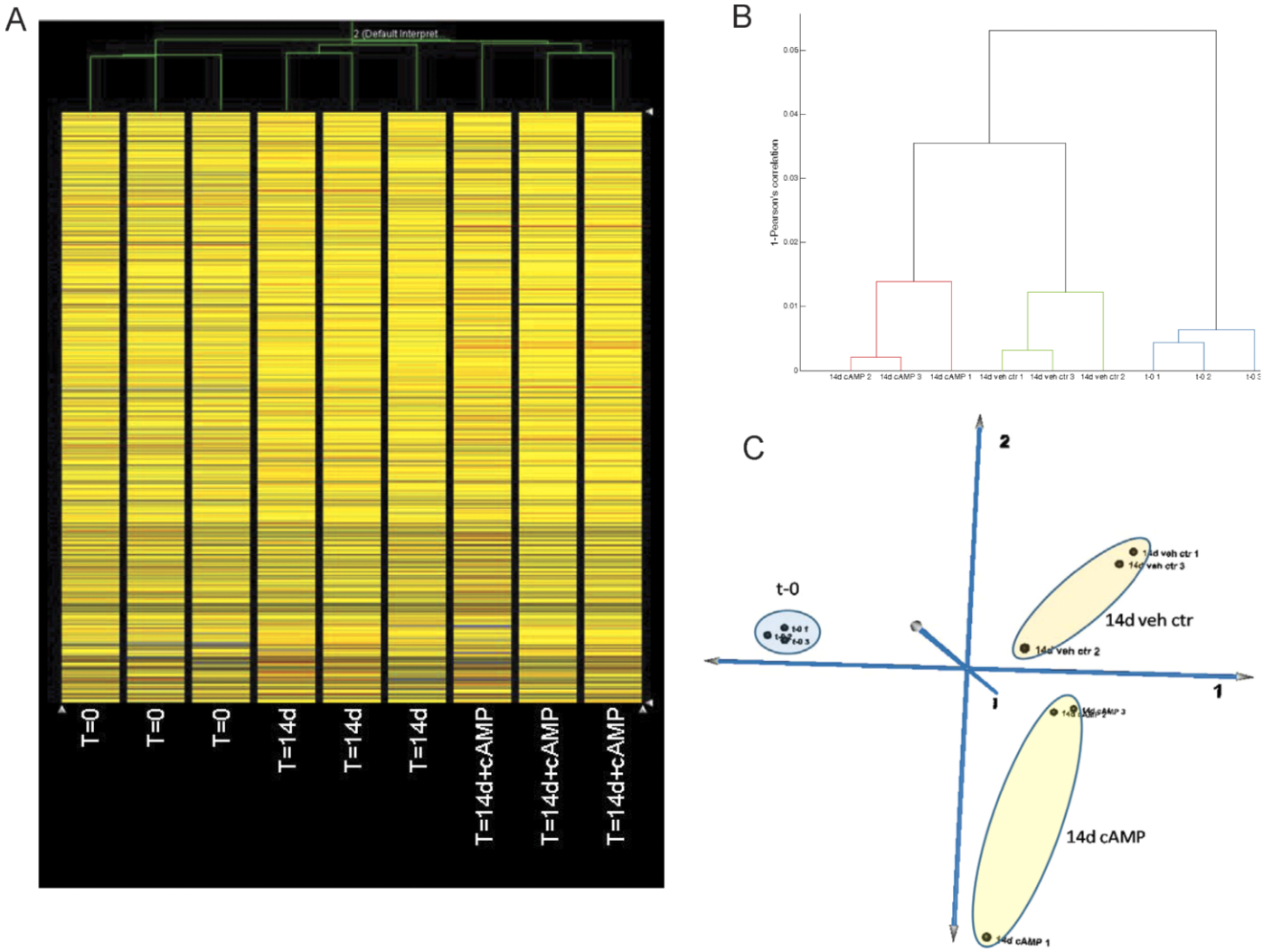


FIG. 4. **A**) Hierarchical clustering analysis of MSC at $t = 0$ (T=0), MSC treated with vehicle for 14 days (T=14d), and MSC treated with 8-Br-cAMP for 14 days (T=14d+cAMP) are also shown. **B**) Dendogram of hierarchical clustering analysis of MSC at $t = 0$ (t-0) and 14 days after vehicle (14d veh ctr) or 8-Br-cAMP (14d cAMP) treatment. **C**) PCA of MSC at $t = 0$ and 14 days after vehicle or 8-Br-cAMP treatment.

Novel Genes Highly Up-regulated in Both MSC Plus cAMP and hESF Plus cAMP

Stanniocalcin 1 (*STC1*), a calcium-regulating glycoprotein strongly expressed in decidualized stromal cells in rat uterus [31], was uniquely regulated in MSC only after cAMP treatment, with 40.2-fold up-regulation (Table 1). Comparably, it was 19-fold up-regulated in hESF from women without endometrial pathology upon decidualization with cAMP [23].

Spermine oxidase (*SMOX*), an enzyme that preferentially oxidizes spermine, a polyamine involved in cellular metabolism and suggested to play a role in immune privilege in the decidua [32], increased 17.4-fold in cAMP-treated MSC compared to cells treated with vehicle for 14 days. It was also 5.8-fold up-regulated in hESF in response to cAMP (Table 1) [23].

SLC16A6 (solute carrier family 16, member 6 [monocarboxylic acid transporter 7], MCT5) belongs to the monocarboxylate cotransporter (MCT) family, several members of which are important for lactate, pyruvate, and ketone body metabolism [33]. It was up-regulated 23.0-fold by cAMP in MSC of the present study. We found expression of *SLC16A6* in normal human endometrium using microarray, real-time RT-PCR, and immunohistochemistry [23, 34] (unpublished data). It was also up-regulated by 8-Br-cAMP in hESF (Table 1) [23].

Comparison of MSC and hESF Gene Signatures

Another important question when studying bone marrow-derived MSC and their differentiation toward the endometrial stromal lineage is how to distinguish the two cells types. Both share the same known stromal cell markers, and identification of genes specific for undifferentiated derived MSC are of great importance for both endometrial and stem cell biology.

Of 19 492 genes on the Affymetrix Human Gene 1.0 ST arrays, 281 genes were different between untreated-uncultured MSC ($t = 0$) and untreated-uncultured hESF ($t = 0$). Based on this gene list (Supplemental Table S5), several genes have been identified as molecular markers that distinguish bone marrow-derived MSC from endometrial stromal fibroblasts (before any differentiation agents). The most highly up-regulated gene (96-fold) was *ENPP2* (autotoxin, ectonucleotide pyrophosphatase/phosphodiesterase 2), which encodes a protein that functions as both a phosphodiesterase and phospholipase. *FOXD4L1* (forkhead box D4-like 1) was up-regulated 78.7-fold in MSC versus hESF; it belongs to FOX gene family. *EFEMP1*

TABLE 1. List of genes regulated in MSC after 14 days of treatment with 1mM 8-br-cAMP vs. MSC treated for 14 days with vehicle (control).[a]

Symbol	Fold change	Entrez gene name	Location	Type
STC1	**40.24**	**Stanniocalcin 1**	**Extracellular space**	**Kinase**
SLC16A6	**23.97**	**Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)**	**Plasma membrane**	**Transporter**
SPON1	21.59	Spondin 1, extracellular matrix protein	Extracellular space	Other
PRL	**21.13**	**Prolactin**	**Extracellular space**	**Cytokine**
IGF1	20.62	Insulin-like growth factor 1 (somatomedin C)	Extracellular space	Growth factor
IGFBP5	20.21	Insulin-like growth factor binding protein 5	Extracellular space	Other
PCSK1	19.81	Proprotein convertase subtilisin/kexin type 1	Extracellular space	Peptidase
SMOX	**17.44**	**Spermine oxidase**	**Cytoplasm**	**Enzyme**
BMP6	16.77	Bone morphogenetic protein 6	Extracellular space	Growth factor
HAS2	14.08	Hyaluronan synthase 2	Plasma membrane	Enzyme
CMKLR1	11.09	Chemokine-like receptor 1	Plasma membrane	G-protein coupled receptor
FAM65B	10.69	Family with sequence similarity 65, member B	Unknown	Other
DBC1	10.64	Deleted in bladder cancer 1	Nucleus	Peptidase
PTHLH	**10.57**	**Parathyroid hormone-like hormone**	**Extracellular space**	**Other**
TFPI2	10.57	Tissue factor pathway inhibitor 2	Extracellular space	Other
EGR2	10.39	Early growth response 2 (Krox-20 homolog, Drosophila)	Nucleus	Transcription regulator
VMO1	9.86	Vitelline membrane outer layer 1 homolog (chicken)	Extracellular space	Other
IGFBP1	**9.02**	**Insulin-like growth factor binding protein 1**	**Extracellular space**	**Other**
PDE1A	8.19	Phosphodiesterase 1A, calmodulin-dependent	Cytoplasm	Enzyme
SFRP1	8.04	Secreted frizzled-related protein 1	Plasma membrane	Transmembrane receptor
FGF10	7.89	Fibroblast growth factor 10	Extracellular space	Growth factor
INHBA	**7.72**	**Inhibin, beta A**	**Extracellular space**	**Growth factor**
C13ORF33	7.69	Chromosome 13 open reading frame 33	Unknown	Other
PDGFD	7.66	Platelet derived growth factor D	Extracellular space	Growth factor
GFPT2	7.45	Glutamine-fructose-6-phosphate transaminase 2	Unknown	Enzyme
PAQR5	6.60	Progestin and adipoQ receptor family member V	Unknown	Other
SESN3	6.45	Sestrin 3	Unknown	Other
BDKRB1	**6.12**	**Bradykinin receptor B1**	**Plasma membrane**	**G-protein coupled receptor**
ETV1	6.09	Ets variant 1	Nucleus	Transcription regulator
MMP16	5.84	Matrix metallopeptidase 16 (membrane-inserted)	Extracellular space	Peptidase
PALMD	5.84	Palmdelphin	Unknown	Other
AREG	5.74	Amphiregulin	Extracellular space	Growth factor
ADAMTS6	5.74	ADAM metallopeptidase with thrombospondin type 1 motif, 6	Extracellular space	Peptidase
NID2	5.52	Nidogen 2 (osteonidogen)	Extracellular space	Other
P4HA3	5.48	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III	Unknown	Enzyme
BDKRB2	5.14	Bradykinin receptor B2	Plasma membrane	G-protein coupled receptor
C17ORF60	5.12	Chromosome 17 open reading frame 60	Unknown	Other
ISM1	4.99	Chromosome 20 open reading frame 82	Unknown	Other
FER1L6	4.92	Fer-1-like 6 (C. elegans)	Unknown	Other
RASD1	4.75	RAS, dexamethasone-induced 1	Cytoplasm	Enzyme
DUSP4	**4.74**	**Dual specificity phosphatase 4**	**Nucleus**	**Phosphatase**
GAP43	4.71	Growth associated protein 43	Plasma membrane	Other
KCTD12	4.71	Potassium channel tetramerisation domain containing 12	Unknown	Ion channel
PITPNC1	**4.54**	**Phosphatidylinositol transfer protein, cytoplasmic 1**	**Cytoplasm**	**Transporter**
IER3	**4.32**	**Immediate early response 3**	**Cytoplasm**	**Other**
ANKH	4.29	Ankylosis, progressive homolog (mouse)	Plasma membrane	Transporter
SMOC1	4.15	SPARC related modular calcium binding 1	Extracellular space	Other
PID1 (includes EG:55022)	4.08	Phosphotyrosine interaction domain containing 1	Unknown	Other
PPP1R3C	3.98	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	Cytoplasm	Phosphatase
PLCB1	3.91	Phospholipase C, beta 1 (phosphoinositide-specific)	Cytoplasm	Enzyme
RAB27B	3.84	RAB27B, member RAS oncogene family	Cytoplasm	Enzyme
VEGFA	**3.67**	**Vascular endothelial growth factor A**	**Extracellular space**	**Growth factor**
CLGN	3.66	Calmegin	Cytoplasm	Peptidase
TNC	3.65	Tenascin C	Extracellular space	Other
ITGA2	3.55	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	Plasma membrane	Other
LGR4	3.46	Leucine-rich repeat-containing G protein-coupled receptor 4	Plasma membrane	G-protein coupled receptor
SLC2A12	3.34	Solute carrier family 2 (facilitated glucose transporter), member 12	Unknown	Transporter
TNFRSF19	**3.30**	**Tumor necrosis factor receptor superfamily, member 19**	**Plasma membrane**	**Transmembrane receptor**
TMOD1	3.30	Tropomodulin 1	Cytoplasm	Enzyme
SGCD	3.10	Sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)	Cytoplasm	Other
JARID2	3.08	Jumonji, AT rich interactive domain 2	Nucleus	Transcription regulator
GLIPR1	2.94	GLI pathogenesis-related 1	Extracellular space	Other
PLAT	**2.92**	**Plasminogen activator, tissue**	**Extracellular space**	**Peptidase**
PEG10	**2.90**	**Paternally expressed 10**	**Nucleus**	**Other**
FZD4	2.87	Frizzled homolog 4 (Drosophila)	Plasma membrane	G-protein coupled receptor
PAPPA	**2.69**	**Pregnancy-associated plasma protein A, pappalysin 1**	**Extracellular space**	**Peptidase**
LYPD1	2.65	LY6/PLAUR domain containing 1	Plasma membrane	G-protein coupled receptor
ENOX1	2.55	Ecto-NOX disulfide-thiol exchanger 1	Unknown	Other

TABLE 1. *Continued.*

Symbol	Fold change	Entrez gene name	Location	Type
GALNT12	2.39	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)	Cytoplasm	Enzyme
TCF21	2.35	Transcription factor 21	Nucleus	Transcription regulator
CDON	2.27	Cdon homolog (mouse)	Plasma membrane	Other
PCDHB4 (includes EG:56131)	2.27	Protocadherin beta 4	Plasma membrane	Other
SVIL	**2.27**	**Supervillin**	**Plasma membrane**	**Other**
GSTM4	2.24	Glutathione S-transferase M4	Cytoplasm	Enzyme
SLC6A9	**2.24**	**Solute carrier family 6 (neurotransmitter transporter, glycine), member 9**	**Plasma membrane**	**Transporter**
TRIM2	**2.21**	**Tripartite motif-containing 2**	**Cytoplasm**	**Other**
F2RL2	2.18	Coagulation factor II (thrombin) receptor-like 2	Plasma membrane	G-protein coupled receptor
TSHZ2	2.18	Teashirt zinc finger homeobox 2	Unknown	Other
IL4R	2.17	Interleukin 4 receptor	Plasma membrane	Transmembrane receptor
MTMR4	2.13	Myotubularin related protein 4	Cytoplasm	Phosphatase
NOTCH3	2.09	Notch homolog 3 (Drosophila)	Plasma membrane	Transcription regulator
GREM2	2.06	Gremlin 2, cysteine knot superfamily, homolog (Xenopus laevis)	Extracellular space	Other
TXNDC13	2.06	Thioredoxin domain containing 13	Cytoplasm	Enzyme
KIAA1632	−2.27	KIAA1632	Unknown	Other
VGLL3	**−2.30**	**Vestigial like 3 (Drosophila)**	**Unknown**	**Other**
TBC1D2	−2.43	TBC1 domain family, member 2	Unknown	Other
KRTAP1–5	−4.83	Keratin associated protein 1–5	Unknown	Other
PSG7	−7.03	Pregnancy specific beta-1-glycoprotein 7	Extracellular space	Other
KRT34	−8.32	Keratin 34	Cytoplasm	Other
PSG4	−8.65	Pregnancy specific beta-1-glycoprotein 4	Extracellular space	Other

[a] Boldface text indicates genes in common with hESF+cAMP vs. hESF treated with vehicle (control; n = 20).

(epidermal growth factor [EGF]-containing fibulin-like extracellular matrix protein 1, fibulin 3), an antagonist of angiogenesis [35], was 43.5-fold up-regulated in MSC versus hESF at $t = 0$. It is a partner with *TIMP3* (tissue inhibitor of metalloproteinases-3) [36], which was 4.2-fold up-regulated under the same conditions. Interestingly, several *MMPs* were significantly down-regulated in MSC versus hESF at $t = 0$, including *MMP1, MMP3, MMP10,* and *MMP12*, which were more than 30-fold down-regulated in MSC.

We found that 481 genes were different between untreated MSC cultured for 14 days (vehicle control) and untreated hESF cultured for 14 days (vehicle control) (unpublished gene list) (Supplemental Table S6). *ENPP2*, *FOXD4L1*, and *EFEMP1* were 34.6-, 90.3-, and 78.8-fold, respectively, higher in cultured MSC versus hESF, indicating the sustained differences between MSC and hESF between $t = 0$ and 14 days. *LOC391706* (ribosomal protein S3a pseudogene 18), *ALDH1A1* (aldehyde dehydrogenase 1 family, member A1), *CNTN1* (contactin 1, glycoprotein gP135), and WNT2 were the most (>390-, >179-, >56-, and >71-fold, respectively) down-regulated genes in MSC versus hESF in both comparisons.

Based on the above, we analyzed the mRNA expression of several potential markers distinguishing MSC from hESF and HDFn. As shown in Figure 6, *ENPP2* and *EFEMP1* mRNAs are highly expressed in undifferentiated, uncultured MSC, and their expression significantly decreases when MSC are differentiated with cAMP. Also, *ENPP2* and *EFEMP1* mRNA expression is very low in highly differentiated fibroblasts, such as hESF, and their expression in HDFn is modest and does not depend on culture conditions or cAMP. Similarly, *ALDH1A1* and *CNTN1* mRNA expression in MSC confirmed the microarray data and demonstrated that expression of these genes in undifferentiated, uncultured MSC is very low compared to that in more differentiated fibroblast cell types (Fig. 6). These data suggest that *ENPP2, EFEMP1, ALDH1A1,*

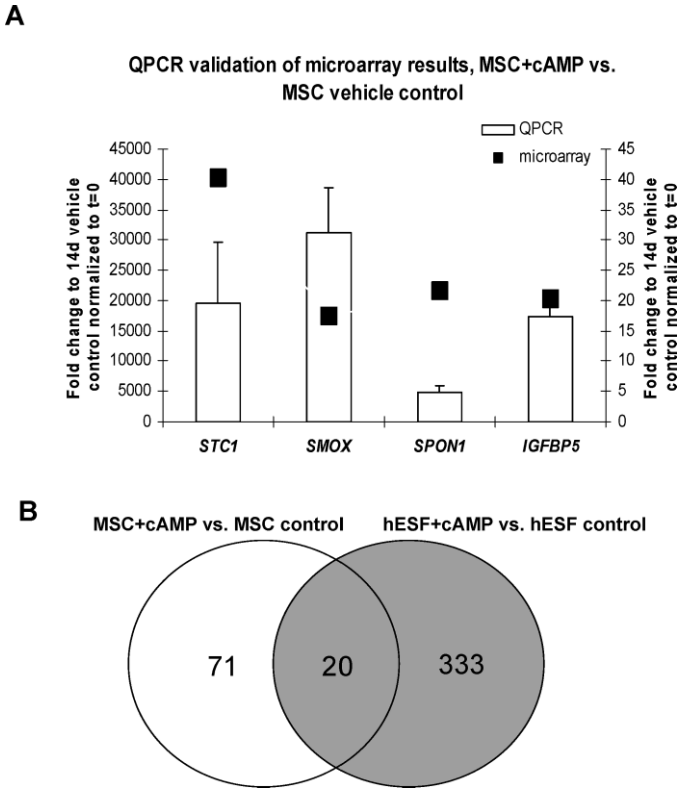


FIG. 5. **A**) Real-time RT-PCR validation of selected genes in MSC treated for 14 days with cAMP versus vehicle control. Correspondent microarray data for stanniocalcin 1 (*STC1*), spermine oxidase (*SMOX*), spondin 1 (*SPON1*), and IGF-binding protein 5 (*IGFBP*5) are presented. Data are presented as fold-change. Error bars represent the SEM. The left *y*-axis addresses quantitative PCR results; the right *y*-axis addresses microarray data. **B**) Venn diagram of common and unique genes expressed in cAMP-treated MSC and hESF compared to their respective controls.

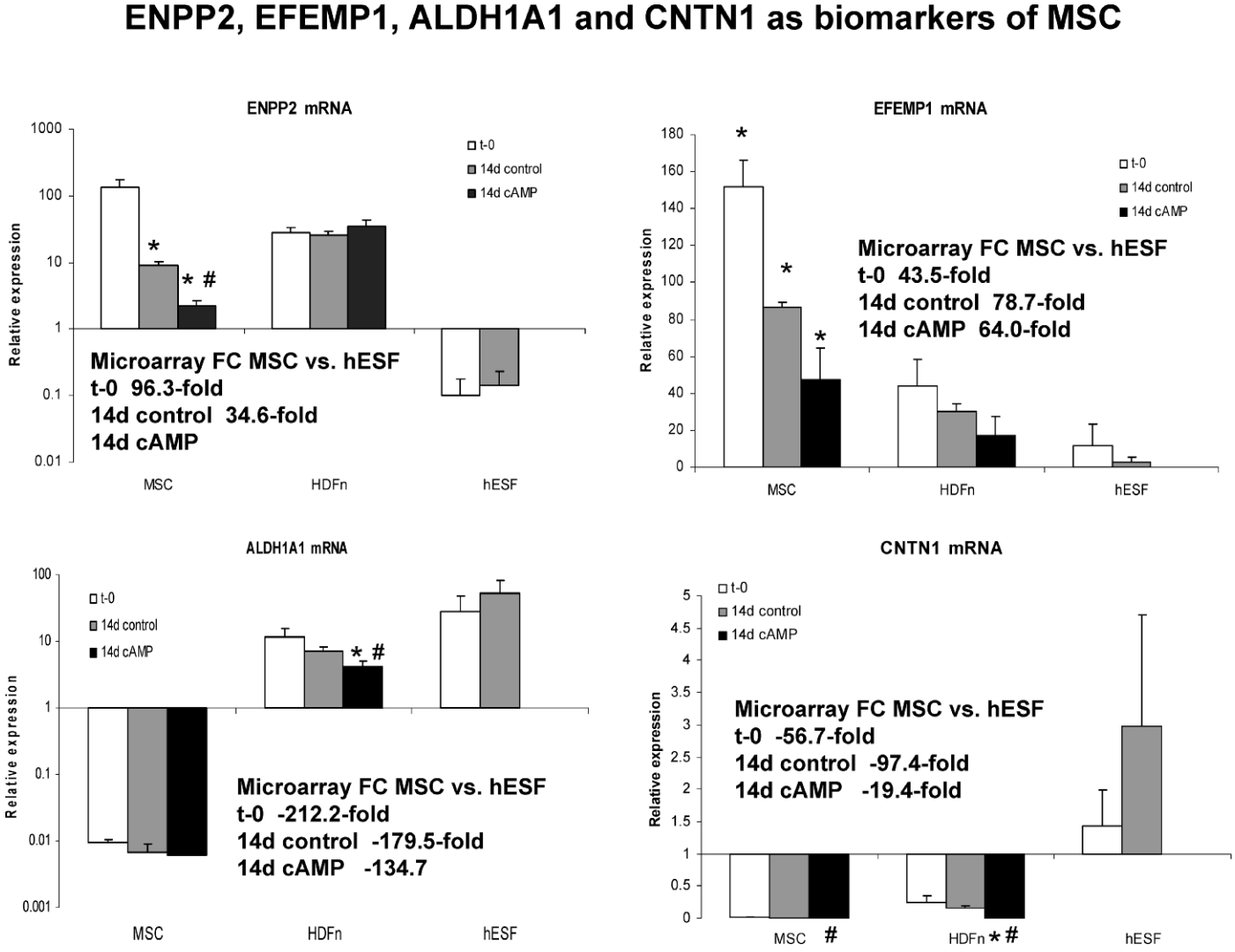


FIG. 6. Relative expression of *ENPP2*, *EFEMP1*, *ALDH1A1*, and *CNTN1* mRNA in MSC (t = 0 [t-0], 14-day control [14d control], and 14 days treated with cAMP [14d cAMP]), HDFn (t-0, 14d control, and 14d cAMP), and hESF (t-0 and 14d control). Inserts indicate the fold-change (FC) expression of genes in the microarray data set in the present study. The *y*-axis is presented as a log scale in the two graphs on the left. An asterisk indicates a statistically significant difference ($P \leq 0.05$) compared to t-0 control for each gene and each cell type; a number symbol indicates a statistically significant difference ($P \leq 0.05$) between vehicle control and cAMP-treated cells. Error bars represent the SEM.

and *CNTN1* may be used as markers of multipotent bone marrow-derived MSC.

DISCUSSION

Activation of the PKA Pathway, Cell Shape, and Decidualization Markers

The present study has demonstrated that PKA pathway activation in bone marrow-derived MSC results in 1) differentiation of this cell type; 2) expression of classical markers of decidualization, PRL and IGFBP1; 3) cell shape changes consistent with the decidualized endometrial stromal fibroblast (hESF) phenotype; and 4) expression of markers of differentiation down the hESF lineage in MSC in response to 8-Br-cAMP, as determined by a transcriptomic approach. Also, untreated MSC expressed mRNA for steroid hormone and gonadotropin receptors. Importantly, of all the different differentiation stimuli investigated, 8-Br-cAMP was the only agent able to stimulate the expression of decidualization markers in MSC and trigger the morphological transformation of these cells. In addition, we have discovered new markers of multipotent MSC that can distinguish them from hESF and, likely, other fibroblast cell types and enable tracking of the differentiation process down various lineages in vitro and in vivo.

As noted, treatment with 8-Br-cAMP resulted in the up-regulation of PRL and IGFBP1 decidualization marker mRNAs in MSC and secretion of the respective proteins. Pathway and network analyses demonstrated progression from activation of metabolism to differentiation in MSC upon culture and treatment with cAMP, accompanied by decidual morphologic transformation of MSC in vitro. Remarkably, comparison of the cAMP-regulated gene lists in MSC versus hESF demonstrated 20 genes in common, all of which are known to be involved in endometrial physiology [23, 34]. Thus, the data strongly support the idea that MSC can differentiate in response to cAMP down the endometrial stromal fibroblast lineage.

The question naturally arises about what stimulus of the PKA pathway initiates the responses observed herein with MSC. (In fact, the stimulus of activation of the PKA pathway is

unclear in human endometrial tissue.) We have considered that differentiation of progenitor cells may begin in the menstrual/postmenstrual repair phase, which is the time of the rise of FSH in the circulation. For that reason, we treated MSC with different doses of recombinant human FSH. However, no effect on decidualization gene expression, similar to the cAMP effect, was observed. Nevertheless, FSH was able to stimulate FSH receptor mRNA and tended to stimulate LH receptor mRNA, although this was not statistically significant. One may speculate that differentiation starts in the previous cycle with the rise of FSH so that cells are fully differentiated by menstruation and the early proliferative phase, when they are needed for regeneration.

Steroid Hormone and Gonadotropin Receptor mRNA Expression

It was unanticipated that multipotent MSC express *ESR*, *PGR*, *AR*, *FSHR,* and *LHCGR* mRNAs, and to the best of our knowledge, this is the first report on the expression of reproductive hormone receptors in human bone marrow-derived MSC. It was reported previously that bone marrow-derived MSC express specific neural proteins before any differentiation [37, 38], indicating that undifferentiated MSC residing in bone marrow express a variety of markers and receptors, probably in anticipation of a stimulus to trigger their differentiation.

Do All Fibroblasts Differentiate Similarly in Response to Identical Stimuli?

Controversial data exist regarding the ability of mature fibroblasts to differentiate in vitro toward different lineages. Some authors report that skin fibroblasts possess stem/progenitor cell properties and that differentiation toward adipocytes or osteoblasts does not distinguish MSC from more mature fibroblasts [39]. Others, however, have demonstrated that human skin or gingival fibroblasts are unable to differentiate in osteogenic medium [40], in contrast to MSC, which do differentiate in this medium [11, 41, 42]. A study by Richards and Hartman [25] demonstrated that dermal fibroblasts express PRL, but not IGFBP1, after prolonged treatment with dibutyryl-cAMP. Cervical fibroblasts decidualize after cAMP plus P_4 treatment for 6 and 10 days [43], and fibroblasts from term human decidua closely resemble endometrial stromal cells by induction of *PRL* and *IGFBP1* expression [44]. However, decidual fibroblasts may be considered to have properties similar to those of hESF because of their ''endometrial niche.''

Herein, we demonstrated that human neonatal dermal fibroblasts are not capable of comprehensive response to 8-Br-cAMP treatment compared to hESF, demonstrating that fully matured fibroblasts are probably restricted in their differentiation ability. This also makes us confident that the potential to differentiate down the endometrial-stromal lineage observed in cAMP-treated MSC is specific to the multipotent bone-marrow derived MSC.

MSC and the Endometrial Niche

The hypothesis that circulating bone marrow-derived MSC are a source of endometrial regeneration is to be considered. Hypothetically, the arrival of circulating MSC to different tissues may be driven by a combination of different chemoattractants and, thereafter, reside in a specific tissue, where they differentiate as needed. Final differentiation toward different lineages may be a result of the niche to which the MSC have migrated and settled. Thus, the niche may ultimately determine the lineage down which the MSC differentiate. Siddappa et al. [45] reported that activation of the PKA pathway by dibutyryl-cAMP in osteogenic medium resulted in increased expression of osteogenic markers and enhanced in vivo bone formation by bone marrow-derived MSC from orthopedic patients. With regard to endometrial stromal fibroblasts, we postulate that they may be derived/repopulated from bone marrow-derived circulating MSC, with the latter potentially differentiating upon arrival to endometrium in response to local stimuli and in the appropriate contextual niche. The longer time required to observe the decidualization response in MSC may result from the fact that MSC in their undifferentiated, naïve state require more time to acquire the endometrial phenotype compared to endometrial-stromal fibroblasts, which are already ''assigned'' a specific pheno- and genotype in endometrial niche.

The question arises whether sufficient numbers of circulating MSC and cells exiting from the bone marrow are available to regenerate and maintain the endometrium on a cyclic basis. It is of interest that in the study by Bian et al. [46], the percentage of circulating MSC ($CD105^+$, $CD34^-$, and $CD45^-$) in control subjects was 0.0028 ± 0.0016 (mean $\pm$ SEM). Similarly, Mansilla et al. [47] demonstrated that in healthy donors, $0.0078\% \pm 0.0044\%$ (mean $\pm$ SEM) of circulating cells are MSC. Furthermore, those studies demonstrated that the number of circulating MSC increases significantly during acute large skin damage (e.g., burns), with bone sarcomas, and with ischemic heart disease [48]. However, no information is available regarding the sufficient numbers of MSC needed to regenerate endometrium each month. Pelliccia et al. [49] showed increased numbers of subpopulations of circulating endothelial progenitor cells in normal postmenopausal women compared to men, whereas Herrmann et al. [50] showed that levels of circulating endothelial progenitor cells are greater in premenopausal women compared to postmenopausal women. Almost all of these studies were performed to determine the basis of cardioprotection in women compared to men and in premenopausal versus postmenopausal women. Whether these results may be applied to the numbers of MSC circulating with the purpose of endometrial regeneration is not known. One may assume that the numbers of circulating MSC would be higher in premenopausal women, particularly at the end of the menstrual cycle. Nevertheless, a quantitative study on the numbers of MSC in the circulation, doubling times in situ in the endometrium, and rates of steroid hormone-dependent differentiation would be needed to attempt to assess whether sufficient numbers of circulating MSC are available to regenerate and maintain endometrial stroma on a cyclic basis.

Unique MSC Biomarkers

The use of markers that will help to distinguish human bone marrow-derived MSC from fibroblasts of any origin, and from hESF in particular, is of value to understand the development and differentiation/regeneration processes of a variety of tissues. Several genes identified herein may serve as potential markers to distinguish MSC from hESF. Ishii et al. [40] showed that several markers differ between MSC and fibroblasts, such as higher expression of tissue factor pathway inhibitor-2, major histocompatibility complex-DR-alpha, major histocompatibility complex-DR-beta, and neuroserpin as well as lower expression of adrenomedullin, apolipoprotein D, C-type lectin superfamily member-2, collagen type XV alpha1, CUG triplet repeat RNA-binding protein, matrix metalloproteinase-1, protein tyrosine kinase-7, and Sam68-like phospho-

tyrosine protein/T-STAR. None of these genes, except for neuroserpin, *MMP1,* and protein tyrosine kinase-7, were regulated in the present study when comparing untreated-uncultured ($t = 0$) MSC and hESF or vehicle-treated cultured cells, suggesting that these genes are not as valuable in comparing MSC with hESF, as they are in other systems (e.g., periodontal ligament cells or human skin and gingival fibroblasts [40, 51]). Wagner et al. [52] demonstrated that ALDH1A1 transcript was down-regulated in human bone marrow-derived MSC versus human newborn foreskin fibroblasts, in agreement with our results. Shahdadfar et al. [53] identified ENPP2 as a human MSC-specific marker. However, we were not able to find data regarding the expression of EFEMP1 and CNTN1 in MSC versus other fibroblast sources. Herein, we suggest that high expression of *ENPP2* (prevents apoptosis [54]) and *EFEMP1*, as well as low or no expression of *ALDH1A1* (data are similar to human limbal stem cells [55]) and *CNTN1* (neural adhesion molecule and neuromuscular junction protein [56]), mRNA are characteristic for multipotent MSC and will aid in distinguishing MSC from endometrial stromal fibroblasts, in particular in studies with endometrial stem/progenitor cells. The level of expression of these markers in stem/progenitor cells isolated from endometrium is currently under investigation in our laboratory.

In summary, marked up-regulation of decidualization markers and changes in cell shape of MSC upon cAMP treatment support the hypothesis that bone marrow-derived MSC may be a potential source of endometrial stem/progenitor cells. Identification of MSC-specific markers distinguishing them from other fibroblasts and, in particular, from endometrial stromal cells is of biologic relevance and practical value to the field of endometrial stem cell research.

NOTE ADDED IN PROOF

Additional information regarding hierarchical cluster analysis of gene expression can be found in an article by Eisen et al., 1988 [57].

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