Characterization of clonogenic stromal cells isolated from human endometrium

R Dimitrov, T Timeva[1], D Kyurkchiev[2], M Stamenova, A Shterev[1], P Kostova[3], V Zlatkov[4], I Kehayov[5] and S Kyurkchiev[5]

Department of Immunochemistry, Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria, [1]Ob/Gy Hospital 'Dr Shterev', 1330 Sofia, Bulgaria, [2]Laboratory of Clinical Immunology, University Hospital 'Sv.I.Rilski', 1431 Sofia, Bulgaria, [3]Ginecological Clinics, National Oncology Hospital, 1756 Sofia, Bulgaria, [4]University Ob/Gyn Hospital 'Maichin Dom', 1437 Sofia, Bulgaria and [5]Department of Molecular Immunology, Institute of Biology and Immunology of Reproduction, Bulgarian Academy of Sciences, 73 Tzarigradsko Shosse, 1113 Sofia, Bulgaria

Correspondence should be addressed to S Kyurkchiev; Email: skyurkchiev@mail.bg

Abstract

Human endometrium is an object of extensive restructuring and remodeling during the female reproductive life and it is quite tempting to assume that these periodic changes happen with the participation of cells that should have the basic characteristics of multipotent cells. The aim of this study was to search for the presence of cells with plastic adherence, clonogenicity, and differentiation in human endometrium. To this end, human endometrial stromal cells were cultured *in vitro* for more than 15 passages. Flow cytometry analysis of the cultured cells showed that they were positive for CD29, CD73 and CD90, which are considered to be the markers of cells with mesenchymal origin. The cells were negative for the hematopoietic cell markers (CD45, CD34, CD14, CD3, CD19, CD16/56, and HLA-DR). Further, it was shown that the cultured cells had 15% clonogenic efficiency and could be induced to differentiate into adipogenic cells containing typical lipid-rich vacuoles. These results demonstrate that the human endometrium contains a low number of cells with the characteristics of endometrial stromal stem/progenitor cells, which seem to belong to the family of the mesenchymal stem cells. It can be speculated that these cells are engaged into the monthly restructuring and remodeling of human endometrium.
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Introduction

Human endometrium is characterized with an exclusively intensive and cyclic regeneration in the course of the menstrual cycle. During the female reproductive period of the woman, the endometrium undergoes hormone-driven dynamic changes including self-renewal, proliferation, differentiation, and shedding off. These characteristics of the endometrium are suggestive of the presence of an adult stem/progenitor cell population, which is responsible for the expressed regenerative capacity of this tissue. So far, mesenchymal stem cells (MSC) have been reported to be present in a number of tissues in adult organisms – intestines (Bjerknes & Cheng 1999), muscles (Jankowski *et al.* 2003), skin (Alonso & Fuchs 2003), blood (Spangrude *et al.* 1991), mouse endometrium (Chan & Gargett 2006), and the nervous system (Morrison *et al.* 1999, Uchida *et al.* 2000, Thomas *et al.* 2001).

Stem cells are generally characterized by their self-renewal capacity by symmetric cell division and their ability to differentiate into various tissues by asymmetric cell division (Gargett 2007). Detection of stem cells in various tissues is rather difficult because of the fact that they are a minor population, and there are still no definite markers for their identification and characterization. For the identification of tissue-specific stem/progenitor cells, the demonstration of their clonogenicity and differentiation capacity is the most commonly used approach. Clonogenicity is the capability of the cells to form cell colonies originating from a single cell.

It is known that the endometrium consists of luminal and glandular epithelial cells, stromal fibroblasts, vascular smooth muscle cells, endothelial cells, and leukocytes forming basal and functional layers. The glandular epithelial layer is formed by epithelial cells that are considered to be in a highly differentiated state. The stroma consists of stromal cells originating from the mesoderm (Gargett 2007). Recently, it was reported that cells with some features of adult stem/progenitor cells such as clonogenicity are present in human endometrium. Using purified single-cell suspensions from endometrial tissue, Chan *et al.* (2004) showed that

0.22% of the epithelial and 1.25% of the stromal cells can form single colonies when seeded at 500 cells/cm^2 for epithelial cells and 300 cells/cm^2 for stromal cells. Cell subpopulations with the features of stem cells have been detected in a number of mammalian species, including humans, using the fluorescent stain Hoechst 33342 and these minor populations are termed side population (SP) cells (Goodell *et al.* 1996). Further, Kato *et al.* (2007) have isolated such cells from human endometrium and showed that they can be categorized as progenitor cells because they can differentiate into epithelial and stromal endometrial cells. Cervello *et al.* described a population of label-retaining cells (LRCs) in mouse endometrium which express markers specific for undifferentiated cells – c-kit and pou5f1 (Chan & Gargett 2006, Cervello *et al.* 2007). These cells are localized in the lower region of the stromal layer and are supposed to be real progenitor cells. So far, the studies reported have been directed to proving the existence of progenitor cells in the endometrium, which have rather limited potential of differentiation as they have been shown to differentiate into epithelial or stromal endometrial cells.

The MSC are multipotent somatic cells capable to differentiate *in vitro* into various cell types from both mesodermal and non-mesodermal origin. Populations of mesenchymal stem cells have been isolated and characterized from a number of different adult tissues such as bone marrow, skeletal muscles, adipose tissue, peripheral blood, connective tissues of the dermis, and umbilical cord blood (Javazon *et al.* 2004, da Silva Meirelles *et al.* 2006). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has clearly defined the strict criteria for the definition of a selected cell population as multipotent mesenchymal stromal cells. First, MSC must be plastic adherent when maintained in standard culture conditions. Secondly, MSC must express CD105, CD73, and CD90, and lack the expression of CD45, CD34, CD14, or CD11b, CD79α, or CD19 and HLA-DR surface molecules and thirdly, MSC must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* (Dominici *et al.* 2006).

The aim of the present study is to demonstrate the presence of a population of stromal stem/progenitor cells in human endometrium with potential for plastic adherence, clonogenicity, and differentiation into one of the mesenchymal cell lineages, which would characterize them as endometrial stromal stem/progenitor cells.

Results

Morphology of cultured cells

Initially, there were both epithelial and stromal cells in the cultures but after the first passage the fibroblast-like cells were growing as single colonies that gradually merged and formed a monolayer while the epithelial cells died around day 7–8 of culture. After 10–14 days, the cultures predominantly consisted of spindle-like cells with round, centrally located nuclei, which formed swirled structures when forming a monolayer. With each passage, the cell population became more homogenous, and after the third passage the spindle-like cells were the only cell type detected in the culture (Fig. 1A and B). In our experiments, at each passage, the cells were seeded at cloning concentrations of 200–400 cells/cm^2 so in that way populations of cloning cells were selected.

Markers expressed by the cultured cells

Cultured endometrial cells at fourth passage were analyzed by flow cytometry as described in the Materials and Methods section. The results clearly demonstrate that the endometrial cells are negative for any of the markers specific to hematopoietic cells such as CD45, CD14, CD19, CD56/16, CD34 and CD3 when analyzed
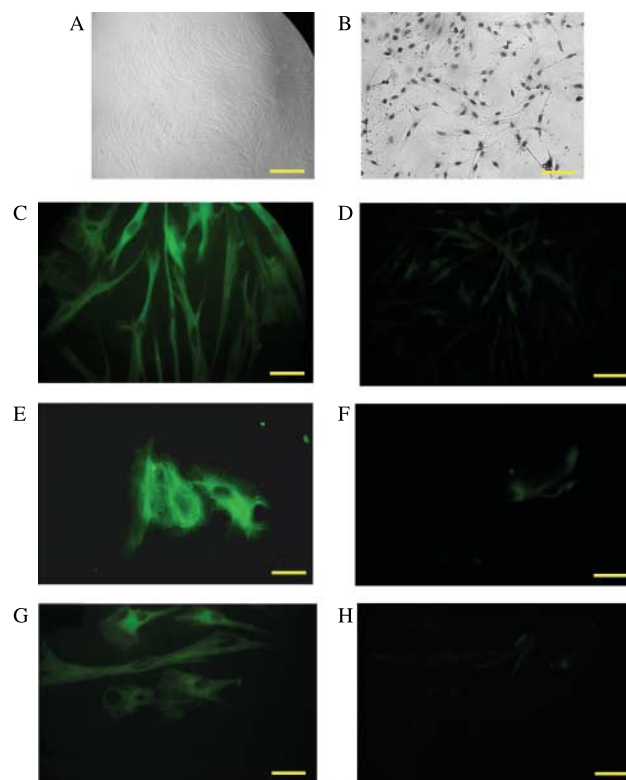

Figure 1 Morphology and indirect immunofluorescence of cultured human stromal endometrial cells. (A) Confluent swirled layer of fibroblast-like cells is formed about 14 days of culture (scale bar=400 μm), (B) after Giemsa staining of sub-confluent cells, the fibroblast-like morphology of the cells is clearly seen (scale bar=100 μm), (C) vimentin positive staining is located in the cytoskeleton of the cells, (D) negative control for vimentin (scale bar=20 μm), (E) positive granular and perinuclear staining for proMMP-3, (F) negative control for proMMP-3 (scale bar=20 μm), (G) positive staining for CD105 is located in the cytoskeleton, (H) negative control for CD105 (scale bar=20 μm). These data are representative for all six samples collected from patients.

by flow cytometry and some cells expressed HLA-DR. The cells were found to be positively stained for CD29, CD73, and CD90, which are markers considered to be specific to cells of mesenchymal origin (Fig. 2).

Further, it was shown by indirect immunofluorescence that the cells were positively stained for vimentin (Fig. 1C), prometalloproteinase-3 (proMMP3; Fig. 1D), and CD105-endoglin (Fig. 1E) as each antigen showed
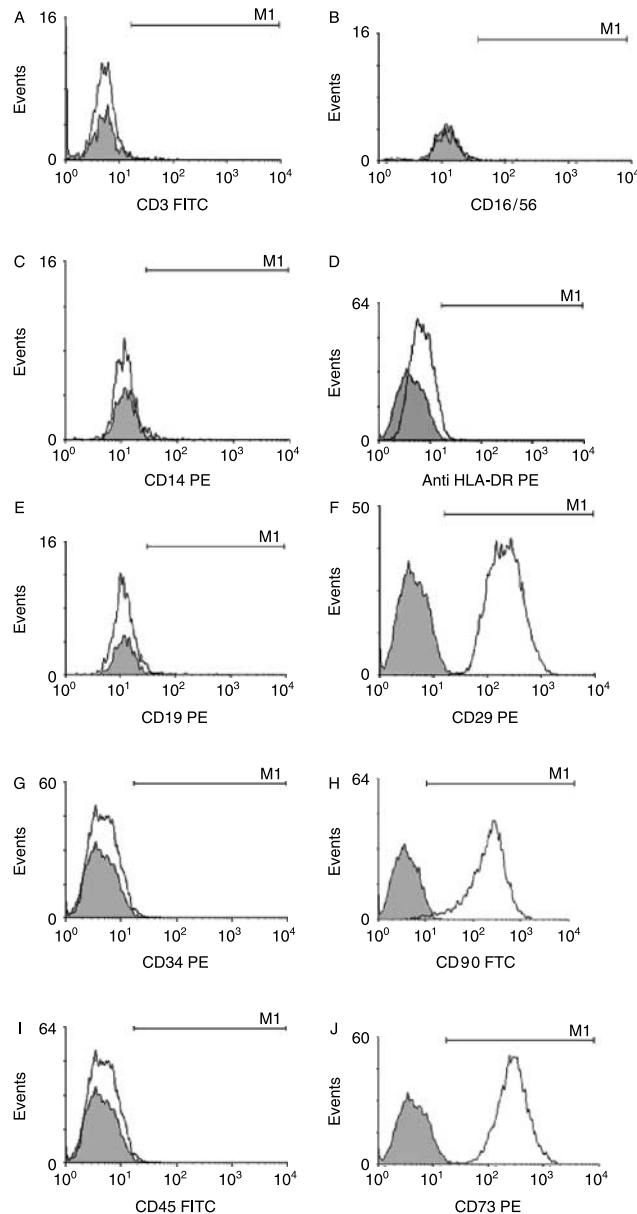

Figure 2 Flow cytometry analysis of cultured endometrial stromal cells. The shaded area is the isotype control. The cell cultures are negatively stained for CD45, CD14, CD19, CD56/16, CD34, CD3, and HLA-DR. Endometrial stromal cells are positively stained with anti-CD90, anti-CD73 monoclonal antibodies, and anti-CD29 antibody. These data are representative for all six samples collected from patients. Markers analyzed are as follows: (A) CD3, (B) CD16/56, (C) CD14, (D) HLA-DR, (E) CD19, (F) CD29, (G) CD34, (H) CD90, (I) CD45 and (J) CD73.

the typical cellular localization. Vimentin was located on the cytoskeleton fibers and proMMP3-positive staining was granular and perinuclear, suggesting its localization in the endoplasmic reticulum. Endoglin was located on the cytoskeleton fibers (versus endothelial cells where CD105 is located on the membrane), which is result of different expression related to function in cytoskeletal organization (Conley *et al*. 2004, Sanz-Rodriguez *et al*. 2004). S100A13 was detected within the cytoplasm of the cells. The cells were completely negative for cytokeratin, metalloproteinases (MMP-1, MMP-2, and MMP-9) and von Willebrand factor. The overall expression or lack of expression of specific markers by the endometrial cells cultured and characterized in these experiments is presented in Table 1. Taken together, the data obtained on the expression of several stem cell markers strongly suggest that the stromal cells are of mesenchymal origin.

Clonogenicity of the endometrial stromal cells

However, the morphology, growth characteristics, and cellular phenotype of the endometrial stromal cells very much resemble the same features of the mesenchymal stem cells and this made us conduct some experiments to look for some other characteristics of the mesenchymal stem cells, such as clonogenicity and differentiation.

To test the clonogenicity, cultured cells at the fourth passage were trypsinized and plated at concentrations 10, 20, 200, or 400 cells/cm^2. The cell growth was monitored by microscopy to ensure that each colony originates from a single cell. After incubation for 15 days, the cell colonies observed consisted of elongated spindle-like cells at the periphery while the central part was formed of rather small round cells (Fig. 3B). The cloning efficiency (CE) for that cell population was 15.4±0.9% (*n*=6), 11.6±0.4% (*n*=6), 2.1%±0.2% (*n*=6), and 1.3±0.16 (*n*=6) when the cells were seeded

Table 1 Overall phenotypic characteristics of the endometrial stromal stem/progenitor cells isolated and cultured in these experiments.

Markers	Immunohisto-chemical characterization	Markers	FACS analysis
Vimentin	Positive	CD45	Negative
MMP-3	Positive	CD14	Negative
Endoglin	Positive	CD19	Negative
S100A13	Positive	CD56/16	Negative
Cytokeratin	Negative	CD3	Negative
MMP-1, MMP-2, and MMP-9	Negative	CD34	Negative
FGF-1	Negative	HLA-DR	Negative[a]
von Willebrand factor	Negative	CD29	Positive
		CD73	Positive
		CD90	Positive

[a]1.2–3.4% of the cells are HLA-DR positive.

at concentrations of 10, 20, 200, and 400 cell/cm^2 respectively (Fig. 3C). There are statistical significances between CE at different cell densities. The endometrial samples were collected at both proliferative and secretory phases and the stage of menstrual cycle did not influence the clonogenic potential of isolated stromal cells. Notably, in the cell density 200 and 400 cell/cm^2, many colonies were observed but it was not possible to definitely discriminate the colonies with single-cell origin.

Differentiation in adipogenic cells

Clonogenic endometrial cells cultured from fourth passage cells were induced to differentiate into adipogenic cells by culturing the cells in the presence of inducing factors as described in the Materials and Methods section. The capacity for adipogenic differentiation of endometrial stromal cells was demonstrated by the formation of neutral lipid-containing vacuoles in the cell cytoplasm, visualized by Oil Red O staining. It should be underlined that the bulk of the clonogenic endometrial cells were differentiated into adipogenic cells (Fig. 4A). This fact clearly demonstrates that the cultured cells were homogeneous populations. Cells isolated from the six
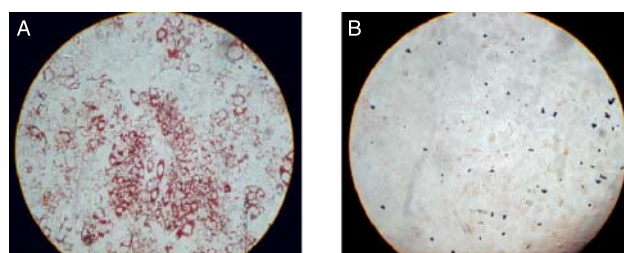

Figure 3 Clonogenicity of human endometrial cells cultured *in vitro*. (A) Wells of six-well plates seeded with 400 cells/cm^2. Separate colonies originating from single cells are seen. (B) Separate colonies consisting of fibroblast-like cells after Giemsa staining are seen. (C) Quantitative data of cloning efficiency for different cell densities ($n=6$). Results shown are means±s.e.m. of $n=6$ patient samples. Statistical significance between cloning efficiencies at different cell densities is shown (*$P=0.00026$; **$P<0.0001$; ***$P=0.0003$).

Figure 4 Adipogenic differentiation was demonstrated by the accumulation of neutral lipid vacuoles stainable with Oil Red O. (A) Cells treated with adipogenic factors and (B) non-induced controls; (magnification, ×200).

samples differentiated in the same manner when processed and cultured under the conditions described. This adipogenic phenotype was not observed in the control cells cultivated only in DMEM-10% FCS (Fig. 4B).

Discussion

Recently, a number of authors have suggested that the high regenerative capacity of human endometrium is possible due to the presence of a minor cell population with the basic features of stem cells (Gargett 2007). However, until now the existence of stem cells in the endometrium was supported only by indirect data.

It is known that the human endometrium grows from 0.5–1 mm right after menstruation to 5–7 mm at the end of each menstrual cycle (McLennan & Rydell 1965), and this process is characterized with intensive cell proliferation and differentiation followed by shedding off in each cycle. To explain this process, it has been proposed that progenitor cells are located in the basal layer of human endometrium (Prianishnikov 1978, Padykula *et al*. 1984, Padykula 1991). The progeny of these cells are rapidly proliferating cells (transient cells) that then move to the functional layer (Padykula *et al*. 1989, Okulicz *et al*. 1997) and actively participate in the regeneration and remodeling of the endometrium. These assertions are based mainly on kinetics studies and zonal distribution in the endometrium.

Another indirect proof is the long-term culture (more than 20 months) of eutopic endometrium-derived stromal cells that have preserved their proliferative and clonogenic capacities (Tanaka *et al*. 2003). Similarly, in our experiments endometrial stromal cells were cultured for 10–15 sequential passages (more than 20 months), frozen, and thawed, and still they preserved their proliferation rate and clonogenicity.

Still, another approach is the use of the LRC technique for the *in vivo* localization and detection of adult stem cells. LRC stromal cells that expressed markers for rather undifferentiated cells such as c-kit and pou5f1 have been detected in mouse endometrium (Cervelló *et al*. 2007). Epithelial endometrial LRC cells are found as scattered
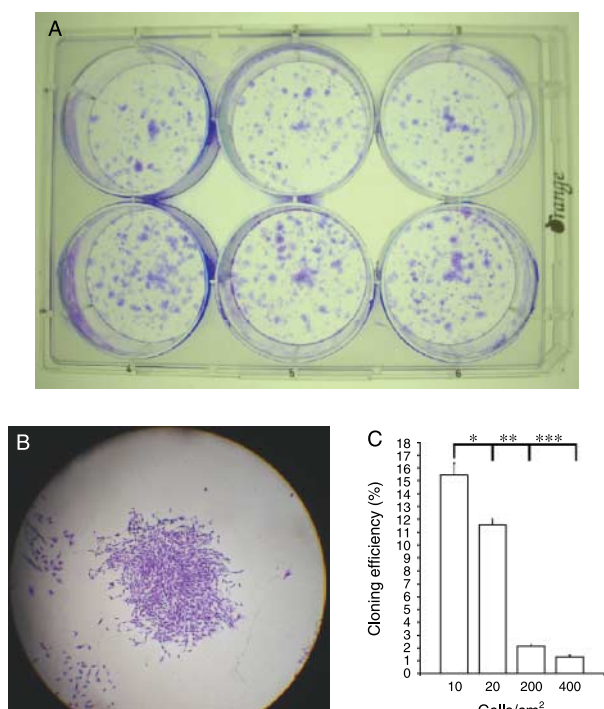
single cells in the luminal epithelia and the endometrial glands, which would suggest their participation in the formation and the growth of the glands in mouse endometrium. Both epithelial and stromal endometrial LRC cells are negative for the expression of CD45, which may suggest that they do not originate from the bone marrow-derived hematopoietic cell lineages according to Chan & Gargett (2006). However, this assertion seems to be an open question because Taylor (2004) has reported that after allogeneic transplantation of bone marrow in patients, donor cells are found in the endometrium of the recipients.

Kato *et al.* (2007) stained the SP cells in human endometrium with fluorochrome and isolated this population. The cells showed morphology of small round cells and could be cultured for a long time and could be differentiated as epithelial CD9+ or stromal CD13+ cells. The cells described are quite different in their morphology and proliferative potential compared with those in our cultures but are similar in the lack of expression of CD34 and CD45 markers. The cells described by Kato *et al.* (2007) could not grow in medium supplemented just with 10% fetal calf serum (FCS) but in the presence of 100 ng/ml interleukin-6, 10 ng/ml thrombopoietin, 100 ng/ml stem cell factor, and 10% FCS on a collagen-coated plate or feeder cells proliferated with rather low rate. So it is clear that this cell population cannot be classified as mesenchymal stem cells since it has not been shown whether they can differentiate into osteogenic or adipogenic cell lines. It is reasonable to assume that these cells represent a population of progenitor endometrial cells with limited differentiation potential.

Identification of stem/progenitor cells by their functional properties such as clonogenicity is a promising approach. Similarly to the findings of (Chan *et al.* 2004, Schwab *et al.* 2005) the cells in our experiments formed individual cell clones originating from a single cell as the CE was 1.3% for 400 cell/cm^2, 2.1% for 200 cell/cm^2, 11.16% for 20 cells/cm^2, and 15% for 10 cells/cm^2. There was a tendency towards a decrease in the clonal efficiency percentages with the increase of the number of seeded cells per cm^2, which might be due to the fact that at high numbers some colonies become confluent and it is difficult to discriminate between single colonies. In general, these findings substantiate the report of Colter *et al.* (2000) that MSC expanded more rapidly when seeded at very low densities (1, 5–3 cells/cm^2). However, the important fact is that the cell populations cultured in our experiments seem to have higher clonogenicity in comparison with the cells described by others (Chan *et al.* 2004, Schwab *et al.* 2005). The reason for this discrepancy might be in the different approaches applied in these experiments, since Chan *et al.* (2004) used freshly isolated endometrial cells while in our experiments a cultured cell population that selects clonogenic cells was used.

In our studies, we have isolated cells from samples consisting of both functional and basal layers of the endometrium. Upon long-term cultures (four to five passages), the cells were shown to be positive for vimentin and proMMP3 and negative for cytokeratin, which identified the cultured cells as stromal endometrial cells. Further analysis by flow cytometry convincingly showed that these cells did not express any of the hematopoietic cells markers (CD45, CD14, CD19, CD56/16, CD4, and CD8) but were highly positive for the set of markers known to be specific for mesenchymal stem cells CD73, CD29, and CD90. A small percentage (1.2–3.4%) of the cultured cells were positive for expression of HLA-DR, which is not surprising since a similar finding has been reported before for mesenchymal stem cells (Potian *et al.* 2003). The expression of these phenotypic markers was stable in cells cultured for four to eight passages. In our experiments, it was not possible to analyze freshly isolated endometrial cells by flow cytometry because the population was heterogeneous since groups of epithelial cells were present. Contrary to this, Schwab & Gargett (2007) have reported that fluorescence-activated cell sorting (FACS) can be applied to isolated typical mesenchymal-like stem cells from suspensions of endometrial cells. They have shown that CD146+PDGF-Rb+ cells are enriched in clonogenic cells with the capacity to differentiate as adipogenic, osteogenic, myogenic, and chondrogenic cells.

Our results, showing that cells isolated from human endometrium after cultivation *in vitro* can differentiate into adipogenic cells full of neutral lipid granules, are in agreement with these findings. A definite limitation of our study is that a single marker for adipogenic differentiation was examined, but this marker is generally accepted in a number of published papers describing the characterization of mesenchymal stem cells isolated from different sources. However, the experiments to induce osteogenic differentiation of the cultured cells (unpublished data) were not successful under the conditions applied in our laboratory. This may imply either the need to find the optimal conditions for their osteogenic differentiation or a lower capacity to differentiate in comparison with bone marrow- or adipose-derived mesenchymal stem cells. Still another explanation might be that there are very low numbers of such cells in the populations cultured for several passages.

There are different speculations concerning the origin of the putative multipotent stromal cells in human endometrium. It has been supposed that low numbers of fetal epithelial and stromal cells reside in the endometrium and participate in the processes of its regeneration and remodeling (Gargett 2007). However, the different clonogenic capacity, the different expression of phenotype markers and the different growth requirements would suggest the possibility for

the existence of at least two different types of multipotent progenitor cells residing in endometrium. Another possibility is that an early undifferentiated population is present, which has a wider plasticity and differentiation potential and higher proliferation rate. If that is the case, it can be speculated that the endometrium forms a specific stem cell 'niche' populated with adult stem/progenitor cells. However, there is obviously a need for further detailed studies in order to identify and characterize multipotent endometrial cells, which make possible the cyclic regeneration and remodeling of human endometrium.

Another field of study is the potential role of multipotent endometrial stem cells in the pathophysiology of diseases such as endometriosis, endometrial hyperplasia, endometrial cancer, and adenomyosis, which are associated with abnormal endometrial proliferation (Gargett 2004, 2006, Du & Taylor 2007). Alterations in the number, function, regulation, and location of endometrial stem/progenitor cells may be involved in the pathology mechanisms of these endometrial diseases. Progress in this field of study will not only increase the understanding of the pathogenesis of these diseases but also has the potential to discover new approaches in the treatment of these diseases.

Materials and Methods

Human endometrial tissues

Samples from endometrium were obtained from six ovulating women (32–52 years old) undergoing hysterectomy for non-malignant uterine tumors – adenomyosis and fibroids. The endometrial samples were collected at both proliferative and secretory phases. The day of the cycle day was established histologically as routinely done, and samples from both proliferative and secretory phases were processed. The samples were put into PBS (pH 7.2) containing 100 IU/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (PAA Laboratories, Vienna, Austria). The samples of endometrium were collected after an informed consent was signed by each patient in accordance with the requirements of the Ethical Committee of the Obstetric and Gynecology Hospital.

Isolation and culture of cells from human endometrium

As soon as the samples were delivered to the laboratory, the endometrial tissue was carefully dissected from the underlying myometrium and minced to 1–2 mm^3 pieces with sharp scissors and incubated with HBSS containing 0.1% collagenase type IA (GIBCO-BRL) in a humidified 5% CO_2/95% air atmosphere at 37 °C for 1 h. The suspension was then filtered through a 150 μm wire sieve to remove mucus and undigested tissue. The filtrates were centrifuged at 250 g for 5 min to collect cells. Cell pellets were resuspended in complete culture medium – DMEM supplemented with 10% charcoal-stripped heat-inactivated fetal bovine serum (PAA Laboratories), streptomycin (100 U/ml; PAA Laboratories), and penicillin

(100 U/ml; PAA Laboratories). The cells were plated in six-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium). The cells were allowed to adhere overnight in a humidified 5% CO_2/95% air atmosphere at 37 °C and non-adherent cells were discarded. The culture medium was replaced every 48 h. For passaging, the cells were washed with PBS (pH 7.2) and treated with 0.05% trypsin/0.2% EDTA (PAA Laboratories) for 15 min at 37 °C, complete DMEM was added to stop the enzyme reaction and the cells were counted in a hemocytometer, centrifuged, and resuspended in complete culture medium.

For immunofluorescence staining, trypsinized cells were plated on cover slips and after 24 h slips were fixed in −20 °C methanol for 10 min and treated with antibodies as described below. Morphology of cultured cells was assessed and documented after staining with Giemza and hematoxylin–eosion following standard laboratory procedure.

Flow cytometric analysis

Immunophenotyping of the cultured cells was performed using flow cytometry. In flow cytometry analysis, 1 ml samples of cells, concentration 3×10^6/ml were used. Flow cytometry analysis was performed with antibodies against CD45, CD34 CD14, CD19, CD56/16, CD3, HLA-DR, CD29, CD73, and CD90, as all antibodies were purchased from Becton Dickinson, (Temse, Belgium). Following washing twice in PBS, the cells were fixed in FIX solution (Becton Dickinson) as recommended by the producer company. The specific fluorescent labeling was analyzed at FACSCalibur flow cytometer (Becton Dickinson) using the Cell Quest software program of the same company as 10 000 events were analyzed.

Indirect immunofluorescence

Cultured cells were characterized by indirect immunofluorescence using polyclonal rabbit anti-human von Willebrand Factor antibody, anti-vimentin, anti-cytokeratin (DAKO, Glostrup, Denmark), anti-proMMP3 (clone 10D6), anti-human metalloproteinase -1, 2, 9, (R&D Systems, Minneapolis, MN, USA), anti-endoglin (CD105), and anti-S100A13 rabbit polyclonal antisera (Molecular Immunology, IBIR-BAS, Sofia, Bulgaria) as primary antibodies. Anti-rabbit IgG and anti-mouse IgG FITC-labeled antisera were used as a second antibody (SAPU, Lanarkshire, Scotland).

After fixation with ice-cold methanol, the cells were incubated for overnight at 4 °C with the corresponding primary antibody. After three rinses in PBS for 5 min, each cover slip was incubated depending on the species specificity of the first-layer antibody with FITC-conjugated second antibody (FITC anti-mouse IgG, SAPU) or FITC anti-rabbit IgG second antibody (SAPU) for 1 h at room temperature and again washed in PBS (three times, 5 min each). The cover slips were mounted in Mowiol-118 mounting medium (Hoechst, Frankfurt, Germany) and read under an epi-fluorescence microscope (Leitz, Wetzlar, Germany).

Clonogenicity of the endometrial stromal cells

To test the clonogenicity of the isolated cells, different number of cells were seeded on fibronectin (10 μg/ml)-coated plastic surface in six-well plates (Orange Scientific) as described by Chan *et al.* (2004). Briefly, cells at the fourth passage were gently treated with 0.05% trypsin/0.2% EDTA (PAA Laboratories) to obtain single-cell suspensions and adjusted to clonal density 10, 20, 200, and 400 cells/cm^2 and seeded in triplicates in six-well plates. Cells were cultured in DMEM (PAA Laboratories) supplemented with 10% fetal calf serum (PAA Laboratories), 2 μM L-glutamine, and antibiotics. The cultures were incubated for 15 days at 37 °C, 5% CO_2 as the medium was refreshed every 3–4 days. Every day the cells were controlled microscopically to ensure the single-cell origin of the clones. At the end of the incubation period (15 days), the cells were washed three times with PBS (pH 7.2) and stained with Giemsa solution following routine histological technique. Clusters of cells were considered colonies when they were visible macroscopically and contained >50 cells. CE was determined from the formula CE (%) = (number of colonies/number of cells seeded)×100.

Differentiation as adipogenic cells

When the clonogenic cells reached more than 80% confluence, they were induced to undergo adipogenic differentiation following the protocol of Kern *et al.* (2006). Briefly, cells were grown in an adipogenic induction medium (DMEM supplemented with 10% FCS, 1 μM dexamethasone (Sigma–Aldrich), 10 μg/ml bovine insulin (Sigma–Aldrich), 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma–Aldrich), and 200 μM indomethacin (Sigma–Aldrich)). After a period of 3 days, the adipogenic induction medium was replaced with an adipogenic maintenance medium consisting of DMEM supplemented with 10% FCS, 1 μM dexamethasone, and 10 μg/ml bovine insulin and cells were then incubated for another 3 days. Non-induced control cells were maintained only in DMEM supplemented with 10% FCS for the same periods of time. After three cycles of replacement of the induction/maintenance medium, at day 18 the cells were stained with Oil Red O (Sigma–Aldrich). For Oil Red O staining, the cells were rinsed with PBS and fixed in 10% formalin neutral solution (Merck) for 30 min at room temperature. The cells were washed with water and then stained with a 0.6% Oil Red O solution (three parts 1% Oil Red O dye in isopropanol and two parts distilled water) for 1 h at room temperature, followed by washing with distilled water to remove the unbound dye (Kume *et al.* 2005).

Statistical analysis

Quantitative data for CE are represented as means ± S.E.M. and analyzed by Mann–Whitney test to determine the statistical significance between different cell densities. Results were considered statistically significant when $P < 0.05$.

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