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ISOLATION OF MULTIPOTENT MESENCHIMAL STROMAL CELLS FROM MINIMAL HUMAN ENDOMETRIUM BIOPSY

A. V. Zlatska[1, 2]
A. E. Rodnichenko[1, 2]
O. S. Gubar[2, 3]
D. O. Zubov[1, 2]
R. G. Vasyliev[1, 2]
S. M. Novikova[1]

[1]State Institute of Genetic and Regenerative Medicine of the National Academy of Medical Sciences of Ukraine, Kyiv
[2]Biotechnology Laboratory "ilaya Regeneration", Medical Company ilaya, Kyiv
[3]Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine, Kyiv

E-mail: alenazlacka@gmail.com

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The aim of the research was establishing a cell culture from a minimal human endometrial biopsy and assessment its conformity with the criteria for multipotent mesenchymal stromal cells. It was shown that cells in the culture possess adhesion to plastic, have characteristic fibroblast-like morphology, express $CD73^+CD90^+CD105^+$, and are negative for hematopoietic markers ($CD34^-CD45^-HLA\text{-}DR^-$), have the ability to directed adipogenic, osteogenic and chondrogenic differentiation. Due to these properties, the cell population isolated from the minimal endometrial biopsy can be attributed to multipotent mesenchymal stromal cells.

Key words: human endometrium, multipotent mesenchymal stromal cells.

The active development of regenerative medicine is associated with the research in the field of cell biology, biotechnology and the search for new therapeutic approaches to the treatment of a number of diseases. The development of auxiliary reproductive technologies has allowed many infertile couples to get the desired pregnancy. A prerequisite for a successful pregnancy is not only the high quality of the embryos, but also a healthy receptive endometrium. It is known that thin (less than 5mm in the middle of the cycle) endometrium reduces the probability of pregnancy up to 0.5–1.0% [1].

Reducing female fertility has a number of causes, among which the proportion of the uterine factor in the isolated or mediated variants is 24–62% [2]. It is known that the frequency of pathological changes in the endometrium in women's infertility is 88%, in cycles using alternative method of auxiliary reproductive technology — ART — 77.5% [3].

The causes of endometrium hypoplasia may be the inflammatory diseases of the pelvic organs, changes in the hormonal homeostasis, medical manipulations, surgical interventions associated with the basal layer of endometrium injury [4].

Gynecologists are working on solving this problem, but the proposed methods do not provide a guaranteed result. Existing methods are based on three main approaches: mechanical (surgical), physical and biological effects on the endometrium.

An alternative method for thin non-peritoneal endometrium restoring is the use of autologous multipotent mesenchymal stromal cells (MSCs).

The popularity of MSCs is due to their immunomodulating properties, significant proliferative potential and the ability to differentiate in cell types — derivatives of mesenchyma [5–7]. The most common source of MSCs is bone marrow [8], although they can be obtained from adipose tissue, pulp of the tooth, skin, cord blood, placenta, etc. [9–13].

Recently, more and more attention has been focused on new sources for MSCs. Endometrium is perspective in this regard as this tissue regenerates on a monthly basis.

Endometrium is a unique structure that is completely restored more than 500 times during the woman's reproductive age [14]. The endometrial basal layer contains a pool of multipotent cells that provides processes for regeneration and reorganization of endometrium tissue. These include stem and progenitor cells — endothelial, epithelial and mesenchymal stromal cells [15, 16].

The first own population of endometrial multipotent mesenchymal stromal cells (eMMSCs) was isolated from the endometrium [17] and later from menstrual blood, in 2004 [18]. High proliferative activity, potential for multiline differentiation and karyotypic stability make this cell type an attractive subject for research and possible use in cell therapy.

The aim of the work was to isolate a population of cells from a minimal endometrial biopsy and to verify their compliance with the criteria for the assessment of MMSCs [19] — adherence to plastic in standard culture conditions, fibroblast-like morphology, expression of a typical phenotype ($CD73^+CD90^+CD105^+CD34^-CD45^-HLA\text{-}DR^-$) and the ability for direct differentiation *in vitro*.

Materials and Methods

eMMSCs obtaining and cultivation

Endometrial samples ($n = 5$) were obtained by biopsy in the proliferative phase of the menstrual cycle from women with endometrial hypoplasia. The age of patients was 34 ± 3.3 yrs. In all cases, voluntary informed consent was signed. The fragments of endometrium were dissociated by enzymatic treatment for 50 min in a solution of 0.1% collagenase IA and 0.1% pronase with the addition of 2% fetal bovine serum (FBS). The resulting suspension of cells was cultured in DMEM/F12 medium with the addition of 10% FBS, 2 mM glutamine and 1 μg/ml of FGF-2 (all — Sigma, USA) in multi-gas incubators at 37 °C, absolute humidity, 5% CO_2 and 5% concentration of O_2. eMMSCs were selected as a cell fraction that adhered to the plastic in 24–48 h after transferring the suspension to a culture vessel.

To study the phenotype and the ability to directed adipogenic, osteogenic and chondrogenic differentiation, cell cultures of the 3rd passage were used.

Directed adipogenic, osteogenic and chondrogenic differentiation

Directed adipogenic and osteogenic differentiation was performed according to generally accepted methods [20].

For confirmation of osteogenic and adipogenic differentiation, cells were fixed in a 10% formalin solution for 20 min, washed with phosphate buffer and stained for 2 min with Alizarin Red S (pH 4.1 for mineralized extracellular matrix detection) or 0.5% Oil Red solution O (for detection of lipid inclusions, all — Sigma, USA). The cells differentiated in the adipogenic direction were additionally contrasted with Romanowsky stain for 20 min.

Chondrogenic differentiation was carried out using the micromass method (300,000 cells were centrifuged to obtain a precipitate in 15-ml test tubes (Nunc, USA) for 14 min, 2000 rpm) in chondrogenic inductive medium containing DMEM-HG with high (4.5 g/l) glucose content (PAA, Germany) with addition of 50 μg/ml ascorbate-2-phosphate (Sigma, USA), 40 μg/ml L-proline (Sigma, USA), 100 μg/ml sodium pyruvate (Sigma, USA), 10 ng/ml *rh*TGF-β3 (Sigma, USA), 10^{-7} M dexamethasone (Sigma, USA), 1% ITS (containing 6,25μg/ml insulin, 6,25 μg/ml transferrin, 6,25ng/ml selenic acid), 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid (BD Biosciences, USA) and incubated for 21 days in a CO_2 incubator at 37 °C and 5% of CO_2. Change of medium was carried out every 3rd day.

As a result, at the 21st day of induction, a dense chondroid was formed at the bottom of the test tube. The chondroid was further cut on the microtome. To determine acid glucose amine glycans (GAGs) the resulting slices (10 μm thick) were stained with a 0.1% solution of Safranin O (Sigma, USA) or a 1% solution of Alcycan Blue (Sigma, USA).

Flow cytometry analysis

The cell phenotype was assessed by fluorescence-activated cell sorting (FACS) on BD FACS Aria flow cytometer (BD Pharmingen, BD Horizon USA). Staining with monoclonal antibodies (PerCP-Cy5.5 mouse anti-human CD105, APC mouse anti-human CD73, FITC mouse anti-human CD90, PE-Cy5 mouse anti-human HLA-DR, APC mouse anti-human CD34, FITC mouse anti-human CD45) was done in accordance to manufacturer's instructions (BD Pharmingen, BD Horizon USA). The analysis was performed using BD FACS Diva 6.1 software (BD Pharmingen, BD Horizon USA).

Microscopy

Axio Observer A1 inverted fluorescence microscope was used, equipped with AxioCam ERc 5s digital camera and ZEN 2012 software (all — Carl Zeiss, Germany).

All numeric data is represented by the mean ±standard deviation (M ± S.D.).

Results and Discussion

When choosing a source for MMSCs one should keep in mind the patient's safety, invasiveness of material sampling and potential for obtaining a sufficient number of cells at the selection stage.

The most common MMSCs source is hypodermic fatty tissue, which is associated with a significant release of cells at the selection stage. So, from 1 g of adipose tissue $0.5–2.0\times10^6$ cells of the stromal-vascular fraction can be isolated in average, which gives 1–10% of stem cells output [21]. Some sources argue that the number of isolated cells can be even larger and will be about $2–6\times10^6$ cells from 1 ml of tissue [22]. The number of MMSCs isolated from 1 g of adipose tissue varies between 0.5×10^4 and 2×10^5 [23]. This is a significant indicator, compared with the MMSCs part in bone marrow, where the MMSCs content is only 0.001–0.01% [24].

These differences are due to the characteristics of the donor, such as gender, age, ethnicity, history of the present disease. In addition, it depends on the type of tissue, location, method of tissue sampling and culture conditions [25].

In this work, after 5–14 days of eMMSCs cultivation, the obtained primary population of cells numbered from $0,6\times10^6$ to 2×10^6 cells. The average cell number at P0 was $1.07\pm0.49\times10^6$ cells in 9±3.8 days after isolation.

One of the MMSCs characteristic features is their ability to adhere to plastic under standard cultivation conditions. One day after transferring the cell suspension into a culture vessel, the cells began to adhere to plastic.

The cells in the culture had a fibroblast-like morphology, actively proliferated and formed a monolayer, as shown in Fig. 1.

The results of immunophenotype study of the P3 cell culture are presented in the Table. Representative FACS histograms are demonstrated in Fig. 2 .

The expression level of MMSCs positive markers was: CD90 — 95.44±3.3%, CD105 — 96.3±2.7%, CD73 — 97.6±1.9%. The percentage of cells expressing hematopoietic markers was: CD34 — 0.92±0.4%, CD45 — 0.36±0.3%, HLA-DR — 0.76±0.2%.

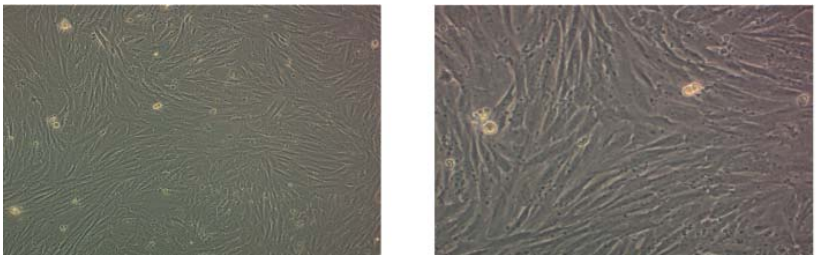

Fig. 1. **eMMSCs morphology at P3: phase contrast microscopy**

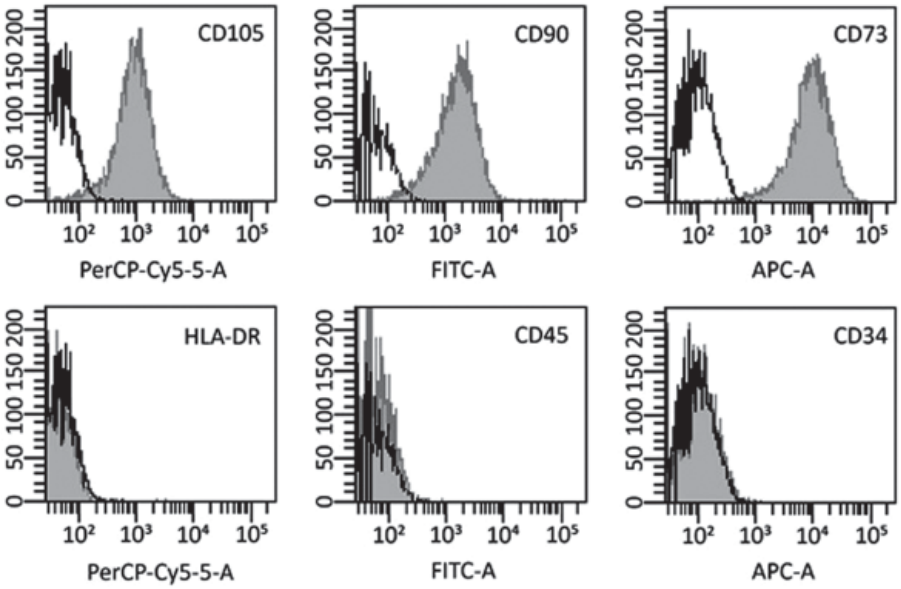

Fig. 2. **Representative FACS histograms**

Table. **Immunophenotype of human eMMSCs**

	CD90	CD105	CD73	CD34	CD45	HLA-DR
No. 1	95,7%	98,6%	99,6%	1,2%	0,1%	0,9%
No. 2	90%	92,6%	94,6%	1,4%	0,9%	0,9%
No. 3	97,8%	94,3%	97,9%	0,6%	0,2%	0,9%
No. 4	98,2%	98,4%	98,4%	0,8%	0,2%	0,6%
No. 5	95,5%	97,65	97,5%	0,6%	0,4%	0,5%
Mean±SD	95,44±3,3%	96,3±2,7%	97,6±1,9%	0,92±0,4%	0,36±0,3%	0,76±0,2%

Note: the results in the table are presented in the form of averages and standard deviations of 5 donors. It cannot be specified the significance level between values as the indicators of phenotypic markers expression. The level of their expression (high or low) is of great interest.

Thus the investigated cell cultures demonstrate the characteristic $CD73^+CD90^+CD105^+CD34^-CD45^-HLA\text{-}DR^-$ phenotype that meet the minimum criteria for the MMSCs definition.

In accordance with the minimum MSC criteria of the International Society for Cellular Therapy, multiple potency is a mandatory property of any MMSCs and is determined by their ability to direct *in vitro* differentiation into mesenchymal cell derivatives (adipocytes, osteoblasts and chondrocytes).

Fig. 3 shows the directed adipogenic and osteogenic differentiation of human eMMSCs. All differentiated cultures have acquired specific characteristics.

In detail, after 14 days of cultivation in adipogenic inductive medium, the cells began to accumulate lipid vacuoles. Interestingly, differentiated eMMSCs formed small lipid vacuoles located in the perinuclear region. In contrast, bone marrow MMSCs and subcutaneous adipose tissue undergo adipogenic differentiation in different way: their lipid vacuoles are significantly larger and are evenly distributed throughout the cytoplasm of the cell.

eMMSC demonstrated typical osteogenic differentiation, the cells matrix began to mineralize from the 14^{th} day of cultivation in the osteoinductive medium. Alizarin Red S staining showed a positive reaction to the Ca-containing mineralized cellular matrix at the 21^{st} day after induction.

As for the chondrogenic differentiation, a dense chondroid was obtained after 21 days of chondrogenic induction. Fig. 4 shows the cytochemical detection of acid glycosaminoglycans using Alcian Blue (*A*) staining and the Romanowsky (*B*) staining.

Thus, isolated from minimal endometrium biopsy, eMMSCs meet minimum ISCT (International Society for Cellular Therapy) criteria for MMSCs, such as adherence to plastic in standard culture conditions, fibroblast-like morphology, expression of a typical phenotype ($CD73^+CD90^+CD105^+CD34^-CD45^-HLA\text{-}DR^-$) and ability for direct differentiation *in vitro*. The possibility of eMMSCs isolation from a minimal endometrial biopsy opens up the new perspectives for their use in regenerative medicine, as well as their potential for drug testing in pharmacology, which makes further investigation of this cell type very promising.

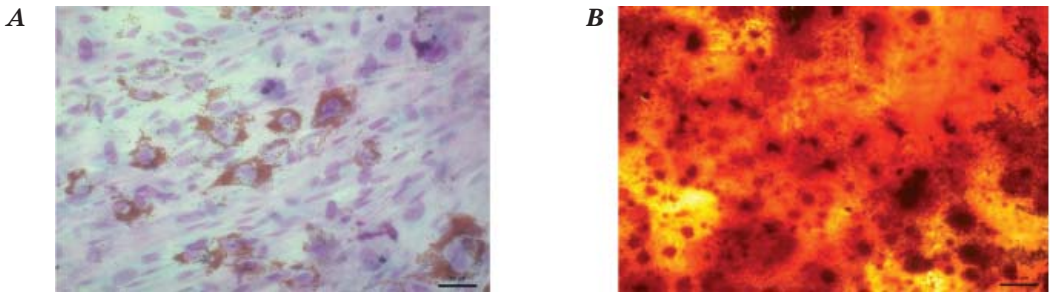

Fig. 3. **Directed adipogenic (*A*, Oil Red O and Romanowsky stain) and osteogenic (*B*, Alizarin Red S stain) differentiation of human eMMSCs:** light microscopy

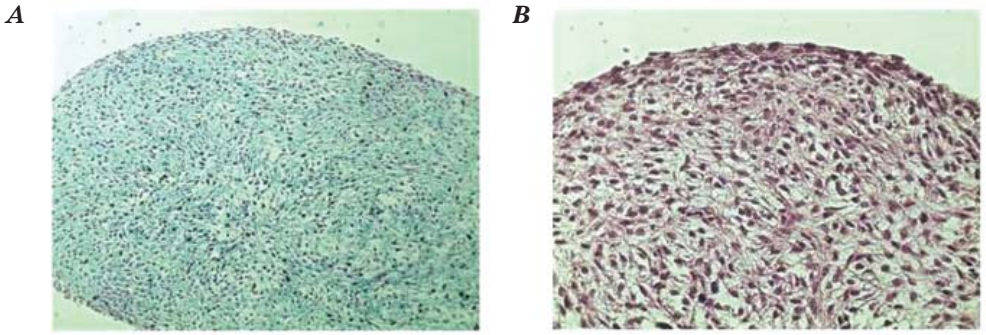

Fig. 4. **Alcian Blue stain, 5× (*A*) and Romanowsky stain, 10× (*B*):** light microscopy

Considering the above-mentioned, human eMMSCs are of specific interest for further investigation for the use in regenerative and reproductive medicine, the pharmacological industry, as well as for the fundamental research.

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ОТРИМАННЯ МУЛЬТИПОТЕНТНИХ МЕЗЕНХІМАЛЬНИХ СТРОМАЛЬНИХ КЛІТИН ІЗ МІНІМАЛЬНОЇ БІОПСІЇ ЕНДОМЕТРІЮ ЛЮДИНИ

О. В. Злацька[1, 2], *А. Є. Родніченко*[1, 2], *О. С. Губар*[2, 3], *Д. О. Зубов*[1, 2], *Р. Г. Васильєв*[1, 2], *С. М. Новікова*[1]

[1]ДУ «Інститут генетичної та регенеративної медицини НАМН України», Київ
[2]Біотехнологічна лабораторія «ilaya Regeneration» медичної компанії ilaya, Київ
[3]Інститут молекулярної біології і генетики НАН України, Київ

E-mail: alenazlacka@gmail.com

Метою роботи було отримання культури клітин із мінімальної біопсії ендометрію людини та перевірка її на відповідність критеріям мультипотентних мезенхімальних стромальних клітин. Показано, що клітини в культурі адгезують до пластику та мають характерну фібробластоподібну морфологію, експресують $CD73^+CD90^+CD105^+$ і є негативними за гемопоетичними маркерами ($CD34^-CD45^-HLA\text{-}DR^-$). Відзначено їхню здатність до спрямованого адипо-, остео- та хондрогенного диференціювання. За цими критеріями популяцію клітин, отриманих із мінімальної біопсії ендометрію, можна віднести до мультипотентних мезенхімальних стромальних клітин.

Ключові слова: ендометрій людини, мультипотентні мезенхімальні стромальні клітини.

ПОЛУЧЕНИЕ МУЛЬТИПОТЕНТНЫХ МЕЗЕНХИМАЛЬНЫХ СТРОМАЛЬНЫХ КЛЕТОК ИЗ МИНИМАЛЬНОЙ БИОПСИИ ЕНДОМЕТРИЯ ЧЕЛОВЕКА

А. В. Злацкая[1, 2], *А. Е. Родниченко*[1, 2], *О. С. Губарь*[2, 3], *Д. О. Зубов*[1, 2], *Р. Г. Васильев*[1, 2], *С. Н. Новикова*[1]

[1]ГУ «Институт генетической и регенеративной медицины НАМН Украины», Киев
[2]Биотехнологическая лаборатория «ilaya Regeneration» медицинской компании ilaya, Киев
[3]Институт молекулярной биологии и генетики НАН Украины, Киев

E-mail: alenazlacka@gmail.com

Целью работы было получение культуры клеток из минимальной биопсии эндометрия человека и её проверка на соответствие критериям мультипотентных мезенхимальных стромальных клеток. Показано, что клетки в культуре адгезируют к пластику и имеют характерную фибробластоподобную морфологию, экспрессируют $CD73^+CD90^+CD105^+$ и являются негативными по гемопоэтическим маркерам ($CD34^-CD45^-HLA\text{-}DR^-$). Отмечена их способность к направленной адипо-, остео- и хондрогенной дифференцировке. По этим критериям популяцию клеток, полученных из минимальной биопсии эндометрия, можно отнести к мультипотентным мезенхимальным стромальным клеткам.

Ключевые слова: эндометрий человека, мультипотентные мезенхимальные стромальные клетки.