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# Isolation of a Population of Cells Co-Expressing Markers of Embryonic Stem Cells and Mesenchymal Stem Cells from the Rudimentary Uterine Horn of a Patient with Uterine Aplasia

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More than 50% cells isolated from the endometrial cavity scraping and the myometrium of the rudimentary horn of an underdeveloped uterus removed from a patient with uterine aplasia and maintained under culturing conditions normal for mesenchymal stem cells (MSC) expressed embryonic transcription factors Oct4 and Nanog, embryonic cell membrane sialyl glycolipid SSEA4, and MSC markers. After 2-3 passages, the cells lost the expression of the early embryogenesis markers, but retained MSC markers. The presence of dormant stem cells in the underdeveloped endometrium and in the uterus indicates that this tissue has a regenerative potential that can be activated and used for completion of organ morphogenesis. This task requires the development of methods of early diagnosis of morphogenesis impairment and tools for safe reactivation of the ontogenesis.

**Key Words:** *Oct4; Nanog; SSEA4; histogenesis; postnatal stem cells*

Mesenchymal stem (stromal) cells (MSC) are a heterogeneous cell population with performance of fibroblast-like multipotent cells adhering to plastic, expressing certain cell surface markers and capable of osteogenic, adipogenic, and chondrogenic differentiation [1]. MSC were first isolated from the bone marrow, but later from almost all tissues. MSC attract special attention, because they were proven to be safe in preclinical and clinical studies and are widely used in the development of cell therapy technologies. By 2022, more than 50,000 scientific reports on MSC studies were published and more than 1000 clinical studies were completed or going on now.

MSC identification remains a challenging task despite large number of studies addressing this topic. Identification of MSC by their CD phenotype has been proposed in 2006 by the International Society of Cell and Gene Therapy (ISCT) [2], mainly as a method of purifying MSC from blood cells by cell sorting. It is still in use, sometimes with minor modifications. Unfortunately, all CD markers expressed by MSC are nonspecific and each of them can be present on other cells. In addition, the set of markers depends on age and the tissue source of MSC [3]. In most experimental and some clinical studies, MSC maintained in adhesive primary cultures and harvested after 3-5 passages are used; under these conditions, the cells have phenotype typical of MSC: $CD105^+$, $CD73^+$, $CD90^+$, $CD45^-$, $CD34^-$, $CD14^-$ or $CD11b^-$, $CD79\alpha^-$ or $CD19^-$, and HLA-$DR^-$. However, cells of different cultures vary by morphology, capacity for osteogenic, adipogenic and chondrogenic differentiation, and other properties [4], and can be

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subdivided into subpopulations based on their transcriptome profiles [5,6].

It is important that in some fresh MSC cultures isolated from the bone marrow and other tissues, cells expressing proteins characteristic of embryonic stem cells, in particular, transcription factors that provide maintenance of pluripotency, were found [1,7]. This indicates that pluripotent cells are possibly present in these cultures and, hence, in the source tissues. Similar cells were also found in the blood [8].

In this regard, studies of MSC isolated from underdeveloped organs can be especially important, because this can be the result of cell differentiation arrest at an early stage. Previously, we reported that histochemistry revealed the presence of cells expressing the Oct4 embryonic transcription factor in the uterine rudiments surgically removed from patients with uterine aplasia [9]. Here we describe an attempt to grow these cells in culture and trace their fate during the culture passaging.

## MATERIALS AND METHODS

The experimental design was considered and adopted by the Local Ethical Committee of the V. I. Kulakov National Medical Research Center of Obstetrics, Gynecology, and Perinatology, Ministry of Health of the Russian Federation (Protocol No. 9, November 22, 2018).

**Biological material.** We examined a sample of the underdeveloped uterus surgically removed from a patient with aplasia of the uterus and vagina at the Department of Operative Gynecology, V. I. Kulakov National Medical Research Center of Obstetrics, Gynecology, and Perinatology. The patient gave informed consent for the use of the removed biomaterial for research purposes. The left rudimental horn of the underdeveloped uterus had an endometrial cavity 0.8×0.5 cm and, according to clinical records, responded to changes of hormone levels occurring throughout the menstrual cycle. The right horn had no endometrial cavity. The rudiment of the left horn was placed in a Petri dish with 0.1% collagenase I solution (Gibco) and the endometrial cavity was carefully scraped out with a scalpel. The resulting suspension was incubated for 30 min at 37°C, then centrifuged, and the pellet was resuspended in the complete growth medium (composition described below). The remaining part of the rudimentary left horn (primarily myometrium), was grinded and incubated with 0.1% collagenase I solution (Gibco) for 60 min at 37°C. The resulting cell suspension was centrifuged and the pellet was resuspended in the complete growth medium. The obtained suspensions (part of suspension of cells from the scrapings of the endometrial cavity and suspension of myometrium cells) were transferred to culture flasks with ventilated caps (75 cm$^2$, Greiner) for growth and further cytofluorimetric analysis or passaging. A small part of the cell suspension obtained from the scrapings of the endometrial cavity was placed on coverslips and, after expansion, subjected to immunocytochemical analysis. The immunocytochemical analysis of the myometrial cells was not performed. The cytofluorimetric analysis of the suspensions of uncultured cells was extremely difficult due to the insufficient number of cells and large content of debris. Immunocytochemical staining was also impossible for the same reasons.

**Cell culture.** Cells were cultured in flasks or on coverslips under standard conditions (37°C, 5% $CO_2$) in complete growth medium: DMEM/F-12 with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all components from Gibco), supplemented with 10% fetal calf serum (FCS, HyClone). After achieving approximately 80% confluence, the cells growing in flasks were detached from the substrate with trypsin-EDTA solution (Gibco) and either subcultured 1:3 to new flasks or coverslips, or examined by flow cytometry. Cells grown on coverslips were used for immunocytochemical studies.

**Flow cytometry.** The cells were resuspended in the staining buffer consisting of PBS (PanEco), 2% FCS (Gibco), and 0.1% sodium azide (AcrosOrganics) at a concentration of $10^5$-$10^6$ cells per 100 μl of the sample. For cell tagging, we used labeled primary mouse monoclonal antibodies to MSC: APC anti-CD73 (BD Biosciences), FITC-conjugated anti-CD90 (BD Biosciences), and PerCP-Cy5.5-conjugated anti-CD105 (BD Biosciences); in addition, antibodies to the SSEA-4 embryonic membrane sialyl glycolipid conjugated with PE (BioLegend) and a mixture of antibodies to blood cell markers (CD34$^+$/CD11b$^+$/CD19$^+$/CD45$^+$/HLA-DR$^+$) conjugated with PE (BD Biosciences) were used. A suspension of untagged cells washed and incubated exactly as antibody-tagged cells served as the control. Staining was carried out for 30 min in the dark at 4°C with periodic shaking of the tubes on a minishaker. The cells were then washed three times in 1 ml of staining buffer and filtered into cytometry tubes (BD Biosciences) with a filter cap (pore diameter 35 μm) to exclude cell aggregates. The Sytox Blue Dead Cell Stain (Invitrogen) was added to the samples 1 min before analysis. Cytometric measurements were performed on a FACSAria III flow cytometer/sorter (BD Biosciences).

**Immunocytochemistry.** Upon reaching 80% confluence, the cells grown on slides were rinsed with saline, fixed with 4% formaldehyde solution in 0.01 M PBS (pH 7.4), and washed with 0.01 M PBS. The cells were kept for 30 min in a blocking solution contain-

ing 5% goat serum and 0.3% Triton X-100 in PBS, then incubated overnight at 4°C with primary antibodies to embryonic transcription factors dissolved in the blocking solution: anti-Oct4 (1:400; Abcam) or anti-Nanog (1:400; Abcam). Cells were washed with PBS (3×10 min) and incubated with second antibodies (anti-rabbit IgG Alexa Fluor 594, 1:500; Abcam) for 2 h at room temperature. The nuclei were counterstained with DAPI (2 μg/ml; Sigma).

**Analysis of the results.** The FACSAria III instrument operation was controlled and the data were analyzed using the BD FACSDiva Software 7 software package.

## RESULTS

We worked with unique biological material and it was impossible to conduct the study on several samples. However, since the results of flow cytometry and immunocytochemistry and the data on various markers of pluripotency were in general coincident, we believe that our main conclusions are reliable and reflect the real situation, at least in some cases of uterine aplasia.

To identify cells expressing proteins characteristic of early embryogenesis by immunocytochemistry, we used antibodies to the two most well-known transcription factors that are actively expressed in embryonic stem cells and induced pluripotent cells. As these antibodies cannot be used in flow cytometry because of the intracellular, predominantly nuclear localization of the transcription factors, we used antibodies to the cell membrane-associated stage-specific embryonic antigen SSEA-4, which is also characteristic of embryonic stem cells.

**Cell cultures before passaging ("zero passage").** Cells isolated from the endometrial cavity and myometrium of the rudimentary uterine horn of a patient with uterine and vaginal aplasia and maintained in culture before the first passaging, looked different under a phase-contrast microscope (Fig. 1, *a-d*). The cells isolated from the endometrial cavity lining were more diverse in shape: some were round or cuboid, while others had fibroblast-like morphology (Fig. 1, *a*, *b*). The number of fibroblast-like cells increased during culturing. Almost all cells from the myometrium had fibroblast-like shape, many with processes, especially at the early stages of culturing (Fig. 1, *c*, *d*), and were characterized by slow proliferation rate. At the early stages of culturing, cells isolated from the scrapings of the endometrial cavity and from the myometrium formed small, gradually growing clusters and then a monolayer that was incomplete due to the presence of

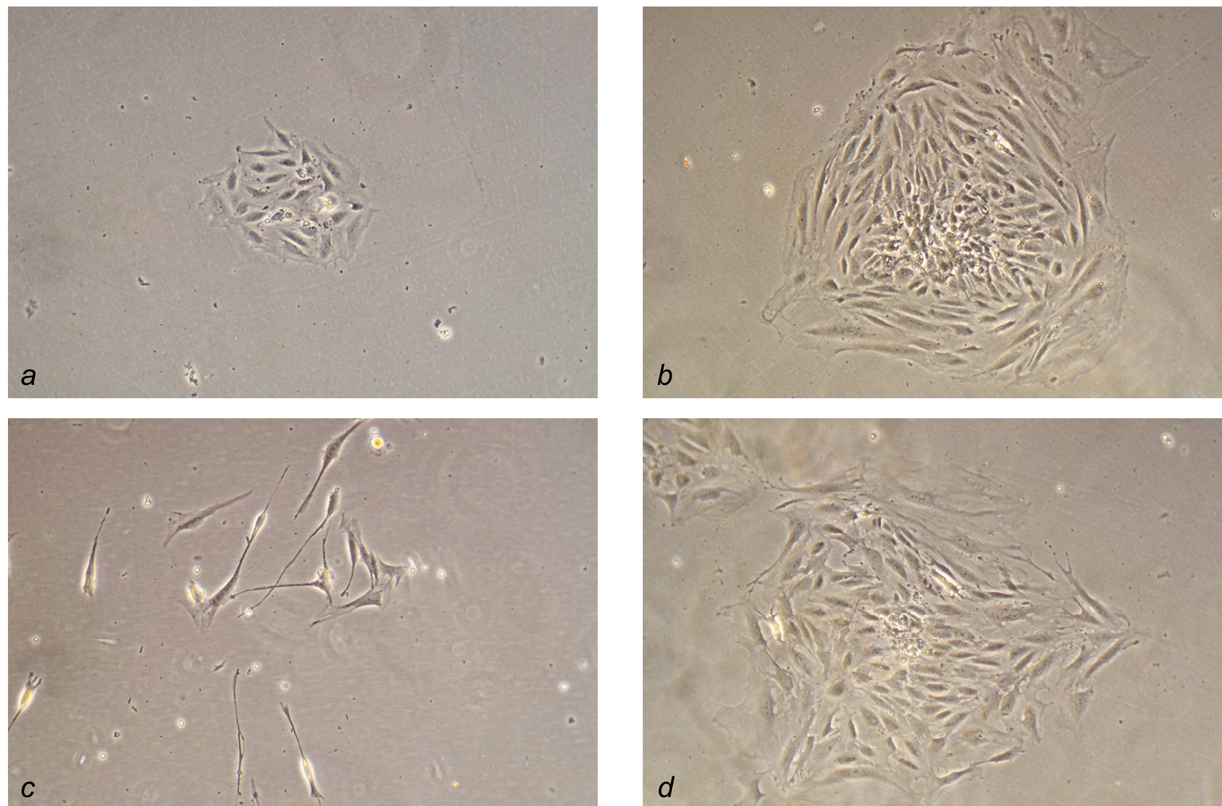


**Fig. 1.** Cells isolated from the endometrial cavity (*a*, *b*) and the myometrium (*c*, *d*) of the rudimentary uterine horn on day 4 (*a*, *c*) and 7 (*b*, *d*) in culture. Phase contrast.

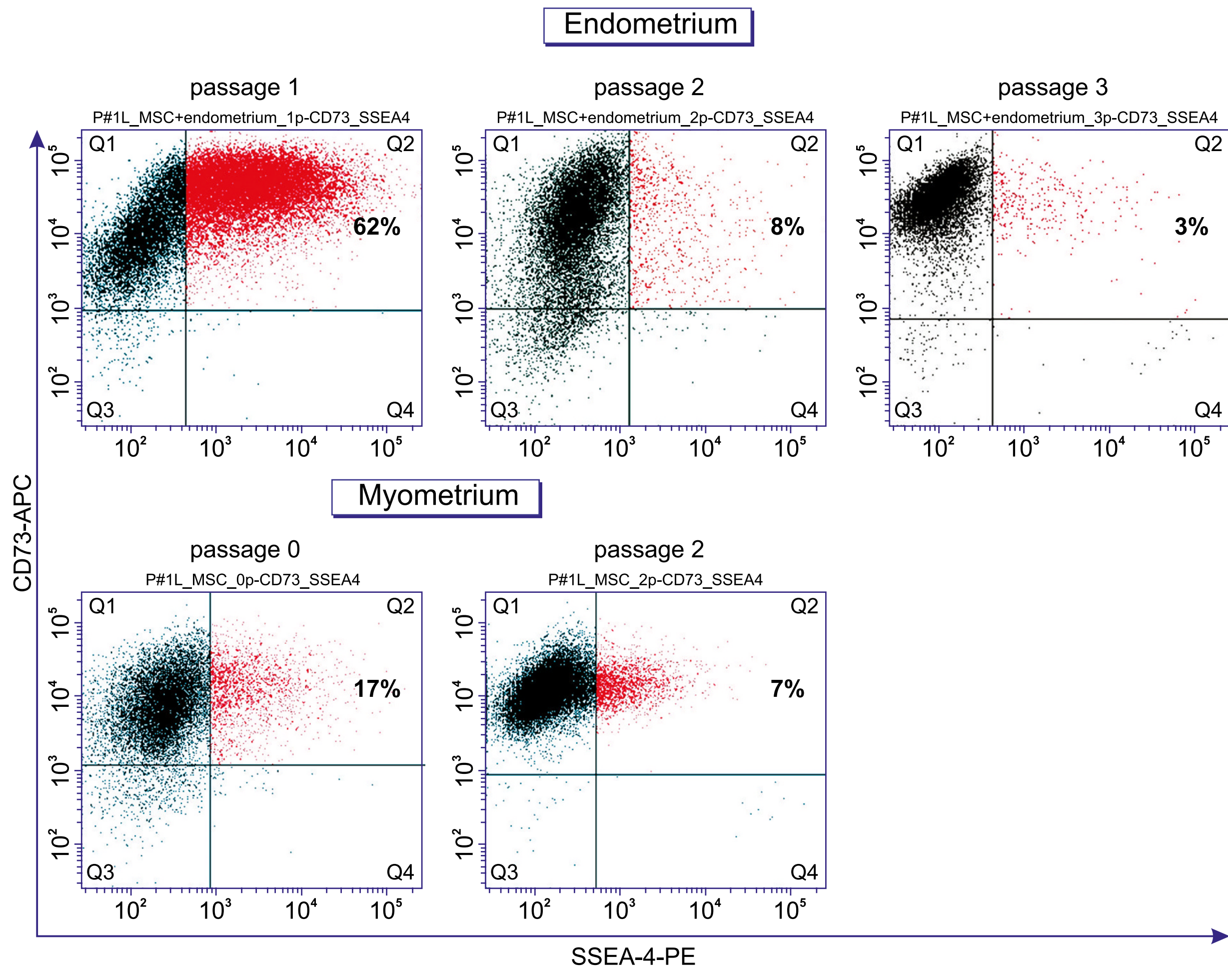


**Fig. 2.** Flow cytometric analysis of co-expression of the MSC marker CD73 and the embryonic marker SSEA4 by the cells isolated from the uterine rudiment. Cells expressing both analyzed antigens are marked in red.

debris and dead cells. After the first passage, the cells from both sources adhered to the plastic and had a fibroblast-like morphology.

In flow cytometry measurements, cell debris and doublets were excluded from analysis based on the direct and side light scattering data, and the percentage of dead cells was taken into account by staining with the Sytox Blue (Thermo Fisher Scientific) fluorescent DNA-binding dye. At passages 0 and 1, the number of blood cells expressing at least one marker from the following set of blood cell markers was additionally evaluated: $CD34^+/CD11b^+/CD19^+/CD45^+/HLA\text{-}DR^+$. Initial cell cultures before the first passage contained large amounts of debris, which prevented the cytofluorimetric study of cells from the endometrial cavity, although it proved possible to study cells isolated from the myometrium (Fig. 2). The suspension of cells isolated from the myometrium contained 1% of blood cells. MSC marker CD73 was expressed by 87% of live cells, CD90 — by 89% of live cells, CD105 — by 66% of live cells. $SSEA4^+$ cells constituted 17%, and all of them co-expressed MSC markers.

Immunocytochemical analysis of cells before passaging was not performed.

**Cell cultures after the first passaging («first passage»).** In the cells culture obtained from the endometrium, blood cells after the first passage constituted approximately 1% of live cells, while 99% of live cells expressed MSC marker CD73, which confirmed predominancy of MSC in this cell culture. The proportion of live cells expressing the SSEA4 embryonic marker was 62% (Fig. 2). Flow cytometry of cells isolated from the myometrium was not performed after the first passage, because of the insufficient number of cells (too many cells were used before passaging).

The immunocytochemical analysis of the cell culture isolated from the endometrial cavity showed that the vast majority of cell nuclei were stained with antibodies to the embryonic transcription factors Oct4 (Fig. 3, *a-c*) and Nanog (Fig. 4, *a-c*). Many stained nuclei were large. Staining with the anti-Nanog

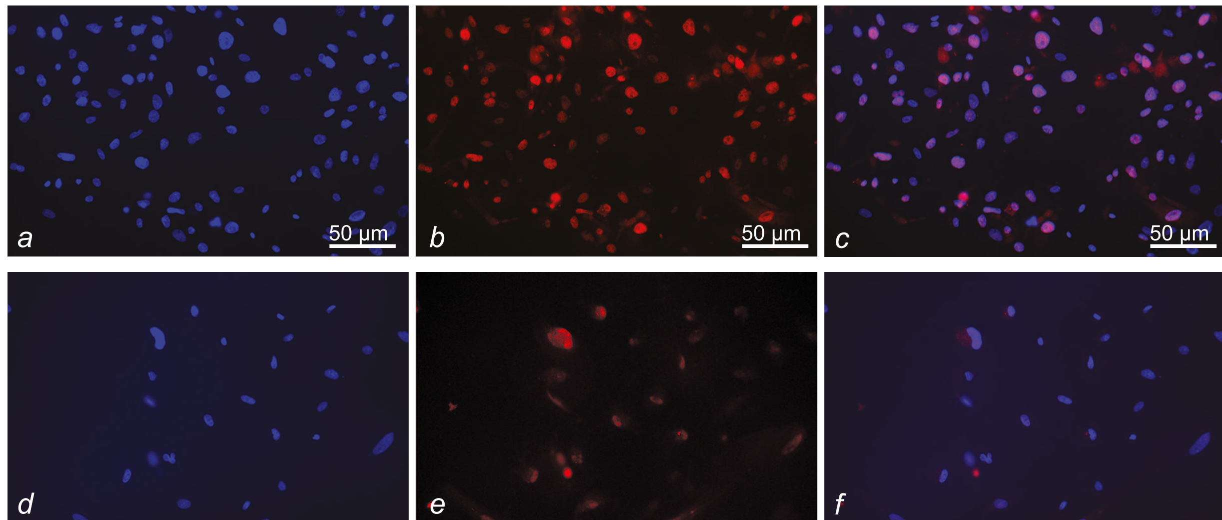


**Fig. 3.** Immunocytochemical study of Oct4 expression in passage 1 (*a-c*) and passage 2 (*d-f*) cultures of cells isolated from the scrapings of endometrial cavity. *a*, *d*) DAPI staining of cell nuclei; *b*, *e*) staining for Oct4; *c*) merged images *a* and *b*; *f*) merged images *d* and *e*.

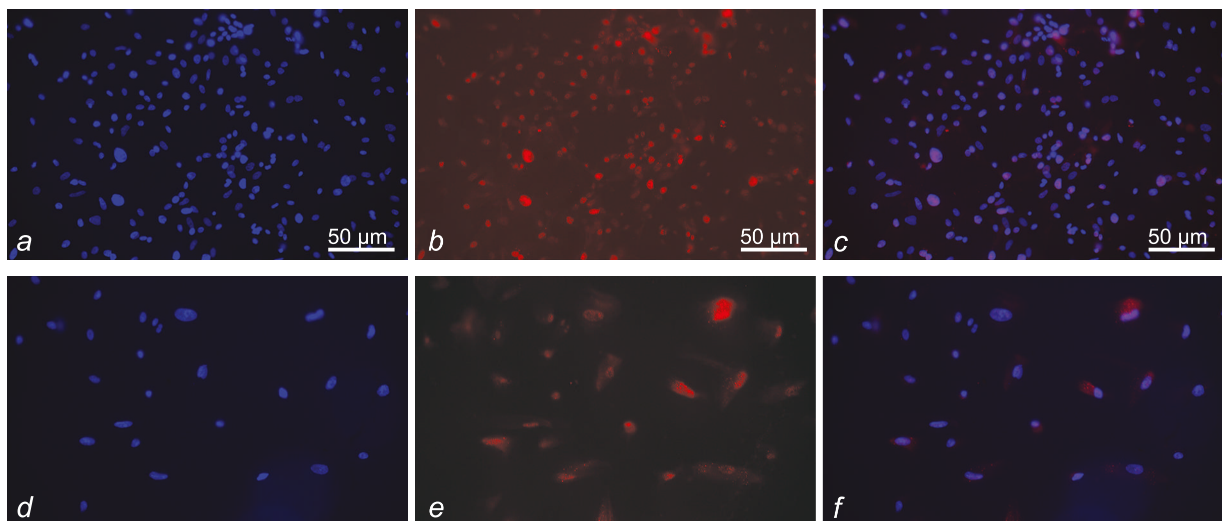


**Fig. 4.** Immunocytochemical study of Nanog expression in passage 1 (*a-c*) and passage 2 (*d-f*) cultures of cells isolated from the scrapings of endometrial cavity. *a*, *d*) DAPI staining of cell nuclei; *b*, *e*) staining for Nanog; *c*) merged images *a* and *b*; *f*) merged images *d* and *e*.

antibodies was less bright compared to the anti-Oct4 antibody staining. Both antigens had a strictly nuclear localization typical of transcription factors. Immunocytochemical analysis of cells isolated from the myometrium was not performed after the first passage.

**Cell cultures after the second and third passaging («second and third passages»).** At the second passage 80-90% of live cells in the culture of cells isolated from the endometrium expressed all three conventional MSC markers: CD73, CD90, and CD105. The proportion of SSEA4$^+$ cells after the second passaging was significantly lower than at passage 1 and amounted to just 8% (Fig. 2). At the third passage, 99% of live cells expressed the MSC marker CD73, and 92% expressed the CD90 and CD105 MSC markers, while the proportion of SSEA4$^+$ cells was only 3% (Fig. 2). In the cell culture obtained from the myometrium, at the second passage, the proportion of live cells expressing CD73 was almost 100%, CD90 — 94%, CD105 — 99%. The proportion of SSEA4$^+$ cells at the second passage decreased to 7% (Fig. 2). It should be noted that at all passages in the cultures obtained from both the endometrium and myometrium the SSEA4$^+$ cells were evenly distributed in the total cell mass by size (forward scatter parameter) and granularity (side scatter parameter).

The immunocytochemical study of the cultures of cells isolated from the endometrial cavity showed that already after the second passage the number of cells expressing embryonic transcription factors Oct4 (Fig. 3, *d-f*) and Nanog (Fig. 4, *d-f*), was significantly decreased compared to cultures after the first passage, and Nanog began to be detected not only in the nuclei, but also in the cytoplasm. After the third passaging, cells expressing Oct4 and Nanog were absent (data not shown).

As shown in this study, the majority of cells isolated from the endometrial cavity of an underdeveloped uterus and maintained in culture under conditions commonly used for the culturing of MSC, initially had mixed epithelial-mesenchymal morphology and expressed such embryonic stem cell markers as the Oct4 and Nanog transcription factors and the SSEA4 membrane-associated stage-specific embryonic antigen. At the same time, most of these cells expressed MSC markers, suggesting their mesenchymal nature or their ability to easily undergo epithelial—mesenchymal transformation. However, cultured cells acquired a fibroblast-like shape typical of MSC after 2-3 passages and progressively lost the expression of the markers of early embryogenesis. The expression of MSC markers remained unchanged. Cells isolated from the myometrium expressed MSC markers and initially included a subpopulation of cells expressing SSEA4 (less than 20%). The number of such $SSEA4^+$ cells also decreased during culturing.

Thus, the studied rudimental uterine horn with an endometrial cavity contained cells expressing embryonic markers, and these cells disappeared in the process of culturing and culture passaging. This raises questions about the nature of cells expressing embryonic markers and why those cells do not persist in culture.

The first question has two supposable answers which are probably both right: embryonic markers-expressing cells are either stem cells which did not get proper signaling directing them to differentiation early in the ontogenesis or they can be “adult pluripotent cells” reported by several research groups [7,10]. Actually, more than 50 papers state that the normal uteri (mainly endometrium) of adult mice and adult women contain small numbers of cells that express embryonic markers and are difficult to isolate [11]. In the underdeveloped uteri, where histogenetic and morphogenetic processes have stopped for some reason, there can be more cells of this kind. However, it should be noted that “adult pluripotent cells” described by different researchers were mostly small and had a high ratio of the volume of the nucleus to the volume of the cytoplasm. In contrast, the embryonic markers-expressing cells described here did not differ in size from cells that did not express embryonic markers, and many of them had a large nucleus.

The most likely reason for the disappearance of cells expressing embryonic markers during culturing can be the cessation of signals from the tissue microenvironment activating intracellular regulatory cascades that maintain cells in an undifferentiated state. It should be kept in mind that Oct4 and Nanog take part in maintaining such cascades in active state [12]. The release of Nanog after the second passaging into the cytoplasm, where it is possibly associated with the centrosome and takes part in the formation of centrioles [13], can be associated with changes in the functions of this protein during culturing. Interestingly, SSEA4 is also involved in the maintenance of cell "stemness" not just in early embryogenesis [14], but also in adult tissues, for example, in male gonads [15]. Thus, it is likely that the embryonic genes-expressing cells present in the underdeveloped uterus are poorly differentiated cells, probably pluripotent stem cells, but this most interesting question requires further investigation.

The presence of dormant stem cells in the underdeveloped endometrium and myometrium of the uterine rudiment indicates that this organ retains certain regenerative potential, which, generally, can be activated and used to complete morphogenesis. Of course, this task requires the invention of methods of early diagnosis of ontogenetic abnormalities and tools for their safe reactivation.

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