

# Mesenchymal stem cells with irreversibly arrested proliferation stimulate decidua development in rats

ALISA P. DOMNINA<sup>1</sup>, POLINA V. NOVIKOVA<sup>2</sup>, OLGA G. LYUBLINSKAYA<sup>1</sup>, VALERIY V. ZENIN<sup>1</sup>,  
IRINA I. FRIDLANSKAYA<sup>1</sup>, VYACHESLAV M. MIKHAILOV<sup>1</sup> and NIKOLAY N. NIKOLSKY<sup>1</sup>

<sup>1</sup>Department of Intracellular Signaling and Transport, Institute of Cytology, Russian Academy of Sciences, 194064 Saint Petersburg; <sup>2</sup>Department of Obstetrics, Gynecology and Reproductology, Faculty of Medicine, Saint-Petersburg State University, 199034 Saint Petersburg, Russia

Received April 21, 2015; Accepted May 16, 2016

DOI: 10.3892/etm.2016.3671

**Abstract.** Stem cell transplantation, which is based on the application of mesenchymal stem/stromal cells (MSCs), is a rapidly developing approach to the regenerative therapy of various degenerative disorders characterized by brain and heart failure, as well as skin lesions. In comparison, the use of stem cell transplantations to treat infertility has received less attention. One of the causes of miscarriages and fetal growth delay is the loss of the decidual reaction of endometrial cells. The present study modeled decidualization processes in pseudopregnant rats. For cell transplantation experiments, the rats were transplanted with MSCs established from endometrial fragments in menstrual blood (eMSCs). These cells express common MSC markers, are multipotent and are able to differentiate into various tissue lineages. Cell therapy frequently requires substantial cell biomass, and cultivation of MSCs may be accompanied by significant changes to their properties, including malignant transformation. In order to minimize the potential for malignant transformation, the proliferation of eMSCs was irreversibly suppressed by irradiation and mitomycin C treatment. Transplantation of the rats with viable, non-proliferating eMSCs stimulated the development of all elements of decidual tissue. Conversely, transplantation of the rats with cells killed using 95% ethanol did not result in the development of decidual tissue. The present study demonstrated the potential for applying eMSCs to the cellular therapy of infertility associated with endometrial disorders characterized by decidualization insufficiency and implantation failure. In addition, the transplantation of viable but non-proliferating cells ensured that their oncogenic potential was limited.

## Introduction

The application of mesenchymal stem cells (MSCs) as a cell therapy for various diseases has been extensively investigated. MSCs possess high proliferative activity and are able to differentiate into various cell types (1,2). The main advantage of MSCs for clinical application is the potential for using a patient's own cells (autologous MSCs) for transplantation (1,2). This reduces the risks of rejection and undesirable immunological reactions. For a long time, bone marrow has been the main source of MSCs. To date, MSCs have been established from adipose tissue (3), umbilical cord blood (4), amniotic fluid (5) and endometrium (6,7). However, the methods used to harvest cellular material from bone marrow and adipose tissue are painful and can be dangerous for donors. In addition, cells from the umbilical cord can only be obtained from newborns.

Human endometrium, which is composed of endometrial glands lined with stromal cells, is a dynamic tissue that undergoes ~400 cycles of regeneration, differentiation and shedding (8). It has previously been demonstrated that endometrium fragments in menstrual blood are a source of endometrial MSCs (eMSC); thus menstrual blood represents a noninvasive and readily available source of MSCs (9,10). Their high proliferative ability during long-term cultivation, genetic stability (11), lack of tumorigenicity and low immunogenicity (12) makes the eMSCs from menstrual blood a promising source of stem cells for future clinical applications.

During pregnancy, specific morphological and biochemical changes, known as decidual reactions, occur in the endometrium. Decidualization of the endometrium is an essential process for embryo implantation, placenta formation and maintenance of pregnancy. Therefore, during all terms of pregnancy, loss of the decidual reactions of endometrial cells may cause miscarriages and fetal growth delay (13,14). Insufficient decidualization of the endometrium may lead to infertility and pathologies such as Asherman's syndrome and endometrium atrophy (15,16). Currently, Asherman's syndrome is treated by surgery, followed by cyclic hormonal therapies over the subsequent 3-6 months. Previous studies have investigated the possibility of using stem cell therapy to correct endometrium failure (17,18). In these efforts, stem cells derived from bone marrow were used. In our previous study, it was demonstrated

---

*Correspondence to:* Dr Alisa P. Domnina, Department of Intracellular Signaling and Transport, Institute of Cytology, Russian Academy of Sciences, Tikhoretsky pr. 4, 194064 Saint Petersburg, Russia  
E-mail: aldomnina@mail.ru

**Key words:** mesenchymal stem cells of endometrium, decidualization, pseudopregnancy, arrested proliferation

that eMSCs transplanted into pseudopregnant rats facilitated the development of all elements of decidual tissue (19).

Cell therapy typically requires substantial cell biomass. The initial content of MSCs in tissues is usually very low; thus, in order to obtain sufficient biomass, MSCs must be expanded in culture. However, cultivation of somatic cells may be accompanied by significant changes to their properties, including malignant transformation. Therefore, the cells used in clinical applications should be carefully evaluated for their safety (1).

The majority of researchers consider that MSCs do not undergo spontaneous transformation during cultivation (1,11). However, the results of previous studies have been controversial. Mouse MSCs were easily immortalized and transformed during long-term cultivation (20,21), whereas the long-term growth of human bone marrow MSCs was not accompanied by transformation (22). Previous reports on the spontaneous transformation of human stem cells from adipose tissue (23), bone marrow (24) and neuronal stem cells (25) are questionable. The first two papers were retracted as, in both cases, stem cell cultures had been cross contaminated with cells derived from immortal cell lines (26). Therefore, it was more likely that cross-contamination with HeLa cells, and not spontaneous transformation, had taken place. Although the spontaneous transformation of human stem cells has not been verified, these papers continue to be cited (1).

The use of MSCs with irreversibly arrested proliferation may reduce the oncogenic risks of transplanted cells. The present study aimed to investigate the effect of human eMSCs with arrested proliferation on the decidual differentiation of the endometrium in a rat model of pseudopregnancy. The proliferation of eMSCs was blocked using mitomycin C exposure or ionizing radiation (IR), both of which are well known inhibitors of the cell cycle (27-30).

## Materials and methods

*eMSC derivation and cultivation.* eMSCs were isolated, as described previously (31). Briefly, menstrual blood containing endometrium fragments was obtained from three female volunteers aged 27 years. Written informed consent was obtained from all donors. A total of 2 ml menstrual blood was collected on the second day of the menstrual cycle and centrifuged at 400 x g for 5 min at room temperature. The resulting pellet containing the endometrium fragments was resuspended in phosphate-buffered saline (PBS) supplemented with 10% antibiotic-antimycotic mixture, incubated for 1 h at 37°C and centrifuged at 400 x g for 5 min at room temperature. Subsequently, the cell pellet was resuspended in Dulbecco's modified Eagle's medium/F12 medium (DMEM) supplemented with 10% fetal calf serum (FCS; GE Healthcare Life Sciences, Logan, UT, USA), 1% antibiotic-antimycotic mixture and 1% glutamax, and seeded into 6-cm Petri dishes (Corning Incorporated, Corning, NY, USA) at a cell density of  $2 \times 10^4$  cells/cm<sup>2</sup>. Cells were cultivated for 3-7 days at 37°C, during which the medium was exchanged several times to ensure that only adhesive cells formed the culture.

*Immunophenotyping.* Immunophenotyping of eMSCs [cluster of differentiation (CD) marker expression] was performed

using an Epics XL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). A single cell suspension was obtained using 0.05% trypsin/EDTA. Cells ( $1 \times 10^6$ ) were suspended in 1 ml PBS containing 5% FCS. Subsequently, according to the manufacturer's instructions, the cells were incubated at room temperature for 30 min with the following mouse anti-human fluorescein isothiocyanate-conjugated monoclonal antibodies: anti-CD34 (555821; 1:250), CD45 (555482; 1:250) and CD90 (555595; 1:250), and phycoerythrin-conjugated monoclonal antibodies: Anti-CD19 (557835; 1:250), CD73 (561014; 1:200), CD105 (560839; 1:250), CD146 (561013; 1:200) and human leukocyte antigen (HLA)-DR (555812; 1:200; all BD Pharmingen, San Diego, CA, USA). All antibodies were pre-diluted for use according to the recommended volume per test. For each test,  $1 \times 10^6$  cells in 100  $\mu$ l PBS were used. For CD73, CD146 and (HLA)-DR the volume per test was 20  $\mu$ l. Cells were analyzed by flow cytometry.

*Immunocytochemistry.* Immunofluorescent staining for nestin was performed according to a standard protocol (31). Briefly, the cells were incubated with rabbit anti-nestin polyclonal antibody (1:100; AB5922; EMD Millipore, Billerica, MA, USA), followed by incubation with cyanine 2-conjugated goat anti-rabbit (1:300; 111-225-144) or DyLight 488-conjugated goat anti-rabbit (1:400; 111-545-003) antibodies (both Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cells were then observed under a fluorescent microscope.

*Adipogenic differentiation.* Cells ( $2 \times 10^4$ /cm<sup>2</sup>) were seeded into Petri dishes coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA). When the cells reached ~80% confluence, 1 mM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 10  $\mu$ g/ml human recombinant insulin (Sigma-Aldrich) and 100 mM indometacin were added to the cells. Cells were cultivated at 37°C in the differentiation medium for 5 days, with half of the medium replaced every other day. Under these conditions, the cells had been differentiated for 3-5 weeks. Lipid drops were visualized with Oil Red staining (Sigma-Aldrich), according to the manufacturer's protocol.

*Osteogenic differentiation.* Cells ( $2 \times 10^4$  cells/cm<sup>2</sup>) were seeded into Petri dishes coated with 0.1% gelatin. After the cells had reached 100% confluence, 100 nM dexamethasone, 10 mM  $\beta$ -glycerol phosphate and 0.2 mM ascorbate 2-phosphate were added to the cells. In this medium, the cells were differentiated at 37°C for 3-5 weeks, with half of the medium changed every 2-3 days. Subsequently, the cells were fixed with 70% cold ethanol for 1 h, stained with Alizarin Red (pH 4.1; Sigma-Aldrich) and observed under a light microscope.

*Mitomycin C treatment of eMSCs.* eMSCs between the third and fourth passages were treated with 10  $\mu$ g/ml mitomycin C (Sigma-Aldrich) for 1.5 h. Treated cells were then washed with PBS three times, resuspended in DMEM supplemented with 10% FCS, 1% antibiotic antimycotic mixture and 1% glutamax, and seeded ( $1 \times 10^5$  cells) into 35-mm Petri dishes.

*Irradiation of eMSCs.* eMSCs ( $10^5$ ) between the third and fourth passages were seeded into 35-mm Petri dishes.

Subsequently, the cells were exposed to 3, 6 and 10 Gy doses of IR using a stationary X-ray device (0,49 Gy/min).

**Cell growth kinetics.** Cell growth properties were assessed by generating growth curves. eMSCs ( $10^5$ ) were seeded into 35-mm Petri dishes, and the number of cells were counted daily using a Goryaev's chamber for 4 days. Two plates were used for each measurement and assays were performed in triplicate.

**Cell cycle distribution analysis.** Adherent cells were rinsed with PBS, harvested using trypsin-EDTA solution and resuspended in PBS at  $1 \times 10^8$  cells/ml. Subsequently, 200  $\mu\text{g/ml}$  saponin, 250  $\mu\text{g/ml}$  RNase A and 50  $\mu\text{g/ml}$  propidium iodide (PI; all Sigma-Aldrich) were added to each sample tube. Following incubation for 60 min at room temperature, the samples were analyzed using an Epics XL flow cytometer. Cell cycle distribution analysis was performed using WinMDI 2.8 (<http://winmdi.software.informer.com/2.8/>) and ModFit LT 4.1 software (<http://www.vsh.com/products/mflit/index.asp>).

**Assessment of cell viability.** Cell viability was evaluated by flow cytometry using PI staining. Briefly, 50  $\mu\text{g/ml}$  PI was added to each sample and mixed gently. Samples were analyzed using the Epics XL flow cytometer.

**Animal model.** A total of 48 adult female albino rats weighing 200–250 g were purchased from Rappolovo Animal Farm (St. Petersburg, Russia) and maintained at the Institute of Cytology (Russian Academy of Sciences, St. Petersburg, Russia) animal care facility at 24°C with free access to food and water and a 14/10-h dark/light cycle, according to the institutional guidelines for the care and use of laboratory animals. Vaginal cytological analyses were performed to assess the estrous cycle. Briefly, a sterile swab was moistened with saline and rotated against the vaginal wall to obtain rat vaginal cells. Vaginal smears were visualized under a light microscope. Pseudopregnancy and an artificial decidual response was induced by electrical stimulation of the cervix during estrus. On the fifth day following stimulation, the rats were anesthetized via an intramuscular injection of Zoletil 100 (5 mg/kg body weight; Virbac, Carros, France) and surgical procedures were performed under aseptic conditions. Briefly, the rats were placed in the dorsal position and double 1.5-cm incisions into the skin and muscles, lateral to the vertebrae, were made. Subsequently, the uterine horns were carefully removed to avoid any trauma.

Rats were divided into four groups (n=12). In the first group, a normal eMSC ( $5 \times 10^5$ ) single cell suspension in 20  $\mu\text{l}$  PBS and 20  $\mu\text{l}$  PBS without cells was injected into the experimental uterine horn and the contralateral control horn, respectively. In the second group, eMSCs were fixed with 95% ethanol at -20°C for 20 min and washed three times with PBS solution. Subsequently,  $5 \times 10^5$  single cell suspensions of ethanol-fixed eMSCs in 20  $\mu\text{l}$  PBS and 20  $\mu\text{l}$  PBS without cells were injected into the experimental uterine horn and the contralateral control horn, respectively. In the second and third groups, a single cell suspension of eMSC ( $5 \times 10^5$ ) previously treated with mitomycin C (group 3) and 6 Gy irradiated (group

4) in 20  $\mu\text{l}$  PBS and 20  $\mu\text{l}$  PBS without cells were injected into the experimental uterine horn and the contralateral control horn, respectively.

Rats were sacrificed via cervical dislocation following administration of diethyl ether (80 mg/kg body weight; Ural Profchem Co., Nizhny Tagil, Russia) on day 11 of pseudopregnancy. To estimate deciduas development in the experimental and control uterine horns, decidual tissue was collected and weighed.

**Histological analysis.** Frozen 10- $\mu\text{m}$  sections of formed deciduas were generated and mounted onto slides. Slides were fixed in an ethanol/methanol mixture for 2 min at -20°C, followed by staining with hematoxylin and eosin. The differentiation extent of the decidua, structural alterations in the decidual tissue, and the presence of inflammation and necrosis were assessed by light microscopy.

**Statistical analysis.** All experiments were repeated at least three times. Data are presented as the mean  $\pm$  standard deviation, when indicated. Statistical significance was evaluated by Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results and Discussion

**eMSC properties.** In our previous study, endometrial MSCs were established from desquamated endometrium in menstrual blood (31). The present study established a novel MSC line from endometrium fragments in menstrual blood, and investigated its potential application for the treatment of infertility.

eMSCs obtained from menstrual blood in the present study met the criteria for human multipotent stromal MSCs suggested by the International Society for Cellular Therapy. eMSCs were adhesive to plastic under standard culture conditions, had a fibroblast-like shape and formed a monolayer with a typical round swirling pattern (Fig. 1A). In addition, the eMSCs were positive for CD146, CD105, CD73 and CD90 expression, but negative for CD19, CD34, CD45 and HLA-DR (class II) expression (Fig. 1B). Furthermore, the cells were multipotent and able to differentiate into mesoderm lineages, including osteoblasts and adipocytes (Figs. 1C and D). Immunostaining for nestin, a neural cell marker, was positive (Fig. 1E).

eMSCs have a high level of proliferation activity, with a doubling time of 22–23 h. Cells undergo >45 population doublings during culture before this phase is terminated in favor of dividing and entering into replicative senescence. Replicative senescence is a common feature of normal human MSCs cultivated *in vitro*. As a noninvasive, easily accessible cell source with a high proliferation activity (higher than bone marrow and umbilical cord blood MSCs), multipotency and ability to undergo long-term cultivation without developing karyotypic abnormalities, MSCs derived from endometrium may be considered to be a promising stem cell source for cell therapy.

**IR and mitomycin C treatment induce the irreversible cell cycle arrest of eMSCs.** Mitomycin C belongs to a class of compounds that cause G1 and G2 cell cycle arrest by activating a DNA-damage checkpoint in the cell cycle, resulting

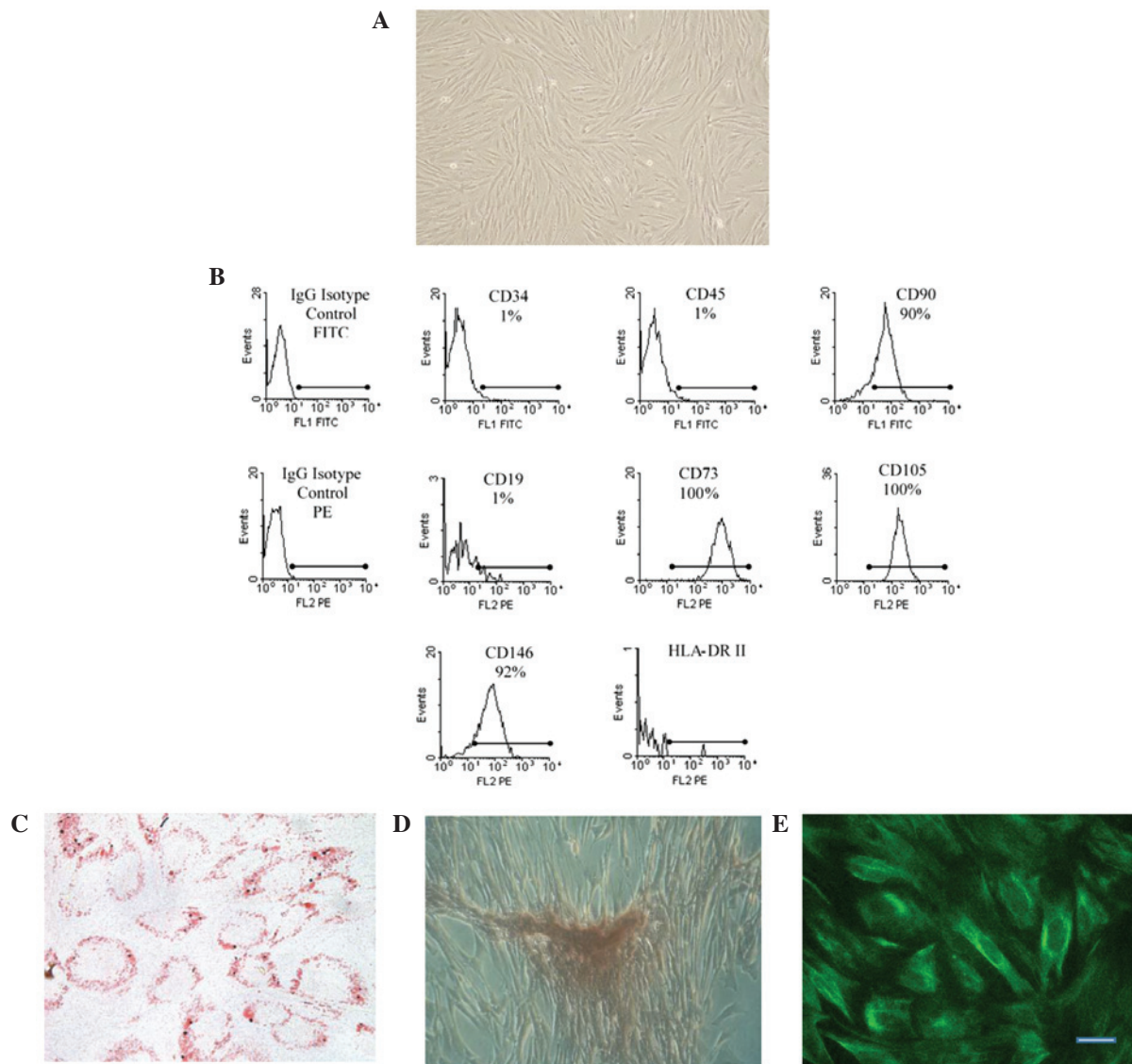


Figure 1. Endometrial mesenchymal stem cell (eMSC) properties. (A) Cell morphology. (B) Flow cytometry analysis of CD marker expression. All plots show relative expression of the surface markers (%). eMSCs differentiated into (C) adipocytes and (D) osteoblasts (magnification, x400). Adipocytes are characterized by accumulation of lipid droplets and calcium deposits, respectively. Lipid drops stained with Oil Red are located in the perinuclear area of the differentiated adipocytes. Calcium deposits were stained with Alizarin Red. The frequency of adipogenic and osteogenic differentiation was 70-80 and 30%, respectively. (E) Nestin expression in native eMSCs (scale bar=25  $\mu$ m). FITC, fluorescein isothiocyanate; PE, phycoerythrin; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

in the inhibition of cell-cycle progression and activation of DNA-repair machinery (29,30). In the present study, the proliferation of eMSCs was inhibited by treatment with 10  $\mu$ g/ml mitomycin C for 1.5 h (Fig. 2). Mitomycin C-treated eMSCs maintained a normal adherent cell morphology (data not shown). The cell viability of mitomycin C-treated eMSCs, as assessed by flow cytometry using PI-staining (Fig. 2A), was only slightly altered, as compared with the untreated cells. However, the growth of eMSCs was entirely suppressed following treatment with mitomycin C. Fig. 2B shows growth curves for normal and mitomycin C-treated cells. The number of eMSCs following treatment with mitomycin C slightly declined over time. Irreversible cell cycle arrest was confirmed by cell cycle distribution analyses via flow cytometry. Fig. 2C shows that mitomycin C-treated cells, unlike normal eMSCs, accumulated in the S and G2/M phases of the cell cycle, and

that cell cycle arrest was not overcome following subculturing of the cells.

IR was also used to induce cell cycle arrest. The present study aimed to identify the minimal IR dose that was able to induce eMSC proliferation arrest without altering the viability of the cells. Previous studies have demonstrated that MSCs from different tissue sources are relatively resistant to IR exposure (27,28,32,33). In addition, no significant apoptosis induction was observed in MSCs exposed to IR doses up to 10 Gy (27).

On the basis of existing literature, the present study selected three IR doses (3, 6 and 10 Gy) to assay the cell cycle distribution and cell viability at 24 and 72 h following irradiation, and 24 h after subculture of the MSCs. Fig. 3 shows the viability and proliferation status of eMSCs exposed to 3, 6 and 10 Gy IR for various durations. The viability of eMSCs irradiated

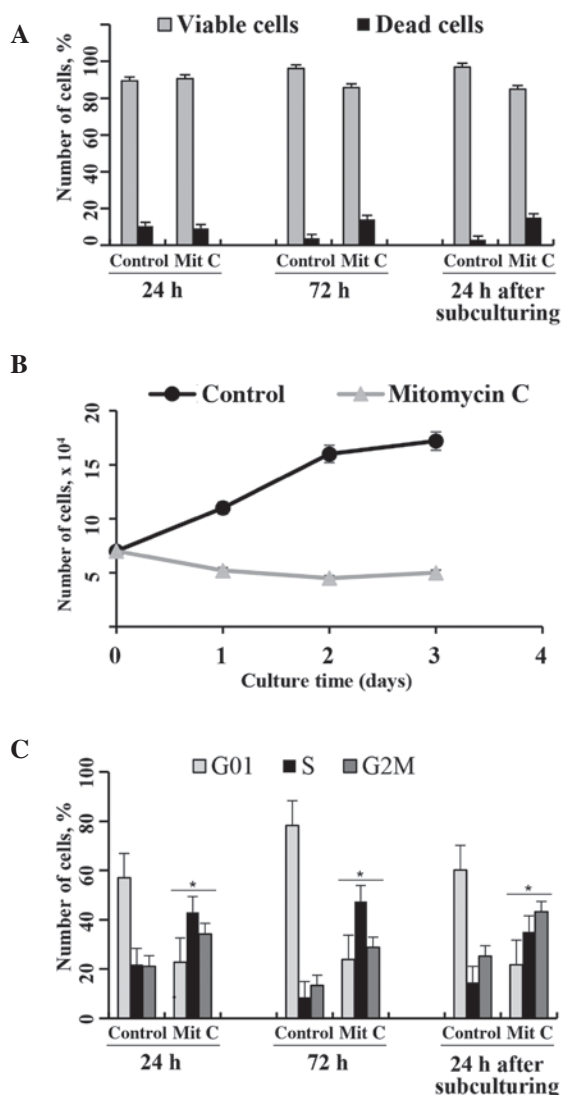


Figure 2. Endometrial mesenchymal stem cells were exposed to 10 µg/ml Mit C for 1.5 h. (A) Cell viability was analyzed by flow cytometry with propidium iodide staining. (B) Proliferation was assessed by constructing growth curves, and (C) the cell cycle distribution was measured by flow cytometry at 24 and 72 h following treatment with Mit C, and 24 h after subculturing. Data are presented as the mean ± standard deviation (n=3). \*P<0.05 vs. control. Mit C, mitomycin C.

with 3 and 6 Gy doses was not significantly different, as compared with normal eMSCs. Conversely, irradiation with 10 Gy markedly reduced the number of viable cells (Fig. 3A).

Cell growth curves (Fig. 3B) show that the eMSCs irradiated with 3 and 6 Gy exhibited cell division arrest, and the number of eMSCs gradually reduced following treatment with 10 Gy. The growth properties of irradiated eMSCs were also assessed by cell cycle distribution analyses via flow cytometry. In all groups, >50% of cells were in the peak corresponding to the G1/G0 phases at 24 h following IR exposure. Percentage of irradiated eMSCs located in the S phase varied from 1.06 to 7.75%, which was markedly lower than in normal eMSCs (21.15%). The main difference between the groups was the number of cells in the G2/M phase. At 24 h following eMSC exposure to 3 Gy, the percentage of cells in the G2/M phase was not significantly different, as compared with the normal eMSCs. Conversely, the cells irradiated

with 6 and 10 Gy were preferentially accumulated in the G2/M phase (45.2 and 55.4%, respectively).

At 72 h, the number of normal and 3 Gy-irradiated eMSCs in the S and G2/M phases were decreased, and those in the G0/G1 phase were increased. During this period, the normal eMSCs had reached confluency and had ceased to proliferate. Conversely, eMSCs irradiated with 6 and 10 Gy exhibited unaltered phase ratios between 24 and 72 h.

On day 4 following irradiation, normal and irradiated cells were subcultured to assess their capacity for proliferation. At 24 h following subculturing, normal and 3 Gy-irradiated eMSCs exhibited increased numbers of cells in the S and G2/M phases (Fig. 3C). These results suggested that eMSCs exposed to 3 Gy had recovered following irradiation-induced stress. Conversely, no changes in the cell cycle distribution were observed in cells exposed to 6 and 10 Gy. No proliferation for these cells was observed at 24 h after passaging. These results suggest that irradiation of eMSCs with 6 Gy to induce irreversible cell cycle arrest while maintaining cell viability is the optimum approach for experiments on transplantation.

Malignant transformation is a potential risk of cell therapy (34). Although it has been widely accepted that MSCs cultured *in vitro* do not undergo malignant transformation, it cannot be concluded that they will not undergo malignant transformation within humans (1,34). *In vivo* conditions may alter the regulation of proliferation in transplanted cells. Therefore, the transplantation of MSCs that remain viable but have lost their ability to divide may significantly reduce their oncogenic potential.

Various preconditioning (pretreatment) strategies have been tested on various stem cells and progenitor cells to enhance transplanted cell viability and function (35). Stem cells and progenitors preconditioned with growth factors, including transforming growth factor- $\alpha$ , insulin-like growth factor-1 and fibroblast growth factor-2, pharmacological agents or ischemia/hypoxia have exhibited improved survival, increased neuronal differentiation, enhanced paracrine effects that lead to increased trophic support, and improved homing to the lesion site (36). The present study focused on cell preconditioning that decreased the oncogenic risk of transplanted cells.

*eMSC transplantation into pseudopregnant rats.* The present study investigated the capacity of eMSCs with arrested proliferation to stimulate decidual tissue development in a rat model of pseudopregnancy. In our previous study, the effect of intact human eMSCs on decidualization processes was analyzed using pseudopregnant rats (19). It was demonstrated that inoculation of human eMSC suspension into the uterus facilitated the development of decidual tissue in pseudopregnant rats, as compared with control PBS injection. Transplantation of rat bone marrow cells into the same model gave similar results, which suggested that the effect of transplanted human eMSCs was not xenogeneic.

The present study compared the development of decidual tissue in rats transplanted with normal eMSCs and those transplanted with eMSCs with irreversibly arrested proliferation. eMSC proliferation was blocked by treatment with mitomycin C or IR. Decidua development on day 11 of

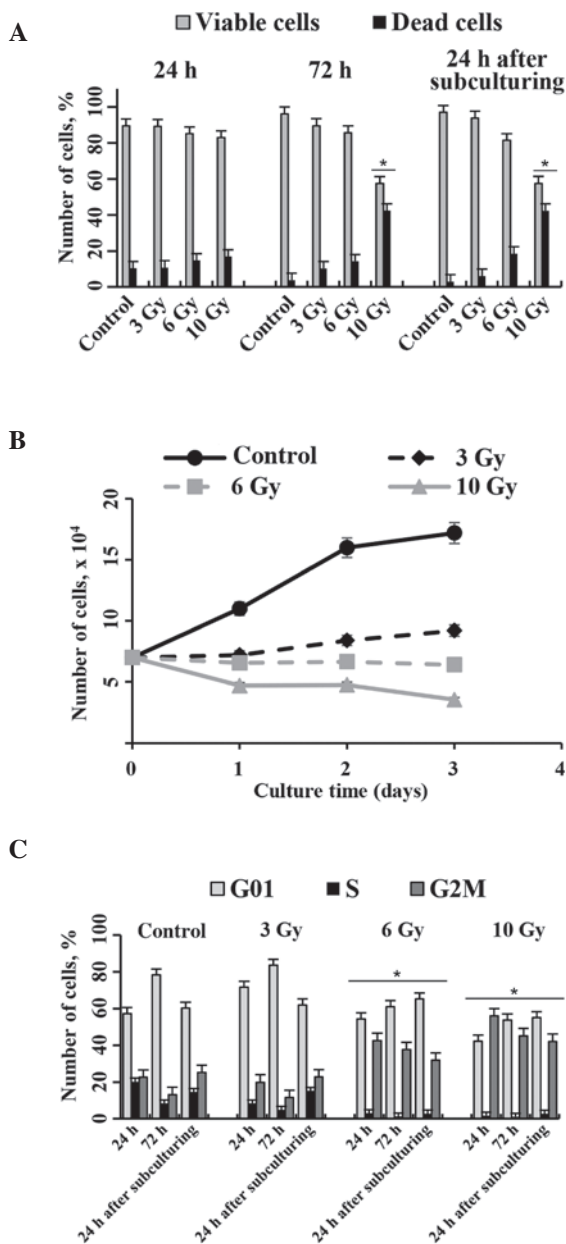


Figure 3. Endometrial mesenchymal stem cells (eMSCs) were exposed to various doses (3, 6 or 10 Gy) of ionizing radiation (IR). (A) Cell viability was analyzed by flow cytometry with propidium iodide staining. (B) The proliferation of eMSCs irradiated with various doses of IR was assessed by growth curves, and (C) the cell cycle distribution was measured by flow cytometry at 24 and 72 h following irradiation, and 24 h after subculturing. Data are presented as the mean  $\pm$  standard deviation (n=3). \*P<0.05 vs. control.

pseudopregnancy was more visible in the uterine horns transplanted with the human eMSCs with arrested proliferation, as compared with the horns injected with PBS control (Fig. 4A). In order to verify that only viable cells are able to promote decidualization, eMSCs killed with 95% ethanol were transplanted into pseudopregnant rats. Fig. 4B shows that, unlike viable eMSCs, there was no difference in size between the decidual tissues derived from the experimental and control horns of rats transplanted with non-viable eMSCs or injected with PBS. Visible differences in the sizes of the experimental and control horns were quantitated by weighing the isolated decidual tissue (Fig. 4C). The weight of decidual tissue

from the experimental horns was significantly increased, as compared with the tissue from the control horns. These results suggested that transplantation of rats with eMSCs with arrested proliferation stimulated decidualization to the same extent as normal cells. Histological analysis of decidual tissue did not detect any changes in cell differentiation or tissue structure following transplantation of normal or treated human eMSCs into the uterus of pseudopregnant rats (Fig. 4D and E). Rodent decidual tissue is formed by large decidual cells (LDCs), small decidual cells and endometrial granulated cells (13). Huge polygonal LDCs of decidual are shown in Fig. 4D, and Fig. 4E shows part of the decidual composed of small decidual cells. The percentage of the total decidual that consisted of LDCs only was 30-40%. Following transplantation, the LDC zone ratio in the decidual section was not altered. These results suggested that transplantation does not modify the tissue structure; the increase in the decidual size resulted from intensive development of all elements of decidual tissue. Furthermore, no leukocyte infiltration into sites of transplanted cells was observed (Fig. 4D and E).

The loss of decidual reactions in endometrial cells is one of the reasons for miscarriages and fetal growth delay at all terms of pregnancy (15). The insufficient decidualization of the endometrium leads to infertility in such pathologies as Asherman's syndrome and endometrium atrophy. The incidence of Asherman's syndrome in women who have undergone a hysteroscopy is 1.55%, and 39% in women who have had recurrent miscarriage (16). At present, Asherman's syndrome is treated by surgery, followed by cyclic hormonal therapies during the subsequent 3-6 months. However, it remains a challenging disease to treat. A few previous studies, including several animal models of Asherman's syndrome (37-39), have investigated the application of cell therapy to the treatment of endometrium-determined infertility. In addition, reports on the successful application of cell therapy to Asherman's syndrome treatment have been published (17,18). The authors described a clinical case of Asherman's syndrome in which intrauterine injection of autologous bone marrow cells led to an increase in the thickness of the patient's endometrium (18). However, to the best of our knowledge, no previous study has investigated the effect of using endometrial cells for the recovery of endometrial disorders, either in animal models or clinical trials.

In conclusion, the present study demonstrated that transplantation of human eMSCs with arrested proliferation into pseudopregnant rats facilitated the development of all elements of decidual tissue. Rats were used to model the endometrial transformation into decidual tissue during the normal pregnancy process. In addition, it was demonstrated that human eMSCs exposed to mitomycin C (10  $\mu$ g/ml) or IR (6 Gy) remained viable but irreversibly lost their proliferation capacity, as assessed by growth curves and flow cytometry. These results suggested that preconditioning eMSCs with division blocking agents may diminish their oncogenic potential *in vivo*. Transplantation of eMSCs killed by ethanol treatment did not promote the decidualization process, thus suggesting that viable MSCs may be required. The results of the present study supported the application of eMSCs to the cell therapy of infertility associated with decidualization insufficiency.

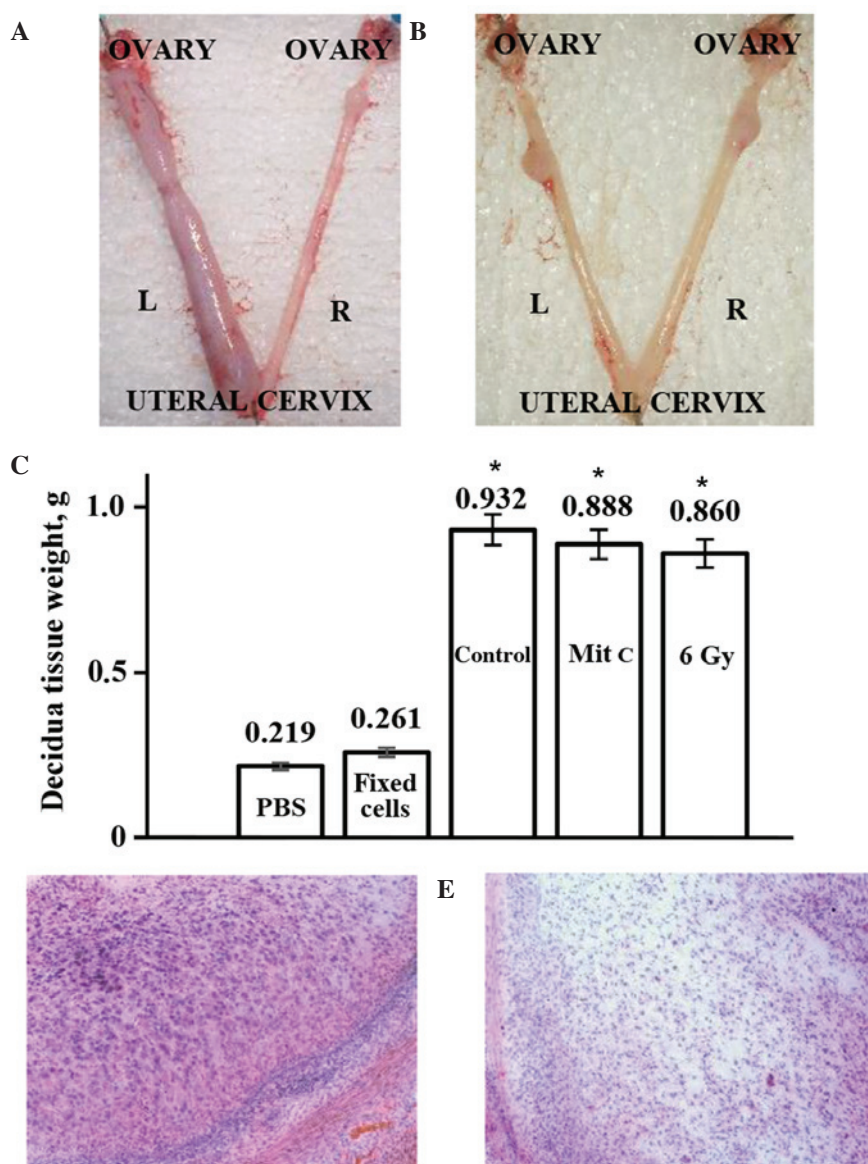


Figure 4. Transplantation of irreversibly arrested endometrial mesenchymal stem cells (eMSCs) into the uterus of pseudopregnant rats promoted the decidualization process. (A and B) The uterus of pseudopregnant rats following inoculation with human eMSCs. (A) Viable eMSCs with arrested proliferation were inoculated into the left uterine horn (L), whereas PBS was injected into the right horn (R) (control). (B) eMSCs killed with 95% ethanol were injected into the left horn, and PBS was injected into the right horn. (C) The weights of decidua tissue in the uterine following injections of normal eMSCs, MitC-treated eMSCs, irradiated eMSCs (6 Gy), ethanol-fixed eMSCs (fixed cells) or PBS. \* $P < 0.05$  vs. PBS. (D and E) Histological sections of a pseudopregnant rat uterus following eMSC transplantation (hematoxylin and eosin staining; magnification, x100). (D) Mesometrial part of the decidua consisting of large decidua cells. (E) Antimesometrial part of the decidua consisting of small decidua cells. MitC, mitomycin C; PBS, phosphate-buffered saline.

## Acknowledgements

The authors would like to thank the Russian Science Foundation (grant no. 14-50-00068) and Russian Federal Agency of Scientific Organizations for their financial support. Professor Irina Fridlyanskaya and Dr Polina Novikova are grateful to the RAS Presidium program of 'Fundamental Sciences for Medicine' for the support.

## References

- Wang S, Qu X and Zhao RC: Clinical applications of mesenchymal stem cells. *J Hematol Oncol* 5: 19-20, 2012.
- Husein KS and Thiernemann C: Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 28: 585-596, 2010.
- Parker AM and Katz AJ: Adipose derived stem cells for the regeneration of damaged tissues. *Expert Opin Bio Ther* 6: 567-578, 2006.
- Harris DT, Badowski M, Ahmad N and Gaballa MA: The potential of cord blood stem cells for use in regenerative medicine. *Exper Opin Biol Ther* 7: 1311-1322, 2007.
- Coppi P De, Bartsch G Jr, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, *et al*: Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 25: 100-106, 2007.
- Cho NH, Park YK, Kim YT, Yang H and Kim SK: Lifetime expression of stem cell markers in the uterine endometrium. *Fertil Steril* 81: 403-407, 2004.
- Gargett CE: Identification and characterization of human endometrial stem/progenitor cells. *Aust Nz J Obstet Gynaecol* 46: 250-253, 2006.
- Gargett CE and Masuda H: Adult stem cells in the endometrium. *Mol Hum Reprod* 16: 818-834, 2010.
- Meng X, Ichim TE, Zhong J, Rogers A, Yin Z, Jackson J, Wang H, Ge W, Bogin V, Chan KW, *et al*: Endometrial regenerative cells: A novel stem cell population. *J Transl Med* 5: 57-66, 2007.

10. Patel AN, Park E, Kuzman M, Benetti F, Silva FJ, Allickson JG: Multipotent menstrual blood stromal stem cells: isolation, characterization, and differentiation. *Cell Transplant* 17: 303-311, 2008.
11. Dominina AP, Fridliandskaia II, Zemel'ko VI, Pugovkina NA, Kovaleva ZV, Zenin VV, Grinchuk TM and Nikol'skii NN: Mesenchymal stem cells from human endometrium do not undergo spontaneous transformation during long-term cultivation. *Cell tissue biol* 7: 221-226, 2013.
12. Murphy MP, Wang H, Patel AN, Kambhampati S, Angle N, Chan K, Marleau AM, Pysznik A, Carrier E, Ichim TE and Riordan NH: Allogeneic endometrial regenerative cells: An 'off the shelf solution' for critical limb ischemia? *J Transl Med* 6: 45, 2008.
13. Mikhailov VM: Life cycle of decidual cells. *Int Rev Cytol* 227: 1-63, 2003.
14. Kim JJ, Taylor HS, Lu Z, Ladhani O, Hastings JM, Jackson KS, Wu Y, Guo SW and Fazleabas AT: Altered expression of HOXA10 in endometriosis: Potential role in decidualization. *Mol Hum Reprod* 13: 323-332, 2007.
15. Csemiczky G, Wramsby H, Johannisson E and Landgren BM: Importance of endometrial quality in women with tubal infertility during a natural menstrual cycle for the outcome of IVF treatment. *J Assist Reprod Genet* 15: 55-61, 1998.
16. Dmowski WP and Greenblatt RB: Asherman's syndrome and risk of placenta accrete. *Obstet Gynecol* 34: 288-299, 1969.
17. Zhao Y, Wang A, Tang X, Li M, Yan L, Shang W and Gao M: Intrauterine transplantation of autologous bone marrow derived mesenchymal stem cells followed by conception in a patient of severe intrauterine adhesions. *Open J Obstet Gynecol* 3: 377-380, 2013.
18. Nagori CB, Panchal SY and Patel H: Endometrial regeneration using autologous adult stem cells followed by conception by in vitro fertilization in a patient of severe Asherman's syndrome. *J Hum Reprod Sci* 4: 43-48, 2011.
19. Domnina AP, Zemelko VI, Mikhailov VM and Nikolsky NN: Stimulation of decidua development by transplantation of endometrial stem cells. *J Biomed Sci Eng* 6: 59-65, 2013.
20. Grinchuk TM, Ivantsov KM, Alekseenko LL, Kozhukharova IV, Zaichik AM, Petrov NS, Mikhailov VM and Popov BV: Characterization of cultured murine mesenchymal stem cell line expressing GFP. *Tsitologiya* 50: 1030-1035, 2008 (In Russian).
21. Popov BV, Petrov NS, Mikhailov VM, Tomilin AN, Alekseenko LL, Grinchuk TM and Zaichik AM: Spontaneous transformation and immortalization of mesenchymal stem cells in vitro. *Tsitologiya* 51: 91-102, 2009 (In Russian).
22. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, *et al*: Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 67: 9142-9149, 2007.
23. Rubio D, Garcia Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC and Bernad A: Spontaneous human adult stem cell transformation. *Cancer Res* 65: 3035-3039, 2005.
24. Røslund GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwicz J, Tonn JC, Goldbrunner R, Lønning PE, *et al*: Long-term cultures of bone marrow derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 69: 5331-5339, 2009.
25. Wu W, He Q, Li X, Zhang X, Lu A, Ge R, Zhen H, Chang AE, Li Q and Shen L: Long-term cultured human neural stem cells undergo spontaneous transformation to tumor-initiating cells. *Int J Biol Sci* 7: 892-901, 2011.
26. Torsvik A, Rosland GV and Bjerkvig R: Spontaneous transformation of stem cells in vitro and the issue of cross contamination. *Int J Biol Sci* 8: 1051-1052, 2012.
27. Sokolov MV and Neumann RD: Lessons learned about human stem cell responses to ionizing radiation exposures: A long road still ahead of us. *Int J Mol Sci* 14: 15695-15723, 2013.
28. Sokolov MV and Neumann RD: Radiation-induced bystander effects in cultured human stem cells. *PLoS One* 5: e14195, 2010.
29. Nieto A, Cabrera CM, Catalina P, Cobo F, Barrie A, Cortés JL, Barroso del Jesus A, Montes R, Concha A: Effect of mitomycin C on human foreskin fibroblasts used as feeders in human embryonic stem cells: Immunocytochemistry MIB1 score and DNA ploidy and apoptosis evaluated by flow cytometry *Cell Biol Int* 31: 269-278, 2007.
30. Hung DT, Jamison TF and Schreiber SL: Understanding and controlling the cell cycle with natural products. *Chem Biol* 3: 623-639, 1996.
31. Zemel'ko VI, Grinchuk TM, Domnina AP, Artsybasheva IV, Zenin VV, Kirsanov AA, Bichevaia NK, Korsak VS and Nikol'skii NN: Multipotent mesenchymal stem cells of desquamated endometrium: Isolation, characterization and application as a feeder layer for maintenance of human embryonic stem cells. *Cell Tiss Biol* 6.1: 1-11, 2011 (In Russian).
32. Cmielova J, Havelek R, Soukup T, Jiroutová A, Visek B, Suchánek J, Vavrova J, Mokry J, Muthna D, Bruckova L, *et al*: Gamma radiation induces senescence in human adult mesenchymal stem cells from bone marrow and periodontal ligaments. *Int J Radiat Biol* 88: 393-404, 2012.
33. Ko E, Lee KY and Hwang DS: Human umbilical cord blood-derived mesenchymal stem cells undergo cellular senescence in response to oxidative stress. *Stem Cells Dev* 21: 1877-1886, 2012.
34. Prockop DJ, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, Simmons PJ, Sensebe L and Keating A: Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 12: 576-578, 2010.
35. Yu SP, Wei Z and Wei L: Preconditioning strategy in stem cell transplantation therapy. *Transl Stroke Res* 4: 76-88, 2013.
36. Yu P, Wei Z and Wei L: Preconditioning strategy in stem cell transplantation therapy. *Transl Stroke Res* 4: 76-88, 2013.
37. Alawadhi F, Du H, Cakmak H and Taylor HS: Bone marrow-derived stem cell (bmdsc) transplantation improves fertility in a murine model of Asherman's syndrome. *PLoS One* 9: e96662, 2014.
38. Kilic S, Yuksel B, Pinarli F, Albayrak A, Boztok B and Delibasi T: Effect of stem cell application on Asherman syndrome, an experimental rat model. *J Assist Reprod Genet* 31: 975-982, 2014.
39. Jing Z, Hong G and Li Y: Development of an animal model for thin endometrium using 95% ethanol. *J Fert In Vitro* 2: 4, 2012.