Short Communication

Magnetic Isolation and Characterization of Porcine Ovarian Putative Stem Cells (Pscs): An *In Vitro* Study

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Abstract

According to the ruling hypothesis the potential source of tumorigenesis might be cancer stem cells (CSCs) resulting from the accumulation of mutations in other types of stem cells (SCs). Therefore we decided to identify and characterize the somatic stem cells commonly called Putative Stem Cells (PSCs) in the porcine postnatal ovary. To isolate ovarian PSCs, an antibody against SSEA-4 conjugated to magnetic beads was used. PSCs were cultured for 7 days. During the culture spontaneously arising embryoid bodies have been observed. Such behaviour under culture condition is characteristic for stem cells. Using immunofluorescence we showed cytoplasmic localization of the Oct 3/4 and Sox-2, pluripotency markers, proteins responsible for maintaining cells in an undifferentiated state. Using immunocytochemistry we showed transmembrane and cytoplasmic localization of the c-Kit receptor and GDF-9 - protein associated with the initiation of cell differentiation in the process of folliculogenesis. We demonstrated also a cytoplasmic and perinuclear location of the Ki-67 protein associated with cell division. PCR method revealed the presence of stem cell markers such as c-Kit, GDF-9 and proliferation marker Ki-67 at the level of the transcript in the putative stem cells (PSCs). Moreover, by the Western blot analysis we also confirmed the presence of markers including c-Kit, GDF-9, Oct-3/4, Sox-2 and Ki-67 in these PSC cells. The results obtained allow to presume that stem cells are actually present in the porcine ovary.

Keywords: Putative stem cells; Oct ¾; Ovary; Pig

Introduction

Ovarian tumors are next to breast cancer the most common cause of cancer death from gynaecologic tumors in females. These tumors are often malignant, with symptoms expressed only when in an advanced stage [1]. One hypothesis suggests that stem cells, progenitor cells, and even somatic cells form cancer stem cells (CSCs) arising from the accumulation of mutations [2,3]. Mature ovaries are organs, in which the presence of stem cells has not been clearly demonstrated so far. Research conducted in recent years has enabled to identify a population of putative stem cells (PSCs) in the ovary characterized by the expression of c-Kit and some markers of stem cells. PSC are a heterogeneous cell population, of small and round cells with a diameter of 5-7 microns, and a large nucleus which fills almost all of the cytoplasm [4]. These cells are similar to very small embryonic-like stem cells (VSEL) found in the bone marrow [5], but also described in the ovary [6]. The population of PSCs seems to be very diverse and covers a lot of cell types with the characteristics of stem cells. Suspected among the source of PSCs are also adult stem cells (ASCs). These ASCs probably are responsible for tissue regeneration in mature organisms, maybe they represent the source of CSCs. ASCs compared to embryonic stem cells (ESCs) and VSEL have limited ability to differentiate. Such cells may differentiate within one germ layer, or a single tissue type, because they are not pluripotent [7,8]. Both CSCs and normal stem cells (SCs) have common surface and nuclear markers, specific for them. The expression of stem cell markers such as c-Kit, Oct-3/4, Sox-2, GDF-9 and SSEA-4 may help in an attempt to identify the pool of PSC. Oct-3/4 and Sox-2 are one of the most important markers of the capacity to differentiate. Certain level of expression of this marker is necessary to maintain these cells in the pluripotent state. Oct-3/4 presence was detected in a variety of somatic stem cells and cancer cells. It seems that the Oct-3/4 may also act to maintain self-renewal of adult stem cells, and furthermore may promote carcinogenesis [9]. Oct-3/4 gene expression in adults usually occurs in the bone marrow of both humans and mice, especially in the hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) [10,11]. SCs usually are located in niches. Niches are specialized microenvironment for SCs in which they persist in the not diversified form. However, the biological, molecular and functional nature of the niches remains largely unknown [12]. Despite that the existence of stem cells in adult mammalian ovaries has recently been confirmed, significant confusion remains about the types of stem cells present and their multi/pluripotency properties. Therefore, the objective of this work was to establish effective methods of PSCs identification and isolation from the postnatal porcine ovary.

Materials and Methods

Sample collection, cell preparation and culture

The research material consisted of immature ovaries of 7 approximately 6 months old pigs collected from a local slaughterhouse. Ovaries were transported to the laboratory in cold PBS (PBS; pH 7.4, PAA The Cell Culture Company, Piscataway, NJ, USA) containing AAS (Antibiotic/Antimycotic Solution; 5 ml/500 ml, PAA The Cell Culture Company). Ovarian cortex was sliced into 1 mm/1 mm pieces with a Tissue Slicer Coronal (World Precision Instruments, USA) and digested enzymatically with Liberase™ Research Grade (Roche, Basel, Switzerland) dissolved in PBS. The enzyme was inactivated with cold PBS, and suspension filtered through 100, 70 and 40 microns filters. In the next step, cells were washed several times in sterile PBS and recovered by centrifugation (90x g for 10 min). Erythrocytes were removed by RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA).

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Cells showing the presence of an SSEA-4 epitope were isolated using antibodies (anti-human SSEA-4) conjugated to magnetic beads (EasySepTM hESC/hiPSC SSEA-4 Positive Selection Kit, StemCellTM Technologies, Vancouver, Canada). Isolation was carried out according to the protocol recommended by the manufacturer. All the steps are depicted on Figure 1. Isolated cells were cultured *in vitro* for 7 days in DMEM/F12 medium (Sigma-Aldrich, St. Louis, MO, USA) with the additions of stem cells factor (SCF - Thermo Fisher Scientific, Waltham, MA, USA) and special supplement B-27 (Gibco® B-27® Supplements, Thermo Fisher Scientific, Waltham, USA). To document the presence of characteristic embryoid bodies, every day at the same time point, pictures of cultures were taken using a Nikon Ti-U microscope with Nikon DS-Fi1c-U3 camera (Nikon, Tokyo, Japan). mRNA and total protein were isolated from some of the cultured cells, the remaining ones were fixed for immunocytochemical studies.

Immunocytochemistry

The cells were washed in PBS and fixed in 4% paraformaldehyde. Then permeabilization of cell membranes was performed using 0.1% Triton X-100 (Sigma–Aldrich; St. Louis, MO, USA) in Tris-buffered saline (TBS; pH 7.4). In the next step, non-specific binding sites were blocked by an incubation with 5% normal goat serum (NGS, Sigma Aldrich), in a humidity chamber for 40 min at room temperature. The remaining cells were incubated with the primary antibody raised against Oct-3/4 (monoclonal mouse Oct-3/4 at dilution 1:50, Stem Cell™ Technologies, Vancouver, Canada), Sox-2 (polyclonal rabbit Sox-2 at dilution 1:100, Abcam, Cambridge, UK), c-Kit (polyclonal rabbit c-Kit at dilution 1:100, Abcam, Cambridge, UK), GDF-9 (polyclonal rabbit GDF-9 at dilution 1:100, Abcam, Cambridge, UK), and Ki-67 (monoclonal mouse Ki-67 at dilution 1:100, Dako, Glostrup, Denmark) overnight at 4 °C in a humidity chamber. After incubation, cells were rinsed a few times in TBST (TBS+0.1% Tween 20, Sigma–Aldrich) and incubated with the suitable secondary antibodies (CyTM3 conjugated goat anti-mouse antibody at dilution 1:300; Jackson Immuno Research Laboratories Inc, West Grove, PA, USA or goat anti-rabbit AlexaFluor488 conjugated, Thermo Fisher Scientific, Waltham, MA, USA) for 1.5 h at room temperature in a humidity chamber in the dark. After the incubation, the cells were washed three times with TBST and TBS. Negative controls included cells incubated with 5% to 10% nonimmune horse or goat serum instead of primary antibody. Then cells were mounted in VectaShield Hard Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Finally, they were

viewed and photographed using an LSM510 METAConfocal Laser Scanning Microscope (Zeiss, Jena, Germany). Cells were viewed under both 20x and 40x objective lenses.
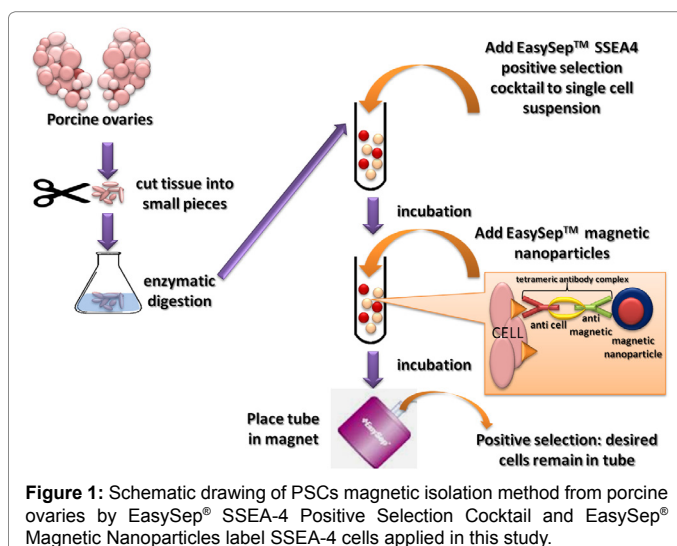
RNA isolation and RT-PCR analysis

Total cellular RNA from PSCs was isolated using TRI Reagent solution (Ambion, Austin, TX, USA) following the manufacturer's instruction. The concentration and purity of RNA was measured by NanoDrop ND2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Samples of RNA from PSCs were frozen and stored at -70ºC. RNA quality was verified by electrophoresis on 1% formaldehyde-agarose gel. A volume equivalent to 1 μg of RNA was taken for reverse transcription. The reaction of reverse transcription was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA was prepared in 20 μl volume of reaction mixtures using the random primers, dNTP mix, RNAse Inhibitor and Multi Scribe Reverse Transcriptase. The reverse transcription wasperformed in a Veriti Thermal Cycler (Applied Biosystems) with a temperature cycling program of 10 min at 25ºC, 2 h at 37ºC and 5 min at 85ºC, with subsequent cooling at 4ºC. In control samples, reverse transcriptase was omitted during the reaction step. Samples of cDNA were kept at -20ºC until further analysis.

Real-time PCR quantification and data analysis

The real-time PCR was performed according to the manufacturer's protocol. For quantitative analysis the mRNA levels of the c-Kit, GDF-9, Ki-67 in each sample TaqMan Gene Expression Assay (Applied Biosystems; assay ID: c-Kit Ss03380145_u1; GDF Ss03391680_m1; MKi67 Ss03389401_m1) was used. The level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems; assay ID: Ss03373286_u1) was estimated for internal control assay. PCR reaction mixtures included 2 μg of cDNA, 1 μl Gene Expression Assay, 10 μL TaqMan PCR master mix (Applied Biosystems) and 8 μL water in a total volume of 20 μL. After a 2 min incubation at 50ºC, the thermal cycling conditions were: 10 min at 95ºC followed by 40 repeats of 15 s at 95ºC and 1 min at 60ºC to determine the cycle threshold (Ct) number for quantitative measurement. PCR products (10 μL) were run on 2% agarose gels containing Midori Green Advance (NIPPON Genetics EUROPE GmbH, Düren, Germany) together with a ready-load 100-bp DNA ladder marker (Promega, Southampton, UK) and bands were digitally imaged under UV illumination. GDF-9, c-Kit, Ki-67 and GAPDH mRNA were detected in the sample by the presence of 93-, 91-, 92- or 83-bp amplification products, respectively.

Western blot

Three different cultures of porcine PSCs were analysed by Western blot assay for the expression of Oct-3/4, Sox-2, c-Kit, GDF-9 and Ki-67 proteins and compared with the positive control (porcine ovarian embryonic stem cells obtained from the foetal - 50 days post coitum-ovaries; oESCs). After termination of culture, PSCs were washed twice with cold PBS, and then proteins were extracted with radioimmuno precipitation assay buffer (RIPA; Thermo Scientific, Inc., Rockford IL, USA) in the presence of protease inhibitor cocktail (Sigma–Aldrich). Then the suspension was sonicated and centrifuged at 10, 000g for 20 min at 4ºC. The supernatant was collected and stored at -20ºC. Protein concentration was determined with DCTM Protein Assay (Bio-Rad Protein Assay; Bio-RadLaboratories GmbH, München, Germany) using bovine serum albumin (BSA) as a standard. Aliquots of cell lysates containing 30 mg of protein were solubilized in a sample buffer consisting of 62.5 mM Tris–HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% b-mercaptoethanol (Bio-Rad Laboratories) and

Figure 1: Schematic drawing of PSCs magnetic isolation method from porcine ovaries by EasySep® SSEA-4 Positive Selection Cocktail and EasySep® Magnetic Nanoparticles label SSEA-4 cells applied in this study.

heated for 3 min at 99.9°C. After denaturation, samples were separated via 10% or 12% SDS-polyacrylamide gel electrophoresis under reducing conditions. Separated proteins were transferred on to a nitrocellulose membrane using a wet blotter in the Genie Transfer Buffer (20 mM Tris, 150 mM glycine in 20% methanol, pH 8.4) for 180 min at constant amperage of 250 mA. After 1 h blocking with 5% non-fat milk in TBS, 0.1% Tween 20 (dilution buffer) at room temperature with gentle shaking, the membranes were treated with the primary antibody (we used the same primary antibody as for immunofluorescence, at dilution 1:500) overnight at +4°C. β-actin was used as an internal control (1:2000; Sigma–Aldrich). The membranes were washed and incubated with a secondary antibody conjugated with the horseradish–peroxidase labelled horse anti-mouse IgG and goat anti-rabbit IgG (Vector Laboratories; dilution 1:1000) for 1 h at RT. The signals were detected by chemiluminescence using ClarityTM Western ECL Blotting Substrate (Bio-Rad Laboratories GmbH, München, Germany). The blots were visualized using the ChemiDocTM and all bands were quantified using the Image LabTM 2.0 Software (BioRad Laboratories).

Results

Cultures

In the PSC cell cultures after 48 h characteristic embryoid bodies were present. These structures were observed until the end of the culture (Figure 2). Location of stem cell markers and proliferation marker to demonstrate cellular expression of stem cell markers and a proliferation marker, immunofluorescent staining was performed. The immunostaining for c-Kit, GDF-9, Oct-3/4 and Sox-2 were detected mainly in the cytoplasm of PSCs cultured for 7 days. The staining was usually dispersed throughout the cytoplasm of some cells near the nuclei (Figure 3). The immunostaining for Ki-67 was detected in the cytoplasm and nuclei of PSCs cultured for 7 days. The staining was usually dispersed throughout the majority cells (Figure 3). Expression of mRNA for c-Kit, GDF-9, Ki-67 on PSCs the expression of mRNA for c-Kit, GDF-9 and Ki-67 in PSCs was revealed using the reverse transcriptase PCR technique. Electrophoresis displayed PCR amplicons of the predicted sizes: 91 bp for c-Kit, 93 bp for GDF-9, 92 bp for Ki-67 and 83 bp for GAPDH in cultured PSCs (Figure 4). The presence of stem cell markers and proliferation marker at the protein level. The total protein has been isolated from PSCs cultured *in vitro* and then Western blot analyses were conducted. In all samples bands confirming the presence of stem cell markers such an Oct-3/4 and Sox-2 at the protein level were detected when compared with porcine oESCs serving as the positive control. Moreover, in all samples bands confirming the presence of c-Kit, GDF-9 and Ki-67 were detected. β-actin was the loading control (Figure 5).

Discussion

The present work is the report proving the presence of PSCs in postnatal pig ovaries. Moreover, the method of isolation relying on SSEA-4-based magnetic bead sorting, which till now was used mainly to isolate cells from physiological fluids (e.g. blood), in our preliminary *in vitro* studies has been successfully adapted. In normal development, SSEA4 is expressed by the majority of pregonadal primordial germ cells (PGCs) and is progressively downregulated when they enter into meiosis in the embryonic ovary [13]. However, it has been stated that the human SSEA4-positive PSCs from ovarian cell cultures are related to ESCs and cells of the germinal lineage [14].

We observed that during the culture PSCs formed characteristic colonies in the form of aggregates. These clusters consisted of small, round cells with a diameter of 5-7 μ. This is in agreement with Bui et
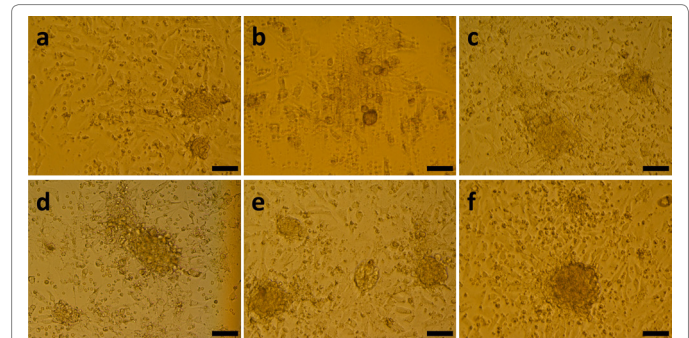

Figure 2: Presence of embryoid bodies in cultured *in vitro* PSCs. The embryoid bodies were present during all days of culture, starting from the second (picture a) to the seventh (picture f). Scale bar on all pictures is 50 mm
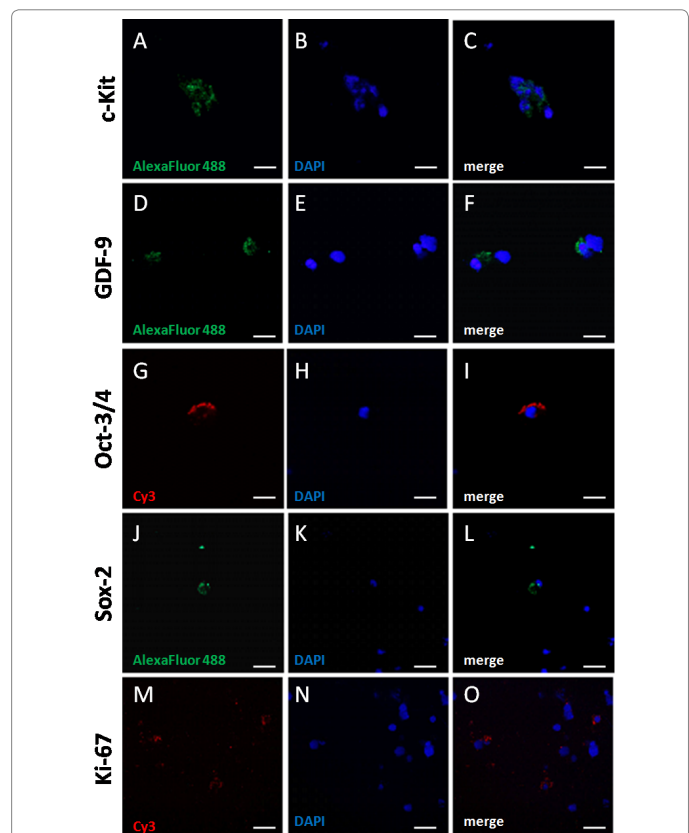

Figure 3: Location of stem cell markers and a proliferation marker: photo A: cytoplasmic immunolocalization of the c-Kit (labelled green by Alexa Fluor 488), photo B: The nuclei labelled blue by DAPI, photo C: superimposed images A and B, photo D: cytoplasmic immunolocalization of the GDF-9 (labelled green by Alexa Fluor 488), photo E: The nuclei labelled blue by DAPI, photo F: superimposed images D and E, photo G: cytoplasmic immunolocalization of the Oct-3/4 (labelled red by Cy3), photo H: The nuclei labelled blue by DAPI, photo I: superimposed images G and H, photo J: cytoplasmicimmunolocalization of the Sox-2 (labelled green by Alexa Fluor 488), photo K: The nuclei labelled blue by DAPI, photo L: superimposed images J and K, photo M: cytoplasmic and perinuclear immunolocalization of the Ki-67 (labelled red by Cy3), photo N: The nuclei labelled blue by DAPI, photo O: superimposed images M and N. No immunofluorescence reaction is seen when the primary antibody is omitted (not shown). Scale bar on all pictures is 10 μm.

al. [4] who reported such a behavior of porcine PSCs under culture condition. This is characteristic for stem cells. It was shown that ESCs form aggregates called embryoid bodies [15]. Embryoid bodies can be obtained particularly easily in ESCs cultured in suspension. Then ESCs spontaneously aggregate to form 3D spatial structures [16]. Oct-3/4,
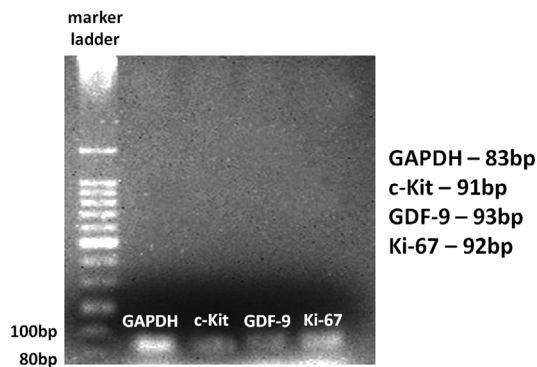
Figure 4: Demonstration of c-Kit, GDF-9, Ki-67 gene expression and GAPDH in PSCs. The sizes of the PCR products are indicated on the right.
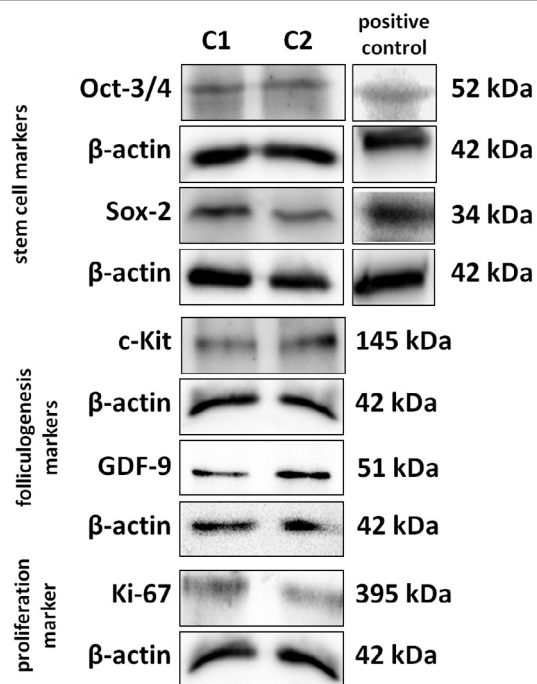
Figure 5: Western blot analysis for: pluripotency markers (Oct-3/4, Sox2), proteins associated with the initiation of cell differentiation during folliculogenesis (c-Kit, GDF-9) and a protein associated with cell division (Ki-67) in two different porcine PSCs cultures (C1, C2) and porcine oESCs (positive control). β-actin was used as a reference.

together with transcription co-activators such as Nanog and Sox-2, inhibits cell differentiation and promotes self-renewal [17]. There are key factors providing the capacity to differentiate. Furthermore, the strong expression of ESC markers i.e. Oct-3/4 and Sox2 in porcine PSCs after 1 week of culture demonstrates that under appropriate conditions the PSCs can dedifferentiate. In our experiment we demonstrated its specific cytoplasmic localization in PSCs after 7 days of culture. Such a result is consistent with reports showing that the location of Oct-3/4 changes over time, from cytoplasmic at the beginning of culture to nuclear thereafter [4]. Such cytoplasmic-to-nuclear (reprogramming) shift of Oct-3/4 expression in PSCs was also demonstrated in the human foetal ovary that showed nuclear localization of Oct-3/4 during the first trimester, with intense cytoplasmic expression during the second one. At week 17 of the foetal development, Oct-3/4 was again identified in the nucleus. Although the significance of cytoplasmic Oct-3/4 expression is unknown, it is notably coincident with major epigenetic changes, such

as the wholesale epigenetic loss of H3K27me3 in human primordial germ cells, followed by the expression of Oct-3/4 in the cytoplasm [18-20]. Moreover, it has been shown that Oct-3/4 overexpression in mouse PSCs enhances their differentiation ability into oocyte-like cells and folliculogenesis, suggesting its potential role in view of reproductive cell therapy [21]. Protein c-Kit is a tyrosine kinase receptor that binds SCF. c-Kit takes part in the regulation of cell cycle control, proliferation, differentiation and migration [22]. This receptor is also involved in the intracellular signal transduction pathways, including those related to the development and progression of cancer [23]. c-Kit can therefore be a marker of both stem cells and cancer cells. The specific expression of Oct-3/4 and c-Kit may indicate that we are dealing with dividing cells, of which a small fraction of has some potential to differentiate. Low but indisputable presence of c-Kit, GDF-9 and Ki-67 at the mRNA level shows that cells with characteristics of stem cells were present in the culture. This is connected with the fact that there are very few cells found in niches. It is difficult to isolate and grow a large population of cells expressing the markers tested. According to literature reports, human cells of the ovarian surface epithelium (OSE) with an epitope of SSEA-4 demonstrate the expression of stem cell markers such as Oct-3/4, Nanog and Sox-2 [14]. Herein our experiments confirmed that PSCs can be isolated by magnetic beads due to the presence of an epitope SSEA-4 also from the porcine ovary. The method of application of antibodies conjugated to magnetic beads was not previously used to isolate stem cells from suspension obtained enzymatically. This is the first such study conducted on pig postnatal ovaries. This is a potentially useful model in this regard because of similarities in organ size, immunology, and whole animal physiology to the human. Enormous hope is connected with ovarian PSCs with regard to cell therapy and neo-oogenesis and this has become one of the most dynamically developing areas of science at the moment.

Acknowledgement

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