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Putative stem cells with an embryonic character isolated from the ovarian surface epithelium of women with no naturally present follicles and oocytes

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Abstract There have been some proposals that stem cells exist in the ovarian surface epithelium (OSE) of the adult human ovary; however, no direct evidence of such cells has been given until now. The aim of this study was to isolate the putative ovarian stem cells (OSCs) from the OSE layer in women with no naturally present oocytes and follicles—20 postmenopausal women and five women with premature ovarian failure. Small round cells with a bubble-like structure and diameters from 2 to 4 μm were isolated from the material obtained by OSE scraping. They expressed early embryonic developmental markers such as stage-specific embryonic antigen-4 and Oct-4, Nanog, Sox-2, and c-kit transcription markers, and they displayed prominent c-kit

immunohistochemical staining. These cells were separated by density gradient centrifugation and grown *in vitro*, where they proliferated. Some of them grew intensively and reached a diameter of approximately 20 μm after 5–7 days. In the OSE cell culture, oocyte-like cells developed, which reached a diameter of up to 95 μm and expressed Oct-4A, Oct-4B, c-kit, VASA, and ZP2 transcription markers, corresponding to early oocytes. They did not express SCP3 meiotic marker. In conclusion, the discovered cells are proposed to represent the adult OSCs with the expression of embryonic stem cell markers. The expression of germ lineage marker c-kit points toward their primordial germ cell ancestry. A new term “embryonic-like stem cells of the adult” is proposed for embryonic-like stem cells that might persist in various tissues and organs of adults. These findings could be used for further studies aimed at the autologous treatment of ovarian infertility and degenerative diseases.

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Introduction

During the past, for at least 50 years many studies have shown that removing the ovarian surface epithelium (OSE) in immature mammals does not later interfere with the follicle/oocyte numbers of the ovary, and that primordial germ cells (PGCs) arise in the early human embryo outside the gonadal ridges (Simkins, 1928, 1932; Byskov, 1986). The PGCs are first identifiable in the human embryo at about 3 weeks in the epithelium

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of the yolk sac, which are then observed, expanded by mitosis, migrating to the connective tissue of the hind gut, and from there into the gut mesentery. Thirty days or so after fertilization, the majority of PGCs pass into the region of the developing kidneys, and into the adjacent gonadal primordia (Johnson and Everitt, 2003). A crucial event for gonadal function is the enclosure of PGCs and somatic cells in specific germ cell compartments (Byskov, 1986). However, several authors have proposed the OSE as a source of oocytes producing stem cells in the fetal and adult mammalian ovary. Because of that, the OSE cell layer was usually named the "germinal" epithelium (Waldeyer, 1870; Kingery, 1917; Allen, 1923; Evans and Swezy, 1931). OSE is a normal, naturally present source of germ cells in the fetal period of life (Simkins, 1928, 1932). Scanning and transmission electron microscopy have revealed numerous germ cells (10 μ m in diameter) within the ovarian OSE of human fetuses from 7 to 24 weeks of intrauterine life (Motta and Makabe, 1986).

There was some experimental evidence showing that the stem cells might also be present in the adult human OSE layer. Wright et al. (1996) have detected telomerase activity in the human fetal, newborn, and adult OSE. Telomerase is a ribonucleoprotein that synthesizes telomere repeats onto chromosome ends and is involved in maintaining telomere length in germline tissues and in stem and cancer cells. Kinugawa et al. (2000) analyzed the telomerase activity in normal human ovaries and in five patients with premature ovarian failure (POF). Telomerase activity was present in the normal ovaries, however, it decreased with age. Two of the POF patients showed high telomerase activity and three showed low telomerase activity. Most epithelia of the female reproductive tract maintain telomerase activity during the reproductive age (Yokoyama et al., 1998). Parrott et al. (2000) have found that normal human OSE express high levels of c-kit receptor and kit-ligand/stem cell factor (SCF) proteins *in situ*, and that *SCF* gene expression in cultured OSE increases considerably. Similarly, Silva et al. (2006) confirmed immunohistologically, that cells in the OSE layer express c-kit *in situ* in goat ovaries.

Bukovsky et al. (2004, 2005, 2006a, 2006b) proposed OSE scraping in order to set-up the cell culture and to differentiate stem cells into different cell types *in vitro*. In the co-culture system, they observed the differentiation of OSE cells into the oocyte-like cells and other cell types, as confirmed by different markers, which provided indirect confirmation of stem cells in the OSE layer in postmenopausal women.

Johnson et al. (2004) have destroyed all primordial follicles in pre-pubertal mice with the alkylating agent busulfan, and confirmed the presence of meiotic active oocytes in ovaries of the same animals in the adult period of life. This could not be possible without stem cells present and capable of differentiating into oocytes. The

same group also reported on putative germ cells, capable of generating oocytes in the bone marrow (BM) of mice (Johnson et al., 2005). BM transplantation restored the oocyte production in wild-type sterilized mice by chemotherapy, as well as in ataxia teleangiectasia-mutated gene-deficient mice, which are otherwise incapable of making oocytes. The appearance of donor-derived oocytes was also observed in female mice following peripheral blood transplantation. Their results identify BM as a potential source of germ cells that could sustain oocyte production in adulthood. Similarly, Ratajczak's group demonstrated that human bone marrow and umbilical cord blood, similar to the pancreas, epidermis, myocardium, and testes in humans and animals contain a population of stem cells, which express early developmental markers, such as stage-specific embryonic antigen-4 (SSEA-4) and transcription factors such as Oct-4 and Nanog (Ratajczak et al., 2007). They hypothesize that these cells are pluripotent stem cells, which are deposited in the organs during embryogenesis, probably during early gastrulation, and that they are direct descendants of the primordial germ lineage. The germ lineage creates soma to pass genes to the next generations and therefore becomes a "mother lineage" for all somatic cell lineages in the adult human body.

It is recently accepted that aggressive ovarian tumors originate from ovarian stem cells (OSCs), which start to proliferate intensively without control (Jiang et al., 2003; Piek et al., 2004; Bapat et al., 2005; Rosen et al., 2005). More than 80% of ovarian tumors originate from OSE. However, there has still not been any direct proof of the existing OSCs in the human OSE. Szotek et al. (2006) have identified breast cancer-resistance protein 1-expressing verapamil-sensitive side populations in human ovarian cancer cell lines. They proposed that in the future, individualized therapy must incorporate analysis of the stem cell-like subpopulation of ovarian cancer cells when designing therapeutic strategies for ovarian cancer patients.

In spite of all extensive experimental work, the presence of stem cells in the OSE of adult human ovaries has not yet been proven. The aim of our work was therefore to isolate and characterize the hypothetical human OSCs with an embryonic character from the adult OSE layer. For this experiment, women without oocyte and follicle formation, i.e. postmenopausal women and young infertile women with POF were chosen in order to see whether OSCs can be isolated from their OSE and grown *in vitro*.

Materials and methods

In this study, 20 postmenopausal women with a mean age of 59 years (50–75 years) and five infertile women with POF and a mean age of 34 years (28–40 years) were included at the University Medical Centre Ljubljana. None of them presented with ovarian cancer and they received no hormonal induction of their ovaries. In the

postmenopausal women, ovaries were surgically removed due to different gynecological reasons (i.e., ovarian cysts, prevention of breast cancer). Patients donated a part of the ovarian tissue (biopsy material) for this research by informed consent. In infertile women with POF, a standard diagnostic laparoscopic biopsy was performed for stem cell retrieval. Likewise, stem cell retrieval was performed after their informed consent.

In each biopsy material, OSE scraping was performed. Ovarian biopsy specimens were gently washed in a saline solution more times so not to impair the OSE layer too much. We tried to remove blood-borne cells as much as possible. Retrieved cell suspension was observed for the presence of potential stem cells and cultured *in vitro* for 20 days. All histological analyses and ovarian cell cultures were performed at the University Medical Centre Ljubljana.

This study was approved by the Slovenian Medical Ethical Committee (Ministry of Health, Republic of Slovenia) and Slovenian Assisted Reproduction Authority.

Microscopy of cell suspensions and isolation of putative stem cells

Several drops of each cell suspension were observed immediately after OSE scraping and then daily during the culture under the heat-staged inverted microscope (Eclipse TE2000-S, Nikon, Tokyo, Japan) at magnification $\times 200$ or $\times 400$, and under the heat-staged light microscope at magnification $\times 1,000$ (Zeiss Imager Z1, equipped with Axiocam HRm camera, Oberkochen, Germany). The types of cells present in a cell suspension were observed visually. We focused on small round cells and their intense proliferation. We tried to isolate putative stem cells from the scraped cell suspension by centrifugation at 2,500 rpm for 10 min, resuspension of the pellet in a fresh pre-incubated medium, and then PureSperm (NidaCon, Mölndal, Sweden) density gradient centrifugation (100%/40%, 1,200 rpm for 30 min). After density gradient centrifugation there was a layer of cells between both fractions. This layer of cells was removed by a 1 ml syringe and checked under an inverted microscope for the presence of putative stem cells.

Histological analysis of ovarian tissue

A routine histology of ovarian tissue was performed on a part of the cortex of each ovarian biopsy material to evaluate the presence of follicles and oocytes in the ovarian cortex after hematoxylin-eosin staining (HE). Another part of the tissue was used to set-up a cell culture just after ovarian scraping and independently of histopathological evaluation. For the evaluation of OSE, the ovarian tissue was formalin-fixed, embedded in paraffin, and 10 μm sections were collected on microscope slides. These sections were de-paraffinized and rehydrated by immersion of slides in a 0.01 M citrate buffer, pH 6.0, at 98°C for 40 min. The slides were then cooled to room temperature, incubated for 20 min with a mouse monoclonal antibody against high molecular weight cytokeratin, clone 34 β E12 (Dako, Glostrup, Denmark) diluted 1:200 in phosphate-buffered saline (PBS), washed, incubated with peroxidase-coupled rabbit anti-mouse immunoglobulins (Dako), and peroxidase visualized by diaminobenzidine solution as recommended by the vendor (Dako). Finally, the slides were dehydrated and mounted in Canada balsam. The sections were evaluated under the light microscope at magnification $\times 400$ for the presence of OSE cells exhibiting brown staining for cytokeratin and for the presence of putative stem cells.

OSE scraping and culture of cells *in vitro*

For the OSE cell cultures, we used Dulbecco's modified Eagle's medium (DMEM) nutrient mixture/F12 ham medium (1:1) with L-glutamine, 15 mM HEPES, and phenol red (Sigma, St. Louis, MO, USA). The medium was supplemented with sodium bicarbonate—3.7 g/l, antibiotics—50 $\mu\text{g}/\text{ml}$ gentamycin, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (DMEM/F12), with 20% (v/v)

fetal calf serum (FCS). The medium had a pH 7.4. Each biopsy material was washed gently with a saline solution to prevent blood contamination, and then placed in a small sterile Petri dish with 3 ml of pre-incubated culture medium. A sterile surgical blade (Feather, Osaka, Japan) was used to scrape the cells from the ovarian surface only, whereas the cortex was deliberately avoided. Five droplets of the obtained cell suspension were put into a pre-incubated culture medium in sterile Nunclon Surface plastic four-well plates (Nunc, Roskilde, Denmark). In each well there was 350 μl of culture medium. The cells were cultured for 20 days in a CO₂ incubator (Hera-Cell Heraeus, Hanau, Germany) at 37°C and at 5% CO₂ concentration in air. The culture medium was not changed during the whole period. The volume of the medium was kept constant by the addition of some drops of medium each other day.

The settled cell cultures were evaluated daily under the inverted microscope (Eclipse TE2000-S, Nikon) with a heat-staged plate at $\times 200$ or $\times 400$ magnification to follow cell attachment and differentiation. The development of oocyte-like cells was followed by the standard morphological criteria (round shape, the presence of zona pellucida and a polar body) and by the size (diameter of around 90 μm), in order to confirm *in vitro* oogenesis.

Release of cells from the cell culture for analysis

For oocyte-like cell analysis, a part of the attached cell culture including oocyte-like cells in plates was washed with PBS and then detached enzymatically by 50% trypsin-EDTA (Sigma) solution in a normal sterile saline for 4 min at 37°C. After cell detachment, some drops of fetal calf serum were added into each well to stop the activity of trypsin. Cell suspension was then centrifuged at 800 rpm and at 37°C for 10 min and the pellet was resuspended in a fresh, pre-incubated medium. Cell suspension was used for analyses.

Immunohistochemistry of c-kit marker

For immunohistochemical analyses of marker expression, putative stem cells just after scraping and putative stem cells after 20 days of culture were fixed in a 4% paraformaldehyde solution in PBS for 30 min, permeabilized with 0.1% Triton-X, washed and blocked in PBS containing 10% normal donkey serum (sc-2044, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The c-kit primary antibodies used in this study were purchased from Santa Cruz Biotechnology Inc. and used at 1:100 dilutions in PBS containing 1% normal donkey serum overnight at 4°C. Thereafter, the cells were washed and incubated for 40 min with a FITC-conjugated secondary antibody (sc-2024, Santa Cruz Biotechnology Inc.) for the marker c-kit. Cell nuclei were counterstained with 4,6-diamidino-2 phenylindole (DAPI; blue). For negative control, the primary antibody was excluded in the first incubation step. Immunohistochemistry for c-kit marker was performed at the Reproductive Genetics Institute Chicago.

Analysis of transcription markers Oct-4, Oct-4A, Oct-4B, Sox-2, Nanog, c-kit, VASA, ZP2, SCP3 expression by reverse transcription-polymerase chain reaction (RT-PCR)

Cell suspensions obtained immediately after ovarian scraping, including putative stem cells, proliferating putative stem cells, and oocyte-like cells after 20 days of culture were analyzed for the transcription markers at the Blood Transfusion Centre of Slovenia. Putative stem cells were analyzed for Oct-4, Oct-4A (stem cell function), Oct-4B, Sox-2, Nanog, and c-kit transcription markers. Oocyte-like cells were analyzed for Oct 4-A and Oct 4-B, c-kit, VASA, ZP2, and SCP3 transcription markers.

Total RNA was isolated from cells using RNAeasy Mini Kit (Qiagen, Hilden, Germany). The quality of the total RNA was evaluated by optic density (*A* 260/280 ratio of > 1.9). RT-PCR

was performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Primer sequences for analyzed genes are presented in Table 1.

Two negative controls were used; the first negative control was DNase/RNase-free water and the second negative control was total RNA isolated from the long lasting cell culture of fully differentiated human adult chondrocytes. As a positive control for embryonic stem cell markers, total RNA from the peripheral blood was used according to Zangrossi et al. (2007), who reported that human peripheral blood mononuclear cells expressed transcription markers, characteristic of embryonic stem cells. As a positive control for germ cell transcription markers, total RNA, isolated from the non-fertilized human metaphase II (mature) oocytes, and the prepared semen from the *in vitro* fertilization program was used.

For the RT-PCR analyses, the products were deduced as correct based on the predicted size of the amplified cDNA (Table 1), because we obtained primers from other authors, who had previously confirmed the correction of PCR product with different methods and reported the real genes and not pseudogenes (Table 1). We also searched the sequences of analyzed genes in the GenBank from National Centre for Biotechnology Information. All primer sequences used in our study were in the range of normal genes and were also used for analyses of embryonic stem cell lines analyzed by The International Stem Cell Initiative (Adewumi et al., 2007).

Flow-cytometry analysis of SSEA-4

To analyze the SSEA-4 on the cell surface (Draper et al., 2002), the cells collected immediately after OSE scraping and the stem cells collected after 20 days of culture were used. The cells in cell suspension were washed in PBS containing 2% fetal bovine serum by centrifugation. The cell suspension was diluted and about 5×10^5 cells/sample were stained in the same medium with mouse monoclonal antibodies specific for SSEA-4 (Abcam plc, Cambridge, UK) for 30 min on ice in the dark. Subsequently, the cells were washed and incubated with FITC-conjugated goat anti-mouse IgG (H+L) antibodies (Abcam plc) for 30 min in the dark. Control samples were stained with isotype-matched control antibodies. After washing, the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), using CellQuest (BD Biosciences) analysis software.

Table 1 Primer sequences used for the RT-PCR

Gene	Primers	Size (bp)	References
<i>Oct-4</i>	Oct-4F gaaggtattcagccaacgac Oct-4R gttacagaaccacactcgga	315	Klimanskaya et al. (2006)
<i>Sox-2</i>	Sox-2F atgcacgcctacgacgtga Sox-2R ctttgcacccctcccattt	437	Bhattacharya et al. (2005)
<i>Nanog</i>	NanogF tgcaaatgtcttctgctgagat NanogR gtcaggatgttgagagttc	285	Klimanskaya et al. (2006)
<i>Oct-4AB</i>	Common downstream antisense primer ccacatcgccgtgtgtatat Oct-4A ctcctggaggccagggaatc Oct-4B atgcatgagtcagtgaacag	381 for Oct-4A and 303 for Oct-4B	Takeda et al. (1992)
<i>VASA</i>	VASA-F gactgcgcttttctctacc VASA-R tttgccgctgttctcttgat	416	Liu et al. (2007)
<i>SCP3</i>	SCP3-F gggaaaccctctgtgga SCP3-R tgtttcaatctctggctctg	486	Liu et al. (2007)
<i>c-kit</i>	c-kitF aaggactgaggtttattctc c-kitR ctgacgttcataattgaagtc	345	Tanikawa et al. (1998)
<i>ZP</i>	ZP2-F gctccaggaccattctc ZP2-R caggtagcagatggagccta	254	Lefievre et al. (2004)

F, forward; R, reverse; RT-PCR, reverse transcription-polymerase chain reaction.

Results

In all patients included in this study, there were no naturally present oocytes and follicles in the ovarian cortex after HE staining of ovarian biopsy materials (Fig. 1A). In all postmenopausal women a clear OSE layer was seen (Fig. 1B). Among women with POF, an OSE layer was present in four out of five women. It was impossible to find putative stem cells in ovarian sections after HE staining. We found them in ovarian sections after cytokeratin staining. The proportion of putative stem cells was estimated up to 10%. Putative stem cells were slightly green colored, with a typical bubble-like structure, and with similar dimensions as the putative stem cells in the OSE scrapings (Figs. 1C,1D). They were not cytokeratin stained. It was difficult to find them among the surface epithelial cells, because the margins of almost all epithelial cells were heavily stained a brown color and they were very tightly packed. We found some OSE regions which were not stained with cytokeratin. It was impossible to estimate the proportion of unstained cells. We also found clearly visible single putative stem cells above the OSE layer (Fig. 1C) or below the OSE layer, and also among epithelial cells in crypts (Fig. 1E). There was no accumulation of putative stem cells around blood vessels, but they were more often present among epithelial cells in the epithelial crypts, which extended into the ovarian cortex.

Cells after OSE scraping

Immediately after OSE scraping in all 20 postmenopausal women and in four women with POF with the confirmed OSE layer, small round cells were found

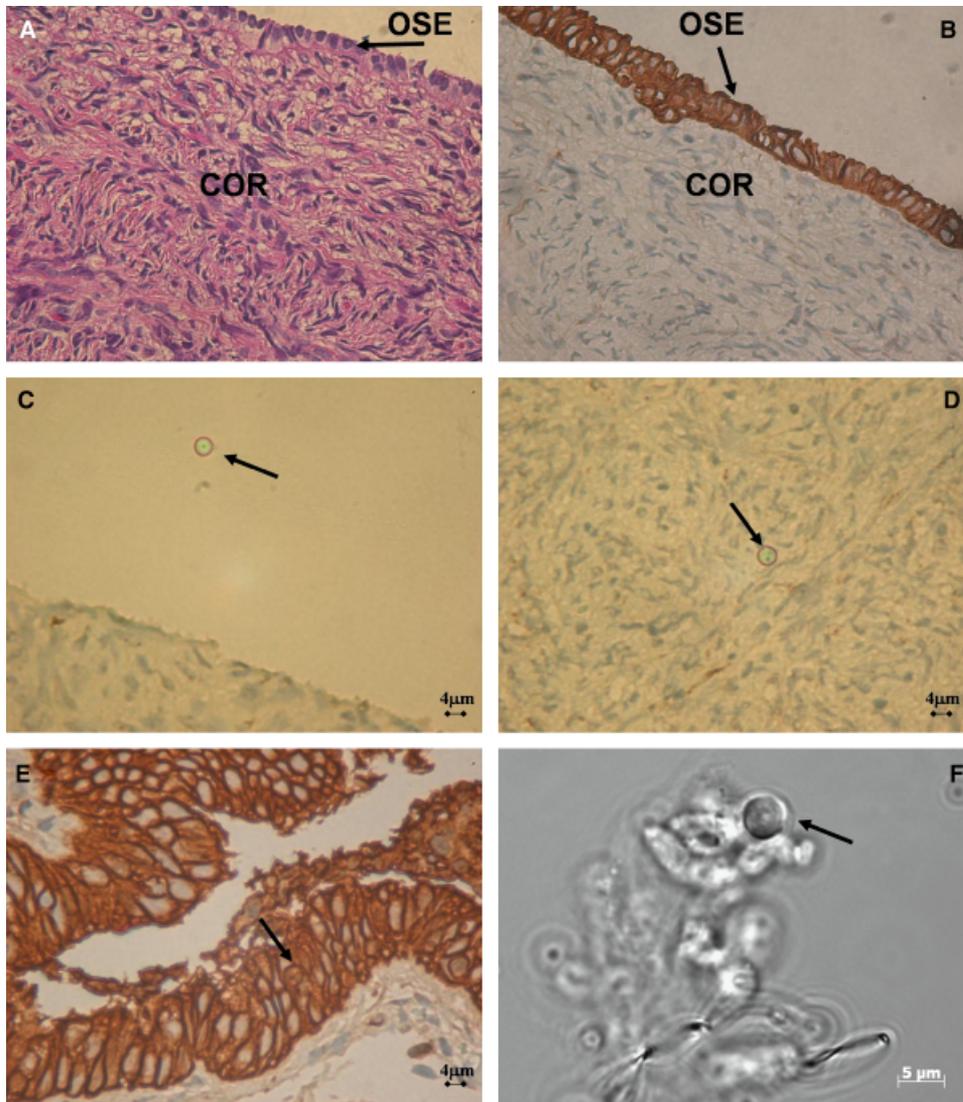


Fig. 1 Ovarian surface epithelium (OSE) and putative stem cells. (A) OSE layer with no follicles and oocytes in the cortex after HE staining of the ovarian section (light microscope, magnification $\times 200$). (B) OSE layer revealed by a brown color after cytokeratin staining of the ovarian section (light microscope, magnification $\times 400$). (C) A small round cell with a bubble-like structure—putative stem cell (arrow) with a diameter of $4\mu\text{m}$ above the OSE layer after cytokeratin staining of the ovarian section (light micro-

scope, magnification $\times 400$). (D) Putative stem cell (arrow) below the tunica albuginea after cytokeratin staining of the ovarian section (light microscope, magnification $\times 400$). (E) Putative stem cell (arrow) in the epithelial crypt after cytokeratin staining (light microscope, $\times 400$). (F) Putative stem cell (arrow) in the cluster of epithelial cells just after OSE scraping (light microscope, $\times 1,000$). COR, ovarian cortex; HE, hematoxylin–eosin; OSE, ovarian surface epithelium.

among collected epithelial cells (Figs. 1F,2). Their diameter was from 2 to $4\mu\text{m}$ and they had a small bubble-like structure inside and slightly yellow appearance (Figs. 2B,2C). We called them putative stem cells. These putative stem cells were floating or were captured in clusters of epithelial cells scraped from the ovarian surface. Concerning the size of putative stem cells, the epithelial cells were mostly of equal size, and sometimes even smaller. Some blood cells were left in the ovarian scrapings, but OSE cells strongly predominated (Fig. 2A). After centrifugation, putative stem cells were isolated successfully from the initial cell suspension obtained by ovarian scraping and a relatively

homogenous cell population was obtained. A layer of cells between both PureSperm fractions consisted of a relatively homogenous population of putative stem cells, which proliferated during the cell culture (Fig. 2D). Their number per observation field under an inverted microscope (magnifications $\times 200$ and $\times 400$) increased considerably.

During the first 3 days of culture, all putative stem cells grew only slightly (from 4 to $6\text{--}8\mu\text{m}$) (Fig. 3). At the same time, some of them started to grow intensively and they reached the diameter of approximately $20\mu\text{m}$ around day 5–7 of culture (Fig. 3C). After reaching this size, they lost their “bubble-like” structure.

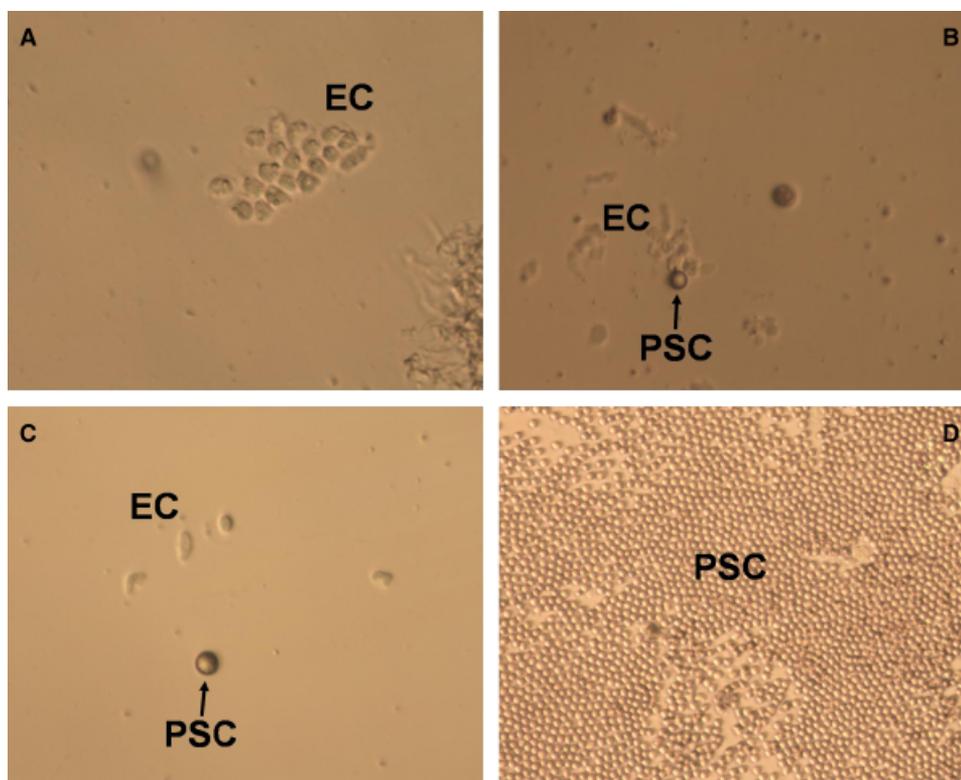


Fig. 2 Ovarian stem cells (OSCs). Small round cells with a bubble-like structure—putative stem cells. (A) Cluster of epithelial cells in a cell suspension immediately after OSE scraping without blood contamination (inverted microscope, magnification $\times 400$). (B) Some epithelial cells and putative stem cells immediately after OSE scraping (inverted microscope, magnification $\times 400$). (C) Epithelial

cells and a putative stem cell immediately after OSE scraping (inverted microscope, magnification $\times 400$). (D) Isolated and proliferating culture of putative stem cells after 20 days of culture (inverted microscope, magnification $\times 100$). EC, epithelial cell; OSE, ovarian surface epithelium; PSC, putative stem cell.

Differentiation of cells in OSE cell culture

An OSE cell culture was successfully set-up in all postmenopausal women and in four out of five patients with POF, in whom the OSE layer was confirmed. The patient with no cell culture development had no confirmed OSE layer.

In each cell culture, cells started to attach to the bottom of the culture plate by day 1 and 2 with an initiation of differentiation. Elongated cells—fibroblasts were the first differentiated cells which developed in the cell culture, and they grew during the culture. Putative stem cells proliferated, and in many occasions they were attached to fibroblasts, as seen by light microscopy. After day 10 of culture some somatic cell types appeared differentiated in a cell culture: myoblast-like cells, neuron-like cells, and a layer of epithelial cells.

Development of oocyte-like cells

On day 5 of the cell culture, oocyte-like cells with a diameter of approximately $20\ \mu\text{m}$ developed among proliferating small cells—putative stem cells in all postmenopausal women and in four out of five women with POF. Some oocyte-like cells were developed from putative stem

cells and they retained small bubble-like structures, which disappeared with further growth (Fig. 4A). Oocyte-like cells mostly grew attached to the fibroblasts, and it appeared that putative stem cells helped them to grow (Fig. 4B). The majority of oocyte-like cells developed attached to the plate bottom. Attached oocyte-like cells grew and reached a diameter of $80\text{--}95\ \mu\text{m}$ by day 20 of culture (Fig. 5A). Some cells developed a zona pellucida-like structure around them (Fig. 5B).

Immunohistochemistry for c-kit marker

Putative stem cells were positive for c-kit just after scraping. After 20 days of cell culture, prominent c-kit staining was present in cells. It was present in the small cells—putative stem cells, and also in bigger cells that have developed from the putative stem cells (Fig. 6A). The nuclei were spread over almost the whole amount of putative stem cells as revealed by DAPI staining (Fig. 6B).

Analysis of transcription markers Oct-4, Oct-4A, Oct-4B, Sox-2, Nanog, c-kit, VASA, ZP2, and SCP3 by RT-PCR

After total RNA isolation from the cells that included a mass of putative stem cells immediately after ovary

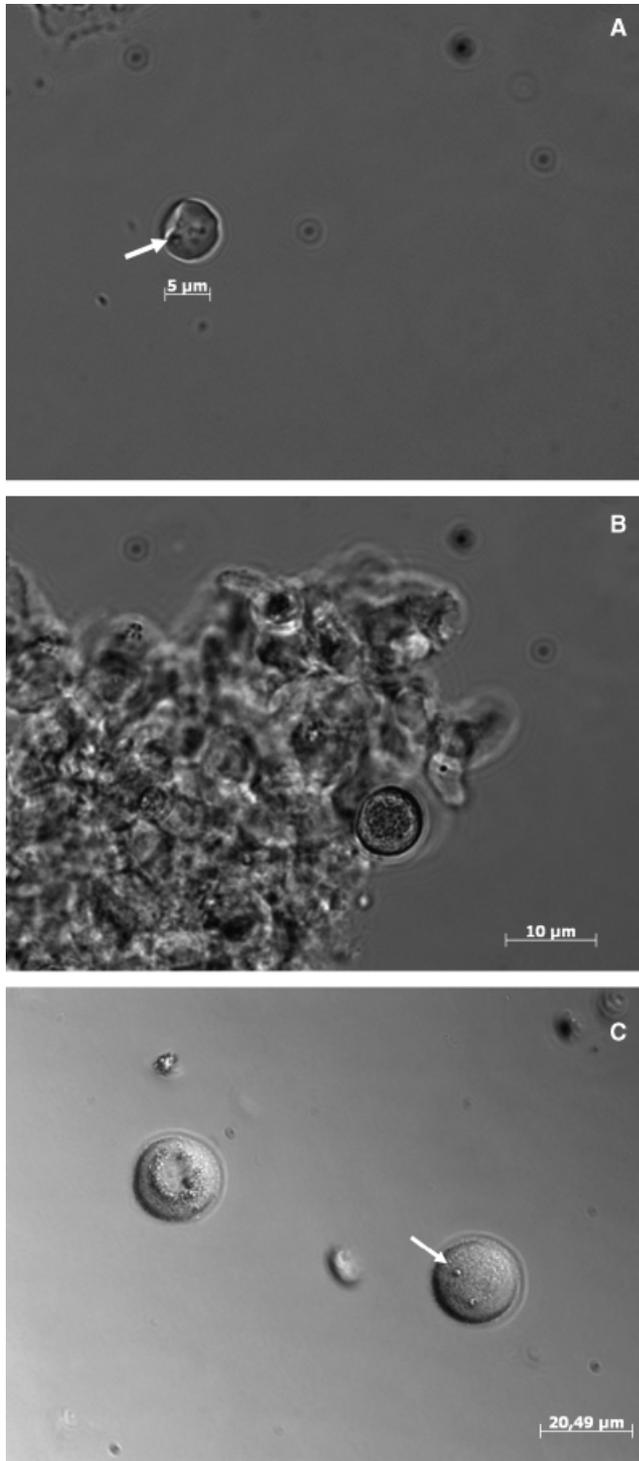


Fig. 3 A small round cell with a clearly visible bubble-like structure (arrow)—putative stem cell growing in the cell culture (A) with a diameter of 6 μm ; (B) with a diameter of 8 μm on day 5 of culture; (C) with a diameter of 20 μm on day 7 of culture (all: light microscope, magnification $\times 1,000$).

scraping there was a strong expression of Oct-4, Oct-4A, Oct-4B, Sox-2, Nanog, and c-kit transcription markers (Figs. 7–9). Similarly, a positive control—total RNA isolated from the peripheral blood expressed Oct-

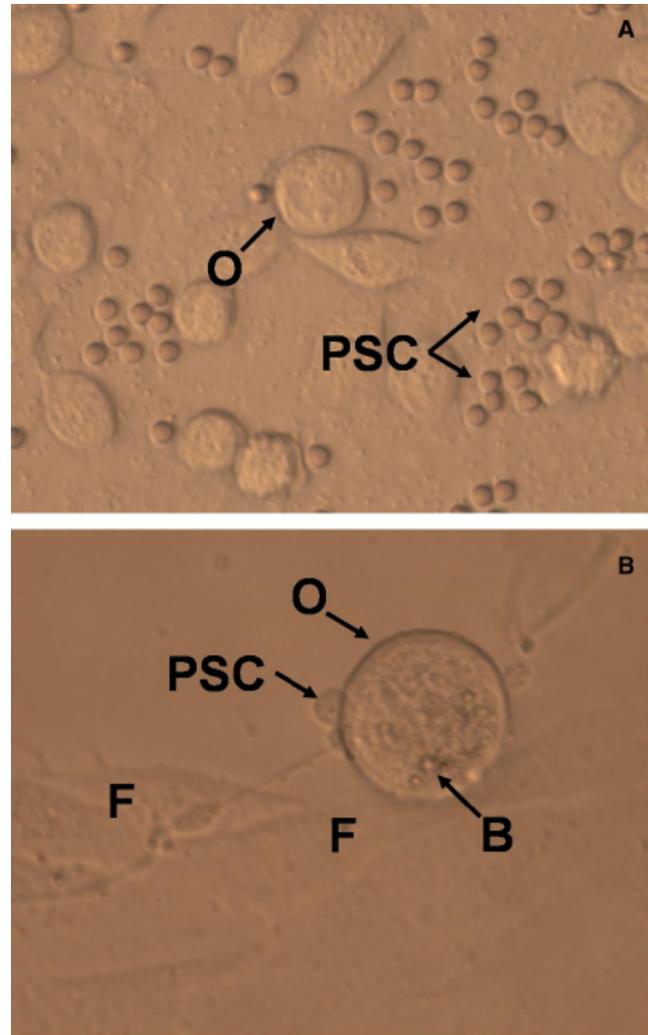


Fig. 4 Oocyte-like cells developing among putative stem cells in the cell culture. (A) Cells of different types, including oocyte-like cell, which attached to the culture plate bottom and started to differentiate among putative stem cells (inverted microscope, $\times 200$). (B) Oocyte-like cell developing in close contact with fibroblasts and putative stem cells on day 5 of culture (inverted microscope, $\times 400$). F, fibroblast; O, oocyte-like cell; PSC, putative stem cells.

4A, Oct-4B, Sox-2, and Nanog transcription markers. After 20 days of the culture of the isolated and proliferated population of putative OSCs, there was a strong *Oct-4* gene and *Oct-4B* gene expression, whereas the expression of *Sox-2* and *Nanog* genes decreased considerably (Figs. 7–9).

After 20 days of culture, total RNA isolated from the oocyte-like cells expressed Oct-4A and Oct-4B (Fig. 10), c-kit (Fig. 11), VASA (Fig. 12), and ZP2 (Fig. 13) transcription markers, whereas it did not express SCP3 transcription marker (Fig. 14).

Flow-cytometry analysis of antigen SSEA-4

Immediately after OSE scraping as well as after 20 days of culture of the isolated cell population, the putative



Fig. 5 Oocyte-like cells after trypsin-EDTA detachment on day 20 of culture. **(A)** With a diameter of 95 μm (inverted microscope, $\times 400$). **(B)** With a zona pellucida-like structure (inverted microscope, $\times 200$).

stem cells expressed the SSEA-4 (Fig. 15). The proportion of SSEA-4-positive cells varied from 10% to 32%. After OSE scraping, the mean proportion of SSEA-4-positive cells was 10%. The proportion of SSEA-4 positive cells after 20 days of culture was increased and was 32% (Fig. 15).

Discussion

To our knowledge, this is the first report on the expression of embryonic stem cell markers in the OSCs obtained from the OSE of postmenopausal and POF women.

The women included in this study were carefully clinically examined and they did not have follicles/oocytes in the ovarian cortex. They received no hormonal induction of their ovaries. We also avoided

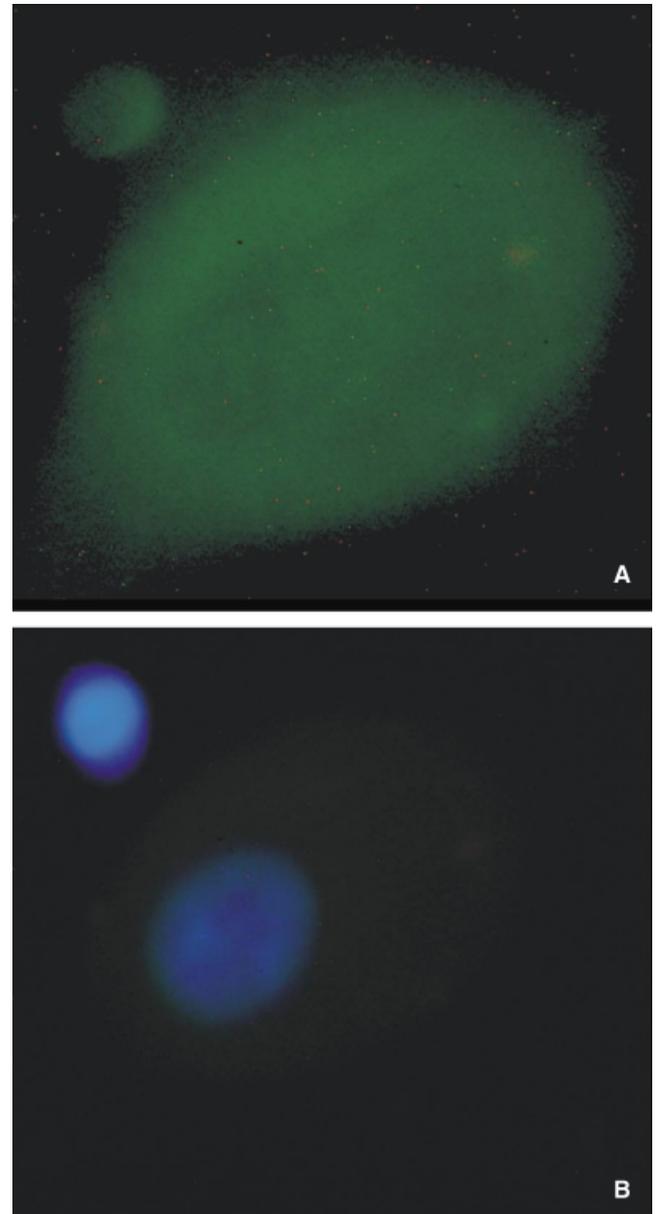


Fig. 6 OSCs stained immunohistochemically for c-kit after 20 days of culture: a small putative stem cell with a diameter of 3 μm , and a bigger cell with diameter of 20 μm . **(A)** After FITC staining. **(B)** After DAPI staining. The nucleus is spread over the whole volume of the small OSC, whereas it covers one-fifth of the bigger oocyte-like cell (both: fluorescent microscope, magnification $\times 400$). OSC, ovarian stem cells.

women with ovarian cancer in order to understand better the normal ovary. Populations of women included in this study are in the center of clinical interest, because the female age and POF are the main factors which limit the success of the current infertility treatment.

In our study, small round cells with a bubble-like structure, with diameters from 2 to 4 μm were found in a cell suspension after OSE scraping. These cells had large nuclei, which spread throughout the whole cell volume with a very small proportion of cytoplasm around them. They had also a slightly yellow appearance. We termed

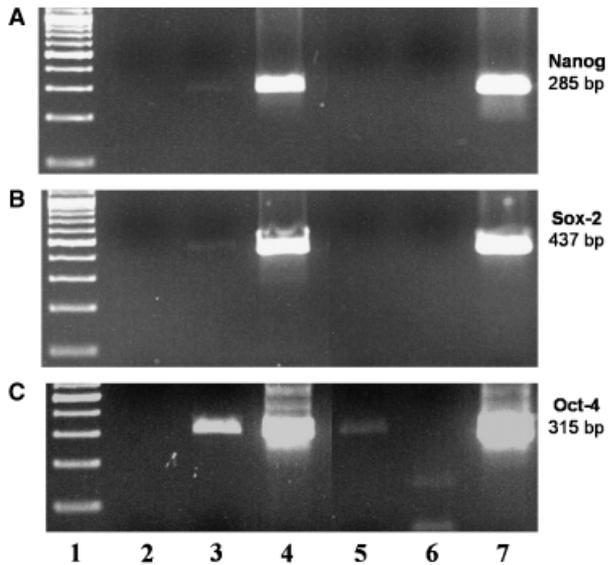


Fig. 7 Transcription marker analysis by RT-PCR. (A) For Nanog. (B) For Sox-2. (C) For Oct-4 transcript marker. Lane 1, molecular marker 100 bp; lane 2, H₂O (negative control); lane 3, proliferated putative stem cells after 20 days of culture; lane 4, putative stem cell just after OSE scraping; lane 5, oocyte-like cells; lane 6, cell culture of fully differentiated human adult chondrocytes (negative control); lane 7, peripheral blood (positive control). OSE, ovarian surface epithelium; RT-PCR, reverse transcription-polymerase chain reaction.

them putative stem cells. These cells were isolated by PureSperm density gradient centrifugation immediately after scraping and they proliferated intensively during the 20 days in culture. We observed that these cells grew a little during the first 3 days of culture *in vitro*. Some of

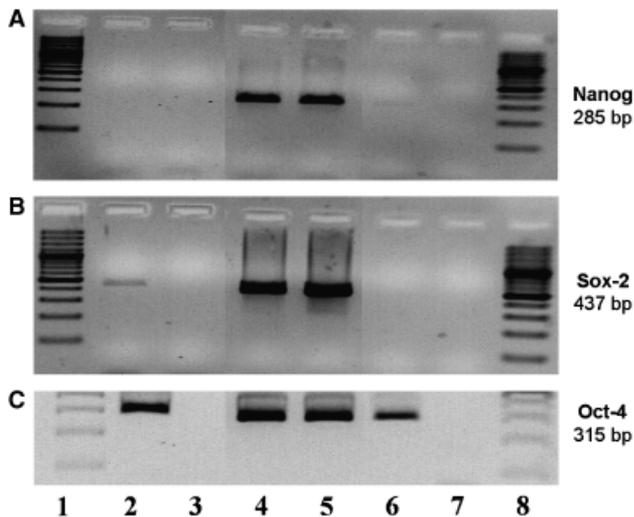


Fig. 8 Transcription marker analysis by RT-PCR. (A) For Nanog. (B) For Sox-2. (C) For Oct-4 transcript marker. Lane 1, molecular marker 100 bp; lane 2, proliferated putative stem cells after 20 days of culture; lane 3, H₂O (negative control); lane 4, peripheral blood (positive control); lane 5, putative stem cells just after OSE scraping; lane 6, oocyte-like cells; lane 7, cell culture of fully differentiated human adult chondrocytes (negative control); lane 8, molecular marker 100 bp. OSE, ovarian surface epithelium; RT-PCR, reverse transcription-polymerase chain reaction.

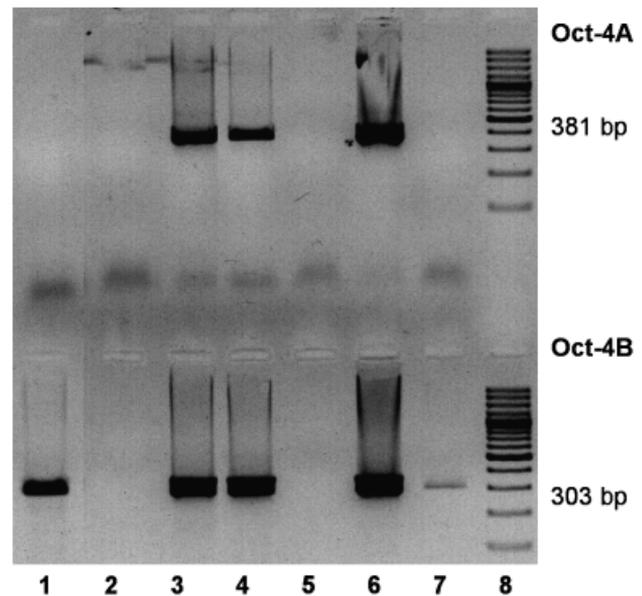


Fig. 9 Transcription marker analysis of putative stem cells by RT-PCR for Oct-4A and Oct-4B transcript markers. Lane 1, proliferated putative stem cells after 20 days of culture; lane 2, H₂O (negative control); lane 3, putative stem cells just after OSE scraping; lane 4, prepared sperm; lane 5, cell culture of fully differentiated human adult chondrocytes (negative control); lane 6, peripheral blood (positive control); lane 7, mature oocytes from the *in vitro* fertilization program; lane 8, molecular marker (381 bp for Oct-4A and 303 bp for Oct-4B). RT-PCR, reverse transcription-polymerase chain reaction.

them reached a diameter of 20 μ m and acquired an “oogonium” like appearance around day 5–7 of culture. The putative stem cells expressed early developmental markers such as a SSEA-4 and transcription factors Oct-4 (new name *POU5F1*)—Oct-4A and Oct-4B, Sox-2, and Nanog immediately after scraping, therefore they could be considered as OSCs with embryonic markers expression. Our finding is in accordance with the results of the International Stem Cell Initiative that characterized 59 human embryonic stem cell lines from 17 laboratories worldwide (Adewumi et al., 2007), and

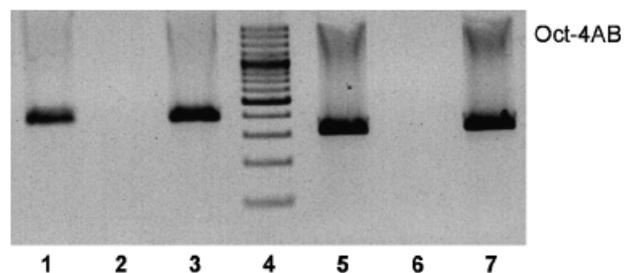


Fig. 10 Transcription marker analysis of oocyte-like cells by RT-PCR for Oct-4A and Oct-4B transcript markers. Lanes 1–4, Oct-4A analysis; lanes 5–7, Oct-4B analysis; lane 1, oocyte-like cells; lane 2, H₂O (negative control); lane 3, prepared semen (positive control); lane 4, molecular marker; lane 5, oocyte-like cells; lane 6, H₂O (negative control); lane 7, prepared semen (positive control). RT-PCR, reverse transcription-polymerase chain reaction.

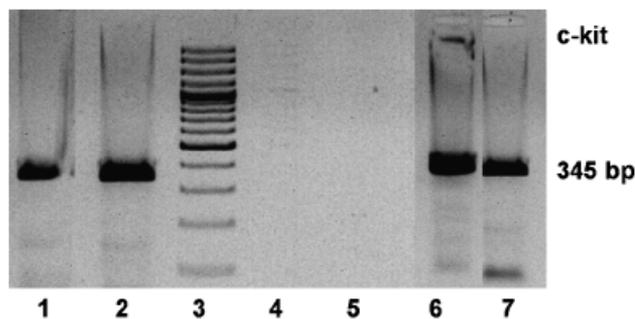


Fig. 11 Transcription marker analysis of putative stem cells and oocyte-like cells by RT-PCR for *c-kit* transcript marker. Lane 1, prepared semen (positive control); lane 2, oocyte-like cells; lane 3, molecular marker 345 bp; lane 4, H₂O (negative control); lane 5, cell culture of fully differentiated human adult chondrocytes (negative control); lane 6, putative stem cells just after OSE scraping; lane 7, mature oocytes from the *in vitro* fertilization program (positive control). OSE, ovarian surface epithelium; RT-PCR, reverse transcription-polymerase chain reaction.

who found that despite diverse genotypes and different techniques used for derivation and maintenance, all lines exhibited similar expression patterns for several markers of human embryonic stem cells, including the glycolipid antigen SSEA-4 and strongly developmentally regulated genes *Oct-4* and *Nanog*. After 20 days of *in vitro* culture, putative stem cells strongly expressed *Oct-4*, whereas they only slightly expressed the *Sox-2* and *Nanog* transcription markers. This could be explained by a decrease in their pluripotency during culture. In the future, we will need to evaluate the dynamics of their embryonic markers expression during cell culture. Our results are in discordance with the recent study of Liu et al. (2007) who did not find positivity for any stem cell and germ cell markers in the healthy adult human ovaries covering an age range from 28 to 53 years, contrary to the testes and fetal ovaries which served as positive controls. However, they have focused on the ovarian cortex, whereas we have focused on the OSE.

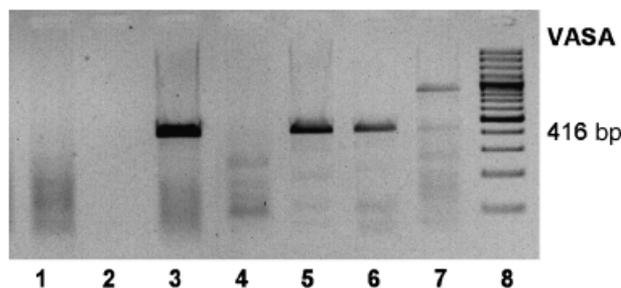


Fig. 12 Transcription marker analysis of oocyte-like cells by RT-PCR for *VASA* transcript marker. Lane 1, putative stem cells just after OSE scraping; lane 2, H₂O (negative control); lane 3, prepared semen (positive control); lane 4, peripheral blood; lanes 5 and 6, mature oocytes from the *in vitro* fertilization program (positive control); lane 7, oocyte-like cells; lane 8, molecular marker 416 bp). OSE, ovarian surface epithelium; RT-PCR, reverse transcription-polymerase chain reaction.

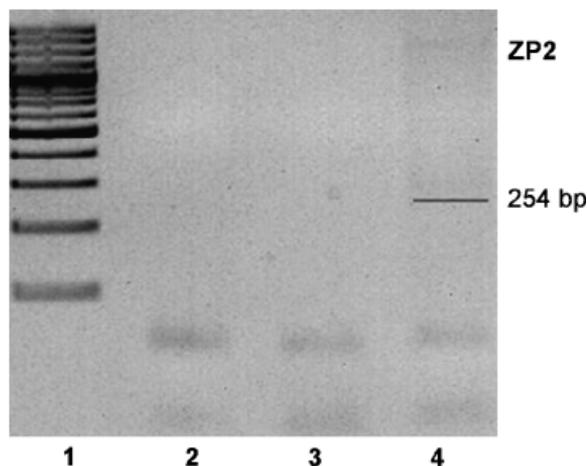


Fig. 13 Transcription marker analysis of oocyte-like cells by RT-PCR for *ZP2* transcript marker. Lane 1, molecular marker 254 bp; lane 2, mature oocytes from the *in vitro* fertilization program; lane 3, H₂O (negative control); lane 4, oocyte-like cells. RT-PCR, reverse transcription-polymerase chain reaction.

Putative stem cells expressed *c-kit* transcription markers just after the OSE scraping. The cultured putative stem cells were also prominently stained for *c-kit* markers as revealed by immunohistochemistry on day 20 of culture. It has been reported that different human stem cell populations expressed tyrosine kinase receptor *c-kit*, for example, the endogenous cardiac stem cells (Barile et al., 2007). Hoyer et al. (2005) evaluated the distribution of the *c-kit* and its ligand *SCF* by immunohistochemistry in PGCs and human embryonic gonads during weeks 5–8 of prenatal life, and in fetal ovaries during weeks 9–36 of prenatal life. Distinct *c-kit* and *SCF* stainings were present in PGCs. *C-kit* receptor and *SCF* as its ligand play important roles during the ascent of primordial germ cells toward the gonadal ridge, and during oogenesis and folliculogenesis in the human fetal ovary. *C-kit* protein is also very important in the adult ovary (Driancourt et al., 2000). During early

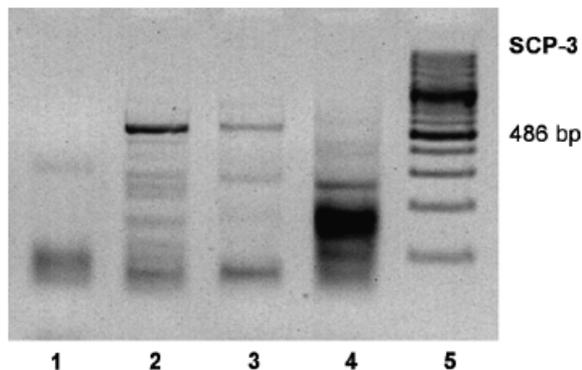


Fig. 14 Transcription marker analysis of oocyte-like cells by RT-PCR for *SCP3* transcript marker. Lane 1, H₂O (negative control); lanes 2 and 3, mature oocytes from the *in vitro* fertilization program (positive control); lane 4, oocyte-like cells; lane 5, molecular marker 486 bp. RT-PCR, reverse transcription-polymerase chain reaction.

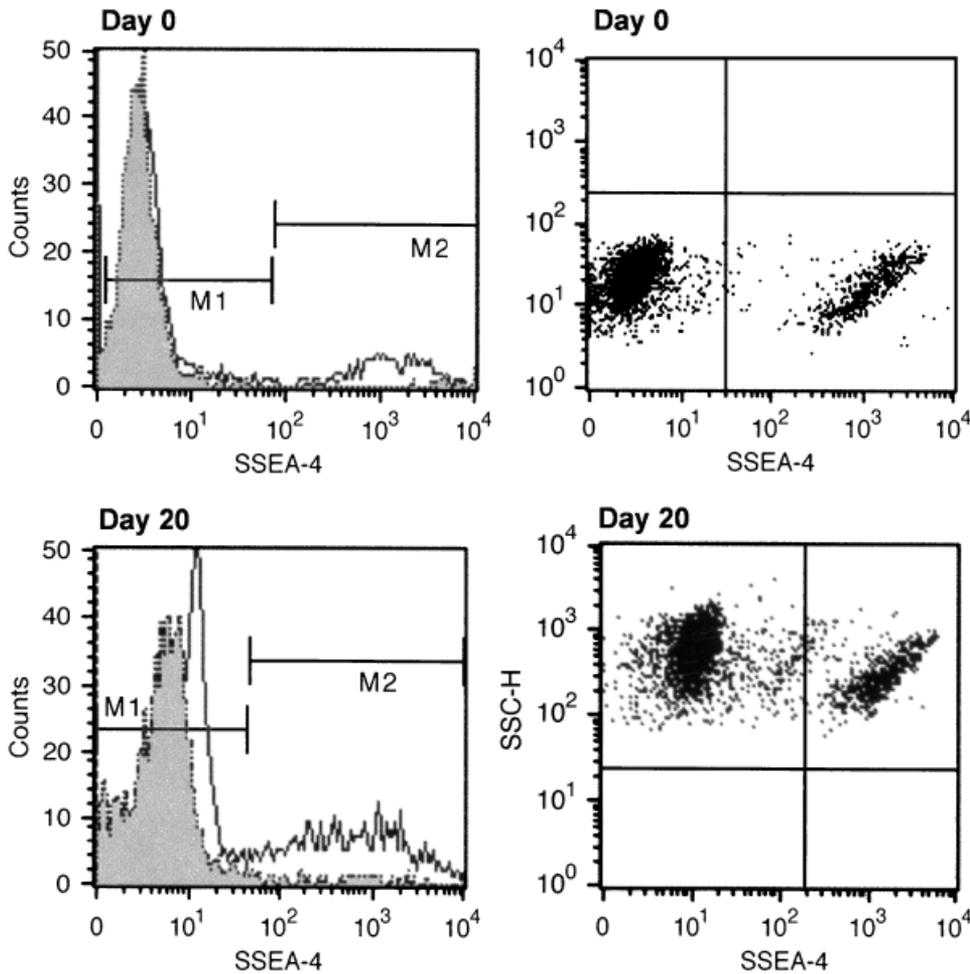


Fig. 15 Populations of putative stem cells (OSCs) immediately after OSE scraping (day 0) and on day 20 of the culture. Both are positive for SSEA-4 antigen as revealed by flow-cytometry analysis. OSC, ovarian stem cells; OSE, ovarian surface epithelium; SSEA-4, stage-specific embryonic antigen-4.

folliculogenesis, the c-kit together with SCF controls oocyte growth and theca cell differentiation, and protects preantral follicles from apoptosis. Formation of an antral cavity requires a functional c-kit/SCF system. In large antral follicles, the c-kit/SCF ligand interaction modulates the ability of the oocyte to undergo cytoplasmic maturation and helps to maximize thecal androgen output. Hence, many steps of oogenesis and folliculogenesis appear to be, at least in part, controlled by paracrine interactions between these two proteins. The recent study of Hoei-Hansen et al. (2007) provides new data supporting the idea that ovarian germ cell tumors develop because of spontaneous c-kit mutation(s) leading to increased survival and proliferation of undifferentiated ongoing.

PGCs can give rise to pluripotent stem cells such as embryonal carcinoma cells and embryonic germ cells (EGCs). Kerr et al. (2008) examined the expression patterns of multiple pluripotent markers in the human fetal ovary, 5.5–15 weeks postfertilization and related this expression with the ability to derive pluripotent EGCs *in vitro*. They identified the specific subpopulations which included OCT4(+)/Nanog(+)/cKIT(+)/VASA(+) PGCs and oogonia. These cells also expressed SSEA4 expression that occurred throughout the

entire gonad. Isolation of these cells from the gonad resulted in EGC colony formation. The number of OCT4(+) or Nanog(+) expressing cells peaked by week 8 and then diminished after week 9 postfertilization, as oogonia entered meiosis. They concluded that PGCs, a unipotent cell, express most of the markers associated with pluripotent cells in the human fetal ovary (Kerr et al., 2008). The cell population, they have analyzed, expressed the same markers, as putative OSCs in this study.

The putative OSCs could be compared to the stem cells found in different human and animal adult tissues and organs (BM, bronchial epithelium, epidermis, myocardium, pancreas, and testes) as reported by Ratajczak et al. (2007), who also reported a novel population of very small stem cells purified from the human bone marrow (Kucia et al., 2006) and cord blood (Kucia et al., 2007), with a diameter from 3 to 5 μm , which is very comparable to the diameter of putative stem cells—OSCs. They called these cells very small embryonic-like stem cells (VSEL). Similar to the putative stem cells—OSCs, their VSEL stem cells were SSEA-4 and Oct-4 positive. They hypothesized that the VSEL stem cells could be descendants of epiblast cells and some rare migrating PGCs (“germ lineage”) and that they are

deposited in the bone marrow early during embryogenesis and could be mobilized to the peripheral blood after tissue or organ injury in an attempt to regenerate damaged organs. These findings could be confirmed by the findings of some other researchers, who published the presence of stem cells with an embryonic character in some adult tissues and organs: Guan et al. (2006), who evidenced a population of stem cells with an embryonic character in adult mouse testes, Beltrami et al. (2007) in different adult human organs and tissues—liver, heart, bone marrow, and McGuckin and Forraz (2008) in human umbilical cord blood. Moreover, the findings of Drusenheimer et al. (2007) provided direct evidence that human bone marrow cells with an embryonic-like character can *in vitro* differentiate into putative male germ cells and identify bone marrow as a potential source of male germ cells that could sustain sperm production.

Repeated rinsing of the tissue obtained by the ovarian biopsy with a saline solution did not ensure that the total clearing of microvascular beds of blood cells in fact occurred. Also the peripheral blood used as a positive control for transcription marker analyses, was positive for the expression of *Oct-4*, *Oct-4A*, and *Oct-4B*, *Sox-2*, and *Nanog* genes, characteristic for embryonic stem cells. An important question arose, whether the putative OSCs were derived from the ovary tissue or simply ended up from the blood circulation. We cannot exclude that OSCs came from the blood stream. In the early human embryo, PGSS originate outside the gonadal ridges. They might be formed from the totipotent embryonic stem cells (Bukovsky and Virant-Klun, 2007), thus expressing embryonic stem cell transcription markers (Kerr et al., 2008). But, when we observed many sections of ovarian tissue after cytokeratin staining in patients included in this study, there was no accumulation of putative stem cells around the blood vessels. We found more putative stem cells among epithelial cells in the epithelial crypts present in the ovarian cortex, and in the region of the OSE layer or below it. The epithelial–mesenchymal transition of adult human OSE stem cells might be explained by the fact that the putative stem cells and epithelial cells in OSE scrapings were of the same dimensions. The difficult question of the real origin of putative ovarian stem cells could be partially answered by the comparison of the population of stem cells isolated from the adult human ovary with the stem cells with an embryonic character isolated from the human bone marrow and adult organs.

In the OSE cell culture system, oocyte-like cells developed. They reached a diameter of up to 95 μm , which is comparable to human oocytes in the *in vitro* fertilization program. No other cells in the body, other than neurons, have the capacity to grow this large. Some oocyte-like cells developed zona pellucida-like structures. Oocyte-like cells expressed *c-kit*, *Oct-4A*, *Oct-4B*, *VASA*, and *ZP* transcription markers. but were not

positive for early meiotic markers such as *SCP3* protein, as revealed by RT-PCR; they resembled early, immature oocytes. Oocyte-like cells were developing in close contact with fibroblasts and putative stem cells, as seen in the figures. It is known that the fibroblasts express aromatase, which further catalyzes the turnover of C(19) steroids into estrogens (Nelson and Bulun, 2001), supporting the development of oocyte-like cells. Fibroblasts also release a fibroblast growth factor FGF (Lavrinos et al., 1994). In addition, the oocyte-like cells in our study developed with the support of accompanying putative stem cells, which possibly acted as granulosa cells and supplied oocytes with additional required substances and organelles. It has already been proposed in the past that OSE stem cells also differentiate into primitive granulosa cells (Bukovsky et al., 2004).

In vitro oogenesis found in this study reflects the processes in the human fetal ovaries. An important question arose, whether a similar process might occur also in the adult human ovary *in vivo*. Bukovsky et al. (1995, 2001) postulated that a similar process is responsible for the *de novo* formation of follicles and oocytes in the adult human ovary. They reported the occurrence of putative germ cells within the OSE of adult human ovaries by using differential interference contrast and immunohistochemistry and concluded the existing possibility that the adult OSE stem cells differentiate from mesenchymal progenitors in ovarian tunica albuginea and that the germ cells originated from OSE stem cells by asymmetric division. In culture, OSE cells undergo an epithelio-mesenchymal transition. The resulting mesenchymal type cells can be stimulated to differentiate back into the epithelial phenotype (Dyck et al., 1996; Auersperg et al., 1999). On the other hand, there were more experimentally and theoretically based studies, which confirmed the opposite, that the end number of follicles and oocytes is set up at the time of birth, and that there is no *de novo* follicle/oocyte formation in the adult human ovary (Zuckerman, 1951; Block, 1952). During adult life, the supply of follicles is said to decline until an advancing age, when the primordial pool is exhausted (Richardson et al., 1987). This difficult and controversial question, which has already provoked many researchers (Albertini, 2004; Gosden, 2004; Telfer, 2004; Byskov et al., 2005), might also be resolved in the future by means of the *in vitro* model.

In conclusion, this study empirically confirms the presence of OSCs in the OSE layer of postmenopausal women and in women with POF. Their embryonic character was proven by the phenotyping and by transcription marker analysis. The expression of germ lineage marker *c-kit* on the OSCs probably indicates their PGC ancestry. The existence of adult OSCs with embryonic stem cell character in the ovaries adds new perspectives to stem cell biology, namely it becomes evident that in the adult, a larger family of similar embryonic-like stem cells might persist in various tissues

and organs. If future studies confirm this hypothesis, we propose a new term for this group of stem cells, i.e., “embryonic-like stem cells of the adult.” Besides using these findings for further studies aimed at the autologous treatment of ovarian infertility, they could also be used for the development of stem cell therapy models for degenerative diseases.

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