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Review

Ovarian pluripotent/multipotent stem cells and *in vitro* oogenesis in mammals

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Summary. There has been a long persisting dilemma about potential ovarian stem cells in adult mammalian ovaries, including human, and now there is steadily increasing experimental evidence on their existence. After some previous indirect evidence about the presence of stem cells in adult mouse ovaries, an important breakthrough was made by Zou and his co-workers who successfully established long-persisting pluripotent/multipotent ovarian stem cell lines in neonatal and adult mice, and were followed by some other important studies in mouse and human. Moreover, oocyte-like cells can be developed *in vitro* from pluripotent stem cells of different origins (embryonic stem cells, induced pluripotent stem cells, fetal skin stem cells, pancreatic stem cells). The aim of this article is to elucidate the fast growing new knowledge on the ovarian stem cells and potential *in vitro* oogenesis in mammals.

Key words: Germinal stem cell, Oocyte, Oogenesis *in vitro*, Ovary

Introduction

Currently there is steadiliy increasing experimental evidence on the existence of pluripotent/multipotent stem cells in neonatal and adult mammalian ovaries. There is also increasing evidence about the potential *in vitro* development of oocyte-like cells from pluripotent stem cells of different origins, e.g., stem cells isolated from the porcine fetal skin and induced pluripotent stem cells in human. The aim of this article is to review the fast growing new knowledge on the ovarian stem cells and potential *in vitro* oogenesis in mammals.

Stem cells in mammalian fetal ovaries

Primordial germ cells (PGCs) are undifferentiated cells in developing fetuses which give rise to gametes, oocytes and spermatozoa, and contribute to new life in the next generation. They arise outside the genital ridge region, and are first identifiable in the human embryo at about 3 weeks in the yolk sac epithelium near the base of the developing alantois. Then the PGC population, expanded by mitosis, migrates by amoeboid movement to the connective tissue of the hind gut and from there into the gut mesentery. From about 30 days after fertilization the majority of cells pass into the region of the developing kidneys, and then into the adjacent gonadal primordia where they join the cells of the sex and medullary cords (Johnson and Everitt, 2000). They arise in the fetal ovaries as small clusters of 40-50 alkaline phosphatase-positive cells (Sabour et al., 2011). Their development, differentiation, and survival (apoptosis) in the fetal gonads are strictly controlled regarding their genetic and epigenetic factors, and are regulated by a combination of different growth factors and other molecular regulators known as the germ cell niche. By establishing long term cell cultures of human fetal ovaries it has been confirmed that bone morphogenetic protein (BMP) is developmentally regulated and one of the key regulators of the PGCs' fate by promoting their apoptosis (Childs et al., 2010). Human PGCs can develop into pluripotent stem cells such as embryonal carcinoma cells (ECCs) and embryonic germ cells (EGCs). PGCs express most, but not all of the markers which are associated with pluripotent stem cells in the human fetal ovary (Kerr et al., 2008). Specific subpopulations of PGCs and oogonia expressing OCT4, NANOG, and c-KIT markers were identified and stage-specific embryonic antigen-4 (SSEA-4) expression was found throughout the entire human fetal ovary by immunohistochemistry.

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Commitment of PGCs to germ cells during mammalian embryogenesis

Molecular mechanisms which enable the commitment of PGCs to germ cells during mammalian embryogenesis are still poorly understood due to the limited amount of available fetal ovarian tissue to perform complex research. Only a few genes (*TNAP, BLIMP1, STELLA,* and *FRAGILIS*) are known to mark the germ cell commitment in the outer layer of cells of the embryo epiblast and can be used with some success to detect PGC formation *in vitro*. Recently, 11 genes specifically expressed in female and male mouse fetal germ cells, but not in ESCs and somatic tissues, were identified (Sabour et al., 2011). These genes were: *Fkbp6, Mov10l1, 4930432K21Rik, Tex13, Akt3, Gm1673, Hba-a1, Pik3r3, Plcl2, Spo11, and Tdrkh.* Three of these genes - *4930432K21Rik, Tex13* (*testis-expressed gene 13*) and *Gm1673* (*predicted gene 1673*) are novel and their functions are still unknown. These genes have already been proposed as ideal and specific markers to identify germ cells developed *in vitro* from pluripotent stem cells (Sabour et al., 2011). *FIGLA* and *NOBOX* genes represent a growing number of oocyte-specific transcription factors which regulate different genes unique to early oogenesis (Pangas and Rajkovic, 2006).

Stemness of PGCs

Because of their stem-like character, human PGCs and EGCs have already been proposed to be researched in terms of cell therapies and potential development into oocytes (Aflatoonian and Moore, 2005).

Mature oocytes can be generated *in vitro* from the mouse oogonia (Qing et al., 2008), human primary oocytes obtained by mechanical disaggregation and cultured in a SCF medium (Brieño-Enríquez et al., 2010), bovine primordial follicles (McLaughlin and Telfer, 2010), and also from mouse premeiotic fetal germ cells. A simple and efficient method as a combination of *in vivo* transplantation and *in vitro* culture (maturation) that can be used to obtain mature oocytes from the premeiotic germ cells of a fetal mouse has been developed (Shen et al., 2006). By using this method, mouse fetal ovaries were isolated and transplanted under the kidney capsule of the recipient mice to initiate oocyte growth from the premeiotic germ cells, and they were recovered 14 days later. Subsequently, the primary and early secondary follicles generated in the ovarian grafts were isolated and cultured *in vitro* for 16 days. The mature oocytes “ovulated” from these follicles and were able to be fertilized *in vitro* and to produce live offspring. The offspring after the *in vitro* fertilization were normal and were able to successfully mate with both females and males. The patterns of the methylated sites of the *in vitro* matured oocytes were similar to those of normal mice. It has been shown that the whole process of oogenesis, from premeiotic germ cells to germinal vesicle-stage oocytes, can be carried out under the kidney capsule of the recipient mice (Shen et al., 2006). Moreover, recently a population of PGCs was collected from the mouse female fetal gonads and, together with gonadal somatic cells, transplanted under the kidney capsule of adult mice (Matoba and Ogura, 2011). The transplanted cells formed ovarian-like tissue under the kidney capsule, and fully grown germinal vesicle oocytes developed within this tissue. PGC-derived oocytes were isolated, matured *in vitro*, and microinjected with normal sperm. In this way the retrieved oocytes were fertilized, and after the transfer of the embryos into the uterus of adult mice, normal pups were born (Matoba and Ogura, 2011). This work demonstrated the flexibility of the PGCs’ development into the competent female gametes and proposed the transplantation procedure as a technical basis for the induction of the development of early germ cells of exogenous origins, such as those from embryonic stem cells. Mouse fetal germ cells were also capable of forming primordial follicles and developing into mature oocytes *in vitro*, which can be successfully fertilized *in vitro* and developed into embryos at the morula/ blastocyst stage (Shen et al., 2007).

All this methodology could not be used in human reproductive medicine, but brought important basic knowledge which could support the successful *in vitro* differentiation of pluripotent stem cells retrieved from different sources into the competent oocytes in future.

Stem cells in adult mouse ovaries

The existence of female germline stem cells (GSCs) in postnatal mammalian ovaries still remains a dilemma and a controversial issue among reproductive biologists and stem cell researchers (Skaznik-Wikiel et al., 2007; Tilly and Telfer, 2009). After some previous indirect evidence about the presence of stem cells in adult mouse ovaries (Johnson et al., 2004, 2005) an important breakthrough was made by Zou and his co-workers (Zou et al., 2009). They found the presence of *Mvh* (mouse *Vasa* homologue) positive cells in the ovarian surface epithelium of neonatal mice ovaries. After immunomagnetic isolation, they established a neonatal mouse germ stem cell (mGSC) line persisting for more than 15 months. At the same time, GSCs from adult mouse ovaries were isolated and cultured for more than 6 months. These GSCs retained high telomerase activity and a normal karyotype during prolonged culture. After infection with a green fluorescent protein (GFP) virus and its transplantation into the infertile mice, transplanted GSCs underwent oogenesis and the mice produced GFP transgene marked offspring (Zou et al., 2009).

By using a transgenic mouse model in which the GFP was expressed under a germ cell-specific *Oct4* promoter, multipotent stem cell lines were isolated and established from the mouse postnatal and adult ovaries (Pacchiarotti et al., 2010). Two distinct populations of

GFP-*Oct4* positive cells were found in the mouse ovaries, based on their distribution and size. A small group of cells with an average diameter of 10-15 μm was located at the ovarian surface epithelium and larger cells with an average diameter of 50-60 μm resembling oocytes were located in the center of the follicular compartment. Flow cytometry analysis revealed that the percentage of GFP-*Oct4* positive cells in the mouse ovaries significantly decreased with age; while 1-2% positive cells were found in the neonatal mice ovaries, only 0.05% was still present in the adult ovaries (Pacchiarotti et al., 2010). These ovarian GSC lines maintained their stem cell characteristics, high telomerase activity, and normal karyotype after many passages for more than 1 year. They formed embryoid body-like structures with a differentiation into all three germ cell layers (endoderm, mesoderm, and ectoderm). The germline stem cells were distinct from the CD133-positive cells circulating in the bloodstream.

Recently, two lines of colony-forming cells isolated from adult mouse ovaries were established on somatic fibroblasts, which expressed markers specific for pluripotent embryonic stem cells and formed embryoid bodies and teratoma after injection into SCID mice (Gong et al., 2010). The embryonic stem cell-like cells in adult ovaries were proposed to be researched also in humans, because they may represent an alternative for establishing autologous stem cell lines from adults without any genetic manipulation.

In all the above-mentioned studies, mouse ovarian stem cells were cultured in comparable culture conditions: in DMEM (Dulbecco's Modified Eagle's Medium) or MEM-α (Minimal Essential Medium-alpha) culture medium containing fetal bovine serum (FBS), antibiotics (penicillin, streptomycin) and some other substances (i.e., sodium pyruvate, non-essential amino acids, L-glutamine, ß-mercaptoethanol, LIF, transferrin, insulin, putrescine, EGF, GDNF, basic FGF), and on mitotically inactivated mouse embryonic fibroblasts (MEFs), as can be seen in Table 1.

The characterization, culture, and *in vivo* and *in vitro* differentiation of putative thecal stem cells isolated from the mouse neonatal ovaries have already been published (Honda et al., 2007).

There is also some evidence that aged mouse ovaries possess not only stem cells, but also dormant premeiotic germ cells expressing *Stra8* and *Dazl* genes that can develop into fully competent GFP-positive oocytes following transplantation into a young host mouse environment, as revealed by the use of aged germline-specific GFP-expressing transgenic mice (Niikura et al., 2009).

Stem cells in adult human ovaries

Very little is known about the presence of stem cells in adult human ovaries. One of the main reasons is that human ovarian tissue is not an easily available material to be researched for the presence of stem cells. In spite of this, Bukovsky and his co-workers scraped the ovarian surface epithelium of postmenopausal ovaries and cultured the scraped population of cells *in vitro* (Bukovsky et al., 2005). Although human ovarian surface epithelium cell cultures have been previously cultured in terms of research of its biology and epithelial cancer formation, Bukovsky's group was the first to observe the development of large oocyte-like cells in postmenopausal ovarian surface epithelium cell cultures in the presence of estrogenic stimuli (phenol red). These large cells with a diameter of 180 μm exhibited germinal vesicle breakdown, expulsion of the polar body, and a surface expression of zona pellucida proteins, as revealed by cytochemistry. This was the indirect evidence of putative stem cells in the ovarian surface epithelium layer of postmenopausal ovaries with no naturally present follicles/oocytes. In the next step, they found the steroid-mediated differentiation of neural/neuronal cells from the epithelial ovarian precursors *in vitro* (Bukovsky et al., 2008).

Some further steps were made by Virant-Klun and her co-workers. They identified an unknown population of putative stem cells in the ovarian sections *in situ* (Fig. 1) and in a population of cells scraped from the ovarian surface epithelium (Fig. 2) of women with no naturally present follicles/oocytes - postmenopausal women and women with premature ovarian failure (Virant-Klun et al., 2008). These cells expressed some transcription factors of pluripotent embryonic stem cells such as OCT4, SOX2 and NANOG, and developed *in vitro* into oocyte-like cells which expressed some oocyte-specific markers. By magnetic-activated cell sorting (MACS), two different types of SSEA-4 positive cells (Figure 2) were isolated by anti-SSEA-4 antibody-coated beads from the scraped ovarian surface epithelium in these women. These cells were positive for SSEA-4 surface antigen. Moreover, they found parthenogenetic blastocyst-like structures (Fig. 3) in the ovarian surface epithelium cell cultures of postmenopausal women with no naturally present follicles/oocytes (Virant-Klun et al., 2009; Virant-Klun and Skutella, 2010).

Furthermore, the multipotent subpopulation of luteinizing granulosa cells was isolated from the follicles, the follicular fluid of infertile women, included in the *in vitro* fertilization programme (Kossowska-Tomaszczuk et al., 2009). These cells were maintained in culture over prolonged periods of time in the presence of leukemia-inhibitory factor (LIF), expressed mesenchymal lineage markers (CD29, CD44, CD90, CD105, CD117, and CD166) and were differentiated *in vitro* into different cell types, such as neurons, chondrocytes, and osteoblasts. After their transplantation into immunodeficient (SCID) mice, these cells survived and generated *in vivo* tissues of mesenchymal origin. There is also some new indirect evidence about the potential stem cells in the ovary in terms of telomerase activity in adult human ovaries (Liu and Li, 2010).

In vitro oogenesis

Enormous effort has been put into the development of oocytes from pluripotent embryonic stem cells (ESCs), which have an unlimited self-renewal feature and are able to differentiate into almost every mature cell type in the body (Oktem and Oktay, 2008). Some studies showed that blastocyst inner cell mass (ICM) cells expressed *NANOS1, STELLAR* and *OCT4* genes, whereas undifferentiated human ESCs expressed all these genes along with the germ cell-specific gene *DAZL* (Clark et al., 2004). It is known that ESCs express some germ cell specific genes such as *OCT4, BLIMP1, STELLA, FRAGILIS, VASA* and *DAZL*. Upon ESC differentation into embryoid bodies they expressed some RNA and protein markers of immature premeiotic germ cells and mature germ cells, including *VASA, BOL, SCP1, SCP3, GDF9,* and *TEKT1* (Clark et al., 2004). ESCs can develop into oocyte-like cells given the right conditions, but most of the studies confirmed that these cells were not mature and fully competent oocytes. Oocytes developed *in vitro* expressed only some oocyte-specific genes, did not extrude zona pellucida and did not progress through the process of meiosis in most cases.

In 2003 it was published in the Science journal that mouse embryonic stem cells (mESCs) had been for the first time successfully developed into oocytes (Hübner et al., 2003). These cells expressed some oocyte specific genes and seemed to enter meiosis; they recruited adjacent cells to form follicle-like structures, and were able to develop into parthenogenetic blastocysts. Therefore, an advanced study of this possible approach to infertility treatment was proposed (Kehler et al., 2005). Later, it was reported that mESCs indeed formed follicle-like ovarian structures with oocyte-like cells,

Table 1. Isolation and culture of mouse stem cells from neonatal and adult ovaries according to the literature.

Source	Isolation	Culture medium and feeder layer	Culture conditions	Cell morphology and function	Cell characteristics	Reference
5 day-old (neonatal) and adult mice ovaries F1 (C57BL/6XCD-1) hybrid mice Mice were injected with BrdU for cell proliferation analysis.	-two-step enzymatic digestion -MACS isolation of Mvh (mouse Vasa homologue) positive cells	MEM-α containing FBS, sodium pyruvate, non-essential amino acids, L-glutamine, ß-mercaptoethanol, LIF, transferrin, insulin, putrescine, mouse EGF, human GDNF, human basic FGF, penicillin STO cell feeder (from mitotically inactivated mouse embryonic fibroblasts)	-medium changed every 2-3 days -cells subcultured using trypsin every 5-8 days Neonatal GSCs: cultured more than 15 months, 68 passages Adult GSCs: cultured more than 6 months, 25 passages	-large round or ovoid cells, nuclear diameter of 12-20 μm, little cytoplasm -present in ovarian surface epithelium -colony-forming cells Offspring after transplantation of GFP marked cells into ovaries of infertile mice	-expressed markers: Oct4, Mvh, Dazl, Blimp1, Fragilis, Stella, Rex1 -AP positive, high telomerase activity, normal karyotype, female imprinting pattern, pluripotent.	Zou et al., 2009
Adult (10-week old) mice ovaries F1 (C57BL6XD BA2) hybrid mice and outbred (ICR) mice	-digestion by trypsin-EDTA and collagenase Type I -filtration and centrifugation	DMEM containing FBS, non-essential amino acids, L-glutamine, ß-mercaptoethanol, mouse LIF, lyophilized mixture of penicillin and streptomycin MEF (mitotically inactivated mouse embryonic fibroblasts)	-on day 7 of primary culture cell colonies mechanically removed and placed on a new MEF monolayer -medium changed daily -sub-passaged every 3 days	-colony-forming cells -in ovarian stroma Differentiated *in vitro* into neuronal cells. Formed EB *in vitro* and teratoma in NOD-SCID mice. EB and teratoma positive for markers of all three germ layers.	-expressed markers: SSEA-1, Oct4, Nanog, Rex1, Cripto, Dnmt3b, Tert, Lif RC, Stat3, Bmp4, Fgf4, Foxd3, Sox2, CD9, Gdf3, integrin α6, integrin ß1, -AP positive, high telomerase activity, normal female karyotype, methylation status different than in ESCs, pluripotent	Gong et al., 2010
2 to 5 day-old (neonatal) and adult mice ovaries OG2 mice	-digestion by collagenase and DNase-I -GFP fluorescent-activated cell sorting (FACS)	According to Okamoto et al., 1990: MEM-α containing FBS MEF (mitotically inactivated mouse embryonic fibroblasts) Every two weeks cells transferred to a new MEF plate.	-on day 7, small colonies manually split for 4-5 passages before using trypsin -half of medium changed every other day	Two distinct populations of cells with different diameters: 1.) 10-15 μm in the ovarian surface epithelium and 2.) 50-60 μm in the center of the follicular compartment. Formed EB *in vitro* and did not form teratoma in SCID mice.	-expressed markers: SSEA-1, Oct4, Vasa, c-Kit, Gcna, Nanog, Gfr-α1, Gdnf -moderate telomerase activity, normal karyotype, formed oocyte-like cells and primordial follicle-like structures, multipotent	Pacchiarotti et al., 2010

BrdU: 5'-bromodeoxyuridine, FBS: fetal bovine serum, DPBS: Dulbecco's Phosphate Buffered Saline, GFP: green fluorescent protein, LIF: leukemia inhibitory factor, EGF: epidermal growth factor, FGF: fibroblast growth factor, EB: embryoid body, AP: alkaline phosphatase.

which did not progress through the process of meiosis (Novak et al., 2006). It was found that whereas some meiotic genes like *Scp3* were expressed in oocyte-like cells, some other meiotic proteins, such as Scp1, Scp2, Stag3, Rec8 and Smc1 were not expressed. It was concluded that ESC-derived oocyte-like cells showed the absence of essential meiotic proteins and failed to progress through meiosis. Oocyte-like cells and follicle-like structures expressing oocyte-specific genes such as *Figalpha* (*Figla*) and *Zp3* have been developed from the mESCs cultured in conditioned medium collected from newborn male testicular cell cultures (Lacham-Kaplan et

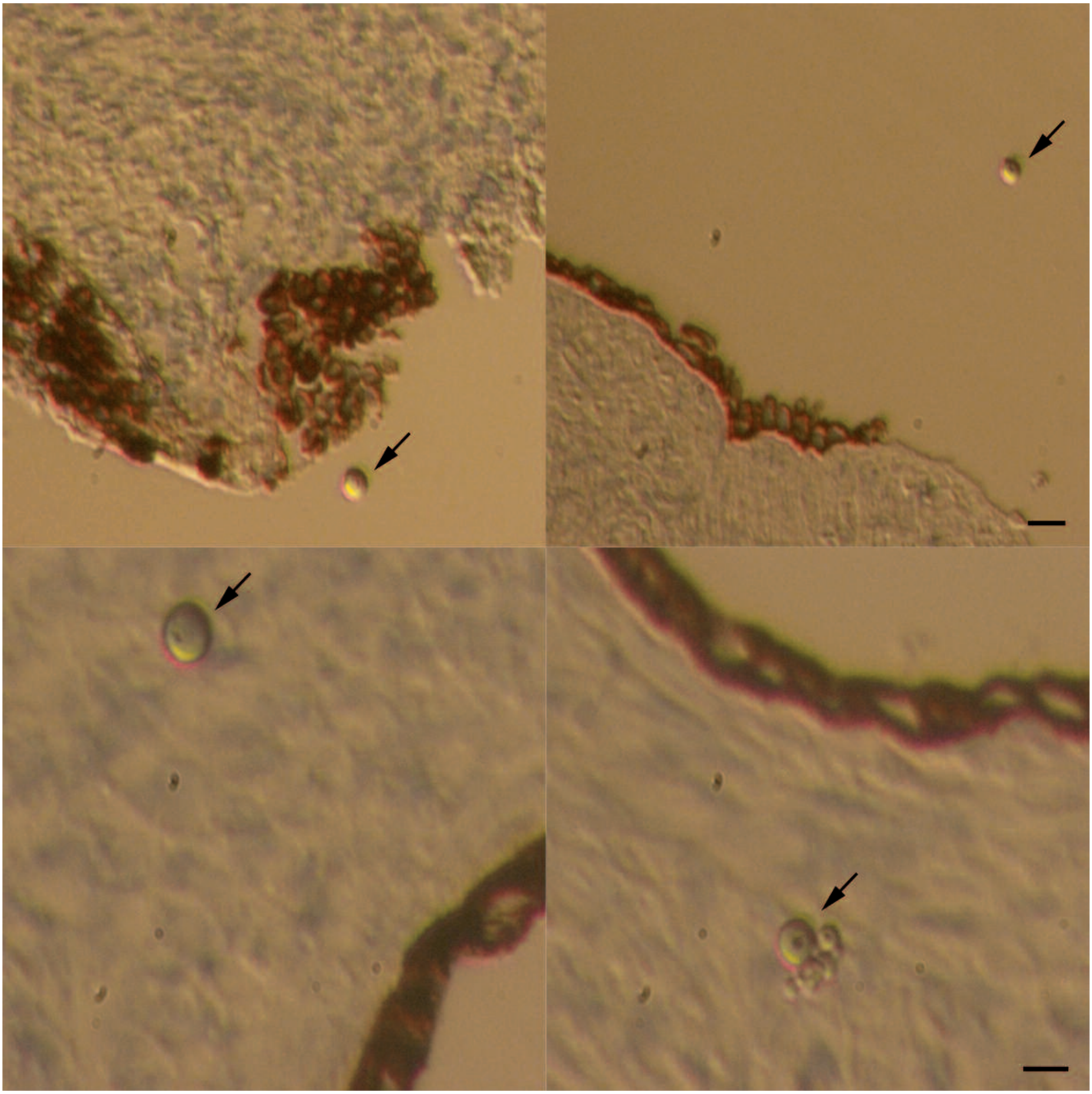

Fig. 1. Putative stem cells (arrows) from the human ovarian surface epithelium (brown stained after cytokeratin staining) in the ovarian sections of patients with premature ovarian failure and no naturally present follicles or oocytes (light microscope, magnifications x 200 and x 400) (Scale bars: 5 μm).

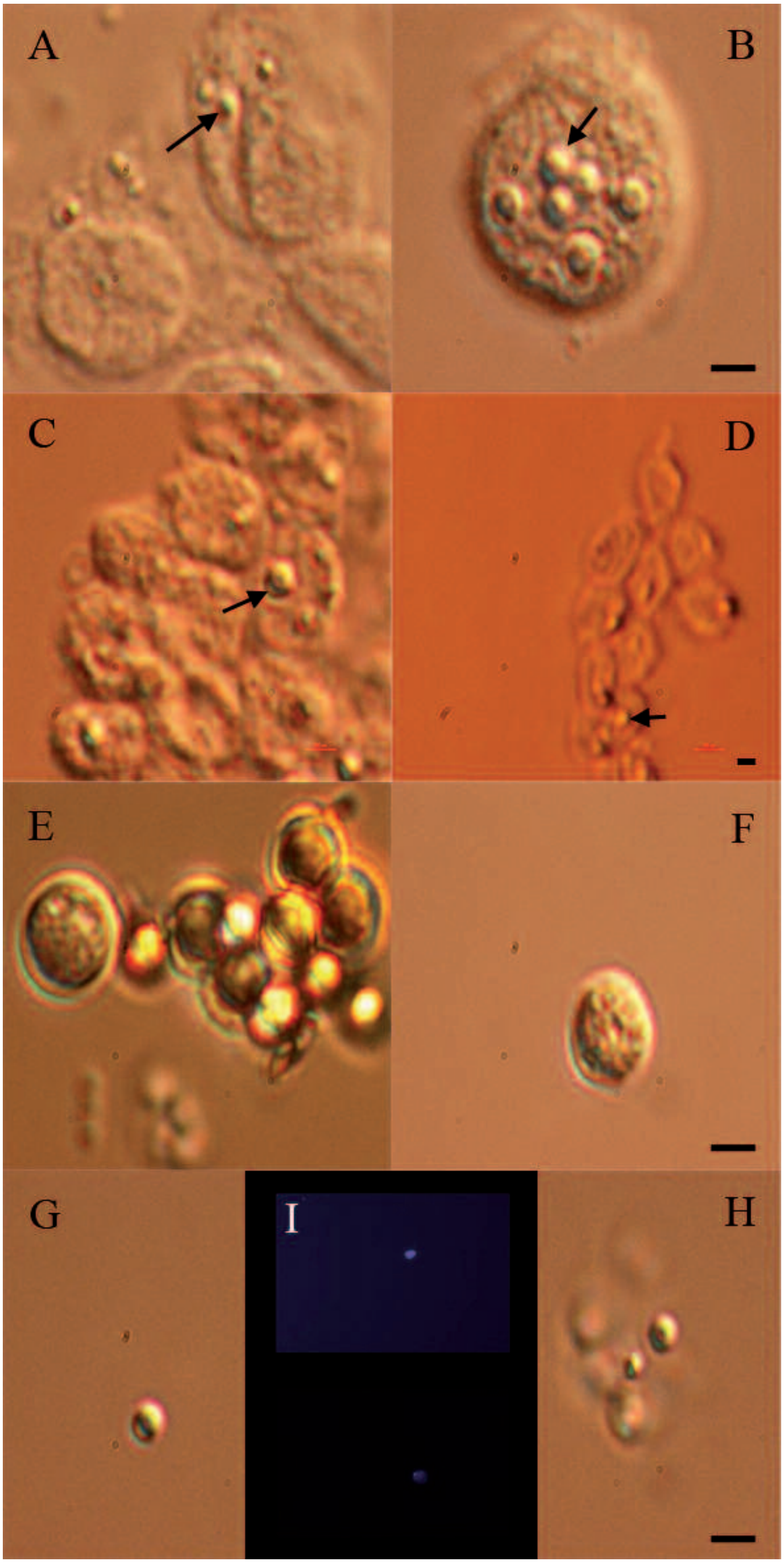

Fig. 2. Putative stem cells (arrows) in the human ovarian surface epithelium scrapings of patients with premature ovarian failure. **A-C.** Putative stem cells (arrows) among clusters of scraped epithelial cells or attached to epithelial cells (inverted microcope, dic-Nomarski, immersion objective, magnification x 6000). **D.** Putative stem cells (arrows) among cluster of scraped epithelial cells (inverted microscope, Hoffman, magnification x 200). **E-H.** Two different populations of SSEA4-positive cells isolated from the ovarian surface epithelium scrapings by anti-SSEA-4 antibody-coated beads and magnet: population of bigger cells (**E, F**) with diameters ~10 μm and smaller cells (**G, H**) with diameters from 2 to 5 μm (inverted microscope, dic-Nomarski, immersion objective, magnification x 1000). **I.** SSEA-4 positive cells isolated by anti-SSEA-4 antibody-coated beads and magnet from the ovarian surface epithelium scrapings (fluorescent microscope, magnification x 200) (Scale bars: 5 μm).

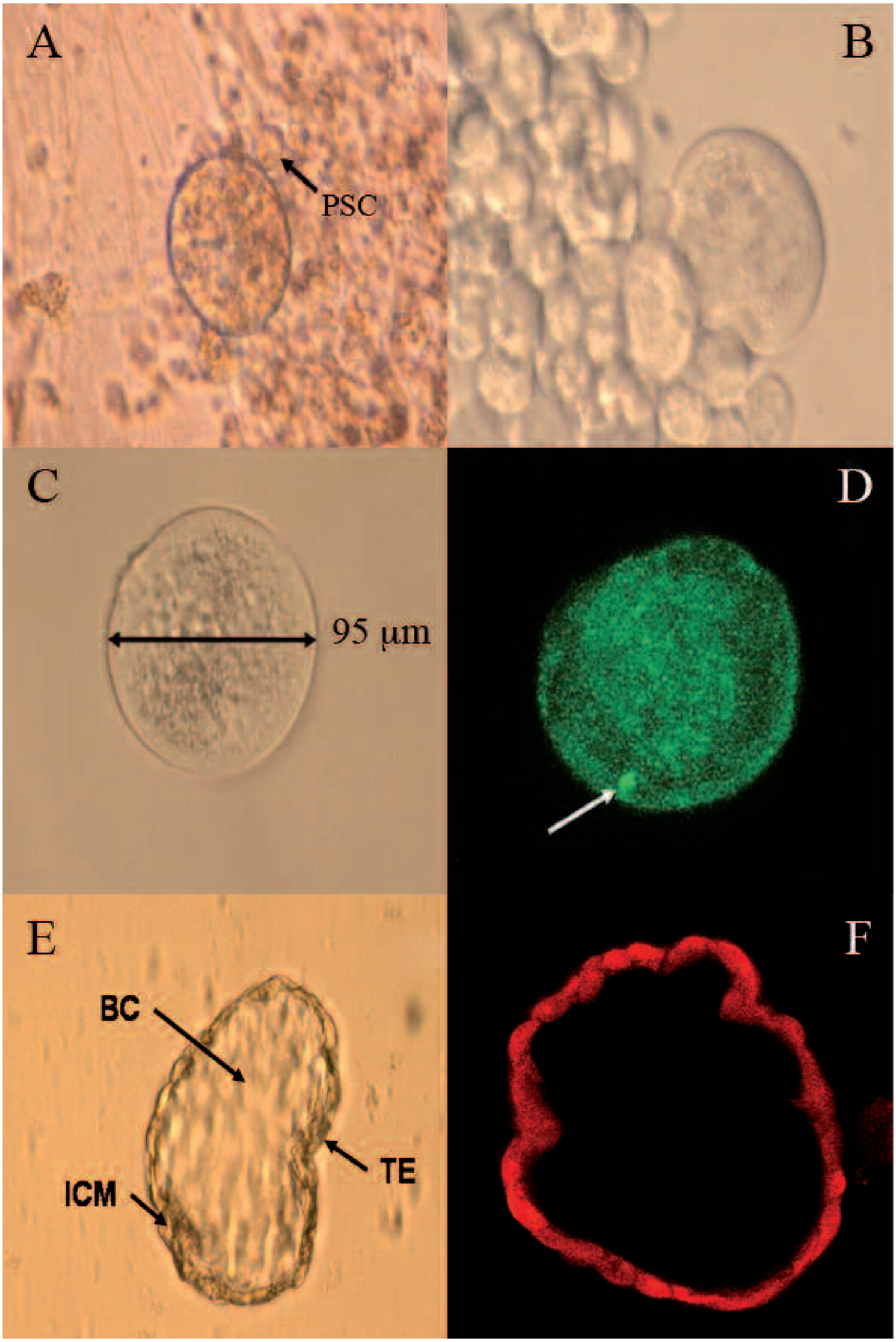

Fig. 3. Oocyte-like cells and blastocyst-like structures developed in the human ovarian surface epithelium cell cultures in postmenopausal women according to Virant-Klun et al., 2009. **A.** Growing oocyte-like cell with attached putative stem cell (arrow) (inverted microscope, Hoffman, magnification x 200). **B.** Oocyte-like cell among other cells detached from the dish bottom by trypsin-EDTA (inverted microscope, Hoffman, magnification x 400). **C.** Oocyte-like cell with a diameter of 95 μm (inverted microscope, Hoffman, magnification x 400). **D.** Accumulation of tubulin resembling the meiotic spindle (arrow) under the surface membrane of the oocyte-like cell (confocal microscope, magnification x 400). **E.** Blastocyst-like structure with inner cell mass-. trophectoderm-, and blastocoel cavity-like structures (inverted microscope, Hoffman, magnification 200x). **F.** Expanded blastocyst-like structure with blastocoel-like cavity monitored by the confocal microscope. Legend: BC: blastocoel cavity-like structure, ICM: inner cell mass-like structure, TE; trophectoderm-like structure.

al., 2006). It was suggested that this methodology should be applied to other mammalian species, including humans.

Not only ESCs isolated from embryos can develop into oocyte-like cells at the appropriate condition. After more publications (Dyce and Li, 2006a,b; Linher et al., 2009) indicating that primordial germ-like cells and oocyte-like cells differentiated *in vitro* from porcine fetal skin-derived stem cells, Dyce and co-workers recently reported the results of a more complex analysis of oocyte-like cells differentiated in this way (Dyce et al, 2010). They found that oocyte-like cells derived from the fetal porcine skin stem cells expressed some oocyte-specific genes like *Oct4, Dazl, Vasa, Gdf9b,* genes *ZpB* and *ZpC* for zona pellucida, and meiotic genes *Dmc1* and *Scp3*, whereas they did not express *Rec8* and *Stra8* meiotic genes. They concluded that skin-derived stem cells from both female and male porcine fetuses were capable of entering into the oocyte differentiation pathway, but their current culture system was inadequate to support the complete development of competent oocytes. Moreover, Danner and co-workers derived oocyte-like cells expressing some germ cell genes (i.e., *Vasa, Gdf9, SCP3, Bnc1*) from rat clonal pancreatic stem cell lines (Danner et al., 2007).

Recently, it was confirmed that induced pluripotent stem cells (iPSs) might also be developed into oocyte-like cells *in vitro*. Mouse iPS cells derived from adult hepatocytes were able to differentiate into oocyte-like cells marked by mouse *Vasa* homolog *Mvh* expression in

Table 2. Differentiation of embryonic stem cells into oocyte-like cells according to the literature.

Source	Stem cell culture medium	Feeder layer	Oocyte-like cell maturation medium	Transfection/other manipulation	Expressed oocyte-specific markers	Reference
mESCs	DMEM containing glucose, FCS, L-glutamine, non-essential amino acids, ß-mercaptoethanol, penicillin, streptomycin, murine LIF	MEFs (mitotically inactivated mouse embryonic fibroblasts) in 0.1% gelatine-coated plates	MEM-α supplemented with BSA, pyruvic acid, transferrin, selenium, insulin, EGF, gonadotropins, hGC and PMSF	Yes. mESCs were transfected with gcOct4-GFP.	Oct4, Vasa, c-kit, Dmc1, Scp3, Gdf9, Zp1, Zp2, Zp3, Figla -formed follicular structures and blastocyst-like structures (Troma1, Hand1, PI-1, Tpßp, Mash2)	Hübner et al., 2003
mESCs line R1, passage 14	DMEM containing glucose, FBS, L-glutamine, non-essential amino acids, ß-mercaptoethanol, penicillin, streptomycin, LIF	MEFs (mitotically inactivated mouse embryonic fibroblasts) in 0.1% gelatine-coated tissue culture plates	Supplemented DMEM medium without LIF and MEFs	Yes. Cells cocultured with a stable mouse 3T3 fibroblast cell line overexpressing BMP4 and transfection to generate a stable mouse BMP4 cell line.	SSEA-1, Scp3, Zp3 -formed follicle-like structures *in vitro* -meiotic proteins Scp1, Scp2, Stag3, Rec8, Smc1 were not expressed, abnormal meiotic chromosomal organization, failed to progress through meiosis	Novak et al., 2008
mESCs	DMEM containing FCS, non-essential amino acids, ß-mercaptoethanol, penicillin, streptomycin, mouse recombinant LIF	0.1% gelatine coated plastic flasks	Testicular Cell Conditioned (TCC) medium prepared from testicular tissue of 1-day-old newborn male mice testes	No.	Oct3/4, Mvh, c-kit (very few), Stella, Dazl, Figla, Zp3, -did not express Zp1 and Zp2 -formed embryoid body-like structures	Lacham-Kaplan et al., 2006
hESCs HES-3 (XX) HES-4 (XY)	Basic medium: 90% DMEM containing FCS, L-glutamine, antimycotics	POF (mitotically inactivated porcine ovarian fibroblasts)	POF-conditioned medium with/without forskolin and retinoic acid	No.	VASA, BOULE, DAZL, GDF3, GDF9, MLH1, PUM2, OCT4 -did not express SCP1 -formed EB-like structures, multilineage differentiation (AFP, NESTIN)	Richards et al., 2010
hOSCs	DMEM/F-12 with phenol red, $NaHCO_3$, FBS, penicillin, streptomycin, gentamycin	Native human ovarian fibroblasts from OSE scrapings	DMEM with phenol red containing $NaHCO_3$, penicillin, streptomycin	No.	ZP, CK5,6,8,17, CK18, VIMENTIN, PS1	Bukovsky et al., 2005
hOSCs	Basic medium: DMEM/F-12 with phenol red, $NaHCO_3$, penicillin, streptomycin, gentamycin	Native human ovarian fibroblasts from OSE scrapings	Basic medium containing human follicular fluid	No.	SSEA-4, c-KIT, OCT4, SOX2, NANOG, c-KIT, ZP2, VASA -SCP3 was not expressed -formed blastocyst-like structures with normal chromosomes X,Y,13,16,18,21,22	Virant-Klun et al., 2008, 2009

hOSCs: human ovarian stem cells from adult ovarian surface epithelium (OSE) scrapings, FCS: fetal calf serum, EB: embryoid body.

feeder-free and suspension cultures (Imamura et al., 2010). Moreover, an international group of experts tried to find the potential of human fetal and adult iPSCs derived from somatic cells to form primordial and meiotic germ cells, relative to hESCs (Panula et al., 2011). They found that approximately 5% of human iPSCs differentiated to PGCs following induction with BMPs. These PGCs expressed GFP from a germ cell-specific reporter and expressed endogenous germ cell specific proteins and mRNAs. In response to the overexpression of the intrinsic regulators, iPSCs formed meiotic cells with extensive synaptonemal complexes (SC) and post-meiotic haploid cells with a similar pattern of acrosin staining as observed in human spermatids. The female germ cells still need to be elucidated.

Factors affecting *in vitro* oogenesis

According to published reports, there are some factors, such as the appropriate selection of the feeder layer, the addition of the granulosa cells and other cells which can secrete BMP4, and the addition of retinoic acid (RA) into the culture medium, which could to some extent induce the differention of ESCs into the germ cells (Zhou et al., 2010). KIT ligand and BMP protein signalling enhances differentiation of hESCs into germ-like cells (West et al., 2010). The loss of KIT ligand in differentiation of cell cultures results in intense down-regulation of germ cell specific genes and a 70.5 % decrease in germ-like cells expressing *DDX4* and *OCT4* (*POU5F1*) genes. On the other hand, endogenous BMP signalling caused germ-like cell differentiation. The inhibition of this pathway causes a significant decrease in the number of germ cell-like cells and in the expressions of germ cell specific genes (West et al., 2010). By eliminating feeders but maintaining their secreted extracellular matrix it is possible to sustain the increased numbers of germ cell-like cells in culture (West et al., 2010).

In most studies, oocyte-like cells were cultured in DMEM culture medium with different supplements (FCS or FBS, glucose, L-glutamine, non-essential amino acids, ß-mercaptoethanol, LIF, $NaHCO_3$, antibiotics, antimycotics) on mitotically inactivated mouse embryonic fibroblasts (Table 2). Only a few of them used some ovarian components in their culture systems, like mitotically inactivated porcine ovarian fibroblasts, natural human ovarian fibroblasts, follicular fluid and granulosa cells.

A comparative evaluation of different *in vitro* systems that stimulate germ cell differentiation in hESCs has already been performed (Richards et al., 2010). Embryoid bodies derived from hESC lines were cultured in six different culture conditions: mitotically inactivated porcine ovarian fibroblasts, a 100% conditioned medium from mitotically inactivated porcine ovarian fibroblasts, a 50% conditioned medium from mitotically inactivated porcine ovarian fibroblasts, forskolin, transretinoic acid, and forskolin combined with retinoic acid. Mitotically inactivated porcine ovarian fibroblasts proved to be the best culture system to induce the hESCs differentiation into the germ cell direction by increasing the expression of several germ cell specific genes in embryoid bodies. Some researchers were able to isolate putative GSCs from embryoid bodies produced by ESCs by using Percoll and Nycodenz density gradient centrifugation (Saiti and Lacham-Kaplan, 2008). In the mouse model, the positive effect of the conditioned medium of mesenchymal stem cells on the *in vitro* maturation of oocytes was confirmed (Ling et al., 2008). In addition to the self-renewal and multiple differentiations, mesenchymal stem cells (MSCs) secrete a variety of growth factors and cytokines beneficial for oocyte *in vitro* maturation. A large-scale production of growing oocytes from neonatal mouse ovaries was developed *in vitro* in a follicle-free culture system and at the sequential provision of essential nutrients and growth factors (Honda et al., 2009). At this point, about 800 otherwise dormant oocytes from a newborn mouse developed, reached the size of oocytes in normal antral follicles, entered the metaphase I meiotic stage, extruded zona pellucida and were capable of fusing with sperm.

It has already been demonstrated that female germ-like cells can be derived from the ESCs through the formation of embryoid bodies. Yu et al. (2009) reported on the transgene expression approach to derive female germ cells directly from the mESCs *in vitro* without the formation of embryoid bodies. They confirmed the development of female germ cells through the ectopic expression of Dazl, which is an important germ-cell specific RNA-binding protein. They proposed *Dazl* as a master gene controlling germ cell differentiation.

Another study confirmed that mouse granulosa cells were effective in inducing the differentiation of mESC-derived PGCs into oocyte-like cells through direct cell-to-cell contacts (Qing et al., 2007).

Oocyte maturation from ESCs by transplantation

Some researchers preferred transplantation, which directs oocyte maturation from the ESCs and could provide a new strategy for female infertility treatment in the future.

Progress has been made by Reijo Pera's group (Nicholas et al., 2009). For the first time, they differentiated mESCs, derived from transgenic mice carrying *Oct4*-GFP, into oocyte-like cells in the presence of a germ cell maturation factor cocktail (FAC) comprising antiapoptotic, germ cell specification and meiotic induction factors, including BMP4 protein, retinoic acid, cytochrome p450, CYP26 inhibitor, stromal cell-derived factor 1 (SDF1), stem cell factor, basicFGF, n-acetyl-cysteine and forskolin. ESC-derived oocytes expressed several oocyte-specific genes as revealed by single-cell gene expression analyses. Genetic analyses have also shown the requirement of *Dazl* expression for *in vitro* oocyte development and limited *in vitro* maturation of ESC-derived oocytes. They observed that approximately 1-3% of *Oct4*-GFP positive cells initiated meiosis, as evidenced by synaptonemal complex protein (SCP) expression and chromosomal localization. However, they detected only partial chromosomal alignment of Scp3 and focal nuclear localization of Scp1 proteins in these oocytes as indicative of a limited meiotic progression. The most significant enhancement of meiosis induction *in vitro* was found when mESCs were differentiated in a co-culture with dissociated mouse embryos although *in vitro* development of follicles was not observed. Because of the incomplete meiotic progression they tried to achieve ESC-derived oocyte maturation by transplantation into a synchronized mouse ovarian niche (Nicholas et al., 2009). To enable the ovarian niche, *Oct4*-GFP oocyte-like cells derived from mESCs were co-aggregated with cells retrieved by enzymatic dissociation of mouse neonatal ovaries and transplanted under the kidney capsule of bi-laterally ovariectomized SCID recipient mice. The formation of primary follicles with oocytes was observed. They concluded that considerable work still remains to be done before safe and effective clinical translation could be realized (Nicholas et al., 2009).

Even *in vitro* maturation of immature - germinal vesicle oocytes retrieved in the *in vitro* fertilization programme is not a sufficiently efficient procedure. ESC-derived oocyte maturation definitely fails in the condition *in vitro*, therefore the transplantation of these oocyte-like cells into an ovarian niche to naturally direct their function and maturity or to ‘wake up’ the ovaries seems to be the best option to achieve the fully competent oocyte, and a potential therapeutic strategy for ovarian infertility.

Oocyte generation in adult mouse ovaries by putative germ cells in bone marrow and peripheral blood has also been evidenced (Johnson et al., 2005; Lee et al., 2007). After bone-marrow transplantation via the tail vein, cell tracking showed that donor mice-derived oocytes were generated in ovaries of recipient mice with ovarian failure due to cytostatic busulfan treatment. In human medicine, there have been some clinical reports about fertility recovery and pregnancy after bone marrow transplantation in patients with ovarian failure due to high-dose chemotherapy (cyclophosphamide, busulfan) or total-body irradiation because of hematologic malignancy (Sanders et al., 1996). Similarly, ovarian recovery and pregnancy have been documented after hematopoietic stem cell transplantation in some Fanconi anemia patients who were infertile due to ovarian damage from myeloablative conditioning (Nabhan et al., 2010). The mechanisms of ovarian recovery in these patients remain unknown.

Ovarian stem cells and cancer

Pluripotent/multipotent stem cells present in adult human ovaries might be somehow involved in the female reproductive function. With inappropriate conditions in the body, they might induce the formation of tumors and the development of cancer in humans. Also, in the most aggressive epithelial ovarian cancers a subpopulation of stem-cell like cells was found which expressed transcription factors LIN28 and OCT4 characteristic for pluripotent stem cells (Peng et al., 2010). Both epithelial ovarian cancer cell lines and the patient’s tumor samples expressed these transcription factors which are usually highly expressed in hESCs. The combined expression of these two proteins in tumor samples was correlated with an advanced tumor grade. When these two proteins were repressed in the same cells by using RNA interference there was a significant reduction of cancer cell growth and survival (Peng et al., 2010). Therefore, it was proposed that these factors of pluripotency may serve as important molecular diagnostics and therapeutic targets for the development of new treatment approaches in patients with aggressive ovarian epithelial cancers. Similary, Ye et al. (2010) found the expression of another pluripotent stem cell marker, SSEA-4 in solid tumors of patients with epithelial ovarian carcinomas. It was proposed that SSEA-4 may play a role during the oncogenesis of epithelial ovarian carcinoma and may be a potential therapy target in these patients. Additionally, OCT4 expression can be found in immature neuroepithelium and reflect the differentiation of neural tissue in immature teratoma of the ovary (Abiko et al., 2010).

Conclusion

Differentiation of oocytes from the pluripotent ESCs/iPSCs has the potential to becoming a future source of oocytes for research use (Marques-Mari et al., 2009) to better understand the molecular mechanisms of oogenesis, oocyte maturation, fertilization, and embryo development. In this way, the pathologies resulting in human infertility would be better elucidated. Additionally, if all complex genetic and epigenetic methodological limitations could be safely solved and complex culture conditions and an appropriate niche could be established, some therapeutic opportunities could be considered. The development of autologous ESCs/GSCs into mature oocytes *in vitro* would enable *in vitro* fertilization and a desired baby with patients with severe ovarian infertility and no naturally present follicles/oocytes (i.e. premature ovarian failure) in the future. It has also been proposed that pluripotent/multipotent stem cells may play a role during the oncogenesis of epithelial ovarian cancers and may be a potential therapy target in these patients to provide more efficient therapy in the future.

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