Stem cells in the ovary

Katarzyna Tomaszczuk – Kossowska

University Hospital of Basel, Switzerland

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Katarzyna Tomaszczuk - Kossowska

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Fakultät auf Antrag von

Prof. Dr. Alex N. Eberle

Prof. Dr. Christian De Geyter

Prof. Dr. Ivan Martin

Prof. Dr. Eberhard Parlow
Dekan

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SUMMARY

For decades, scientists have thought that female mammals are born with a lifetime supply of oocytes in the ovary, irreversibly destined to decline after birth. However, in recent years a significant controversy with regard to the potential replenishing effects of cells from the bone marrow and blood on ovarian follicular renewal has been stirred up. Although these claims have been met with harsh skepticism, if they prove to be true, the current understanding of the female reproductive system must be revisited. Although these observations and allusions have been limited to the mouse system only, they have opened new discussions about the potential consequences of bone marrow transplantation and even blood donation to the replenishment of the female genital system in general. Still, these findings have not been replicated in other research laboratories so far and the proof that oogenesis can be renewed after birth from cells originating in the bone marrow is still lacking.

In contrast to the ongoing controversy with regard to the possibility of ongoing renewal of oogenesis in the ovary and the possible existence of adult germ stem cells, the existence of somatic stem cells in the ovary has not been hypothesized for a long time.

The first part of this study has been performed to confirm the presence of pluripotent or multipotent stem cell populations among granulosa cells collected from mature human ovarian follicles. This work includes attempts to promote the growth of GCs over prolonged time periods *in vitro*. Previous studies have demonstrated that this is not possible with culture media which contain FSH and androgens. We identify the

specific markers for mesenchymal stem cells and mature GCs and differentiate luteinizing GCs into other cell types of the mesenchymal lineage.

Graafian ovarian follicles consist of follicular fluid, one single mature oocyte and several hundred thousands of granulosa cells (GC). Until now, luteinizing GCs are considered to be terminally differentiated, destined to undergo death after ovulation. Present concepts of luteal function, endocrine regulation of early pregnancy and the recruitment of new ovarian follicles are all based on the cyclical renewal of the entire population of GC.

The first part of this study has been performed to confirm the presence of pluripotent or multipotent stem cell populations among granulosa cells collected from mature human ovarian follicles. This work includes attempts to promote the growth of GCs over prolonged time periods *in vitro*. Previous studies have demonstrated that this is not possible with culture media which contain FSH and androgens. We identify the specific markers for mesenchymal stem cells and mature GCs and differentiate luteinizing GCs into other cell types of the mesenchymal lineage.

In the second part we demonstrate a three-dimensional (3D) pellet culture system containing type I collagen, which together with LIF allowed not only the survival and growth of primary human GCs, but supported a significant subpopulation of GCs to maintain their phenotype and functionality for prolonged time periods.

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ABBREVIATIONS

3D – three dimensional

APC - Allophycocyanin

ART – assisted reproduction technology

bFGF - basic fibroblast growth factor

BM – bone marrow

BMST – bone marrow stromal cells

BMT – bone marrow transplantation

BSP – bone sialoprotein

cDNA – single strand DNA

COC – cumulus oophorus oocyte complex

COLL1 – Collagen 1

COLL 2 – Collagen 2

CT - chemotherapy

DMEM – Dulbecco's modified Eagle's medium

DMSO - dimethylsulphoxide

DNA - Deoxyribonucleic acid

E2 - estradiol

ECM – extracellular matrix

EGF - epidermal growth factor

FACS – Fluorescence Activated Cell Sorter

FITC – fluorescence isothiocyanate

FCS – fetal calf serum

FSH – follicle stimulation hormone

FSHR – follicle stimulation hormone receptor

GAG - glycosaminoglycan

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GC – granulosa cells

GFP – green fluorescent protein

GSC – germline stem cells

H&E - hematoxylin and eosin

HCG – human chorionic gonadotropin

HGF - hepatocyte growth factor

HLA-ABC - human leukocyte antigen class I

HMG – human menopausal gonadotropin

ICSI – intracytoplasmic sperm injection

IGF-1 - Insulin-like growth factor 1

IVF – *in vitro* fertilization

IVM – *in vitro* maturation

ITS-1 – Insulin, Transferrin, Selenium

LH – luteinization hormone

LHR – luteinization hormone receptor

LIF – leukemia inhibiting factor

LIFR – leukemia inhibiting factor receptor

MSC – mesenchymal stem cells

OC - osteocalcin

OCT-4 – denominated POU5F1

OP - osteopontin

OSE – ovarian surface epithelium

P4 - progesterone

PCR – polymerase chain reaction

PE - Phycoerythrin

pI – propidium iodide

POF – premature ovarian failure

POU5F1 – POU domain, class 5 homebox 1

PB – peripheral blood

RNA - Ribonucleic acid

SE – surface epithelium

SOX-9 - sex determining region Y box 9

TA – tunica albuginea

TGF beta (TGF-β) - transforming growth factor beta

VEGF - vascular endothelial growth factor

1 INTRODUCTION

1.1 THE BASIC DOGMA OF REPRODUCTIVE BIOLOGY

A central dogma of reproductive biology has been that females of most mammalian species have the capacity of producing a defined stock of oocytes during fetal development only, each oocyte being enclosed by somatic cells in globular structures referred to as ovarian follicles [Zuckerman, 1951; Zuckerman and Baker, 1977]. Despite repeated but short-lived criticisms [Pansky et al., 1953; Vermande-Van Eck, 1956; Artem'eva, 1961] Zuckerman in 1951 settled the debate in favour of a quantified reserve of oocytes fixed at birth: "The basic biological doctrine that during the life of the individual there neither is nor can be any increase in the number of primary oocytes beyond those originally laid down when the ovary was formed" [Zuckerman, 1951].

In part through a finely tuned hormonal regulatory network, only a small fraction of the postnatal oocyte pool acquires the opportunity to become ovulated at some point during reproductive life. The fate of the vast majority of ovarian follicles enclosing the oocytes consists of atretic degeneration. The number of ovarian follicles steadily declines throughout adulthood to the point of exhaustion around age 50 culminating in menopause, which is defined as the last menstruation in a woman's life time [Richardson et al., 1987].

1.2 HISTORY OF THE BASIC DOGMA OF A PREDETERMINED NUMBER OF OVARIAN FOLLICLES AT BIRTH

Today most scientists accept the dogma that all oogonia, i.e., the female germinal stem cells, become extinct early in life as they become transformed into oocytes. However, during the last 150 years and still now, neo-oogenesis in adult mammals is being discussed. Waldeyer in his publication ''Eierstock und Ei'' from 1870 maintained the idea that no new formation of oocytes took place in adult mammals and birds, but that the oocytes arose in the ovarian surface epithelium, the so-called "germinal" epithelium, during a limited period early in life [Waldeyer, 1870]. His hypothesis was overturned by Kingery [Kingery, 1917], who claimed that all oocytes formed during fetal life degenerated and was replaced by oocytes arising in the adult ovarian "germinal" epithelium. Allen [Allen, 1923] supported this theory and stated that mitotic divisions in the "germinal" epithelium resulted in new oocytes. In 1921, Pearl and Schoppe cited a "basic biological doctrine that during the life of the individual there neither is nor can be any increase in the number of primary oocytes beyond those originally laid down when the ovary was formed" [Pearl et al., 1921]. This concept was solidified as a dogma in 1951 in a paper that critically evaluated, and effectively dispelled, any work contrary to the belief that mammalian females are endowed with a finite and non-renewing germ-cell reserve during the perinatal period. The discussion of neo-oogenesis in the adult mammalian ovary was almost ended by the studies of Zuckerman and his group [Zuckerman, 1951] and the Peters' group [Peters et al., 1962]. Peters et al. showed that the premeiotic S phase leading to the formation of oocytes in the mouse only takes place during a limited prenatal period and that these oocytes remain in the adult ovary.

Accordingly, a central dogma of mammalian reproductive biology stipulates that females are born with a finite, non-renewing pool of germ cells (oocytes), all of which are arrested in meiosis I and are enclosed by somatic cells in structures referred to as follicles [Zuckerman, 1951; Borum, 1961; Franchi et al., 1962; Peters, 1970; McLaren, 1984; Anderson et al., 1992]. Oocyte numbers progressively decline throughout postnatal life [Faddy et al., 1976; Faddy et al., 1987; Faddy, 2000] through mechanisms involving apoptosis [Perez et al., 1999; Tilly, 2001] eventually leaving the ovaries barren of germ cells [Gosden et al., 1983]. In humans, exhaustion of the oocyte reserve occurs around the fifth decade of life, leading to menopause [Richardson et al., 1987]. The process that is believed to occur in female mammals with respect to germ-cell development differs from that of several invertebrate organisms, including *Drosophila melanogaster*, in which germinal stem cells maintain oocyte production in adult ovaries. [Lin, 1997; Spradling et al., 2001; Deng and Lin, 2001].

It seems that few species have been shown to represent exceptions to the prevailing dogma. In pro-simian monkeys (e.g. lower primates), oocytes in transitory stages of the first meiotic prophase are confined to “germ cell nests” and are present both in juvenile and in adult ovaries, as first described in 1920 [Gerard, 1920], later confirmed by many others [Ioannou, 1967]. Also the armadillo’s ovary contains germ cells in meiosis but in this species they are confined to cord-like structures within the cortex [Enders, 1960].

From a phylogenetic viewpoint, it seems contradictory that mammalian females, including humans, would evolve a uniquely retrogressive reproductive mechanism, requiring preservation of their gametes from the fetal period for up to several decades.

Such long-lasting preservation could cause an accumulation of spontaneous or environmentally induced genetic alterations of oocytes in resting primary follicles [Bukovsky et al., 2005].

Interestingly, the conclusion reached by Zuckerman [Zuckerman,1951] remained for the most part unchallenged for more than 50 years despite the fact that, as emphasized by Zuckerman himself later in his career [Zuckerman, 1971], his conclusion was not based on even one single experiment proving that female mammals are incapable of oogenesis during postnatal life. Instead, he arrived at this conclusion based solely on an absence of data he felt, that would be inconsistent with this idea [Tilly et al., 2007].

Although this dogma has persisted, newer studies provide some evidence that may challenge the validity of the prevailing belief, which represents one of the most basic underpinnings of reproductive biology.

1.3 THE ONGOING CONTROVERSY SURROUNDING THE FINITE, NON-RENEWABLE OVARIAN FOLLICLE POOL AFTER BIRTH

Oogenesis has been demonstrated in cultured mouse embryonic stem cells [Hubner et al., 2003] and mitotically active germ cells have been reported in ovaries of adult pro-simian primates [Ioannou et al., 1967] and mice [Johnson et al., 2004]. Mesenchymal cells in the ovarian tunica albuginea (TA) differentiate into surface epithelium, a source of germ cells entering blood vessels and contributing to follicular renewal in adult human females [Bukovsky et al., 1995; Bukovsky et al., 2004]. These reports all represent challenges to established dogma on the fetal origin of mammalian oocytes [Bazer et al., 2004; Gosden, 2004].

Recently, Johnson and colleagues [Johnson et al., 2004] describe signs of preservation and proliferation of germline stem cells in postnatal mouse ovaries, thus opening the possibility of a postnatal contribution to the oocyte reserve. Another report based on the mouse model has recently suggested that, the number of primordial follicles per ovary do not decline between day 7 to day 100 after birth and that a significant depletion only occurred 200 days after birth [Kerr et al. 2006]. The authors propose that immature germ cells present in the adult may be able to generate *de novo* oocytes in a favourable environment.

Johnson et al. [Johnson et al., 2004] based their conclusions of oocyte and follicular renewal from existing germline stem cells (GSC) in the postnatal mouse ovary on three types of observations: discordance in follicle loss versus follicle atresia in the neonatal period, detection of proliferating GSC with meiotic capacity and neo-folliculogenesis in ovarian chimeric grafting experiments with adult mice.

Based on the hypothesis of an extra-ovarian source of germ-line precursors able to migrate to the ovaries and contribute to new oocytes, these reports have led to the proposal that bone marrow or peripheral blood cell transplantation might provide novel treatments for premature menopause or chemotherapy-induced sterility [Johnson et al., 2005b]. However, interpretation of these results demand caution, as it was revealed that some cells associated with the ovulated oocytes and stained with markers of haematopoiesis, were in fact contaminated cells arising from blood circulation [Eggan et al., 2006].

1.4 ARGUMENTS PUT FORWARD TO SUPPORT RENEWAL OF THE FOLLICLE-POOL AFTER BIRTH

The hypothesis that oocyte and follicle renewal may still be ongoing in the postnatal mouse ovary was verified by several experimental approaches [Johnson et al., 2004].

Mitotically active germ cells were suggested to be present in the ovaries of both young and adult mice, which, based on rates of oocyte atresia and clearance, are needed to continuously resupply the follicle pool [Johnson et al., 2004]. Histological analysis of young and adult mouse ovaries revealed the presence of large egg-shaped cells in the surface epithelial cell layer covering the ovary, similar to germ cells of fetal mouse ovaries [Crone et al., 1965; Morita et al., 1999]. These large ovoid cells were of a germline lineage as the presence of germ cells in various stages of mitosis was annotated. These data are considered by some scientists to build a strong case for germ-cell proliferation and follicle renewal in the postnatal mouse ovary.

The renewal of the postnatal ovarian follicle pool was then verified using chemotherapy (CT): prepubertal female mice were treated with the germ-cell toxicant, busulphan, which is known to eliminate the primordial follicle reserve without inducing atresia. After treatment with CT, cells expressing the meiotic entry marker were demonstrated in young and adult mouse ovaries. Ovaries of females treated with CT possessed, however, less than 5 % of the normal primordial follicle pool. These data were put forward to support the concept that proliferative germ cells not only persist in the postnatal ovary but also are required to renew the follicle pool [Johnson et al., 2004].

Wild-type ovaries were then grafted into transgenic female mice with ubiquitous expression of green fluorescent protein (GFP) [Hadjantonakis et al., 1998]. Grafted wild-type ovarian fragments penetrate into the GFP-positive ovarian tissue and follicle-enclosed wild-type germ cells gradually became indistinguishable from the GFP-positive germ cells that formed follicles. These data were proposed as a strong argument for the existence of proliferative germ cells that sustain oocyte and follicle production in the postnatal mammalian ovary [Johnson et al., 2004]. Considering results from past studies of mammalian stem-cell migration to their natural niches after introduction into a host [Nagano et al., 2003; Szilvassy et al., 2003; Torrente et al., 2003; Oh et al., 2003], the data presented above were interpreted such that germ-line stem cells continue to exist in the postnatal mouse ovary.

1.5 THE EXTRAGONADAL SOURCE OF GERMLINE STEM CELLS AFTER BIRTH

After a further set of experiments Johnson and his collaborators [Johnson et al., 2005] claimed that adult mouse ovaries rapidly generate hundreds of oocytes within 24 hours. The expression of germline markers in bone marrow (BM) was taken as an argument to suggest the presence of an extragonadal source of germ cells. Consequently, Johnson and colleagues began to claim that BM transplantation would be able to restore the production of oocytes in wild-type mice sterilized by CT, as well as in mice, which, due to genetic disease are incapable of producing oocytes. Based on gene expression analyses and bone marrow transplantation (BMT) experiments using CT-sterilized recipients, these authors presented the idea that a putative germ-line stem cell reservoir supporting oogenesis in adult female mice resides in the BM.

The same authors also presented the idea that the peripheral blood (PB) contained an additional source of germ-line stem cells in female mice. After transfusion of peripheral blood (PB) harvested from transgenic females with germline-restricted green fluorescent protein (GFP) expression GFP-positive oocytes were demonstrated in the ovaries of CT-treated recipient females. These findings nurtured the hypothesis that putative germ cells in BM release progenitor cells into the peripheral circulation which then may migrate to the ovaries [Johnson et al., 2005a].

1.6 THE OVARIAN SURFACE EPITHELIUM AS A SOURCE OF OVARIAN GERMLINE STEM CELLS

In adult human females the ovarian surface epithelium (OSE) is thought to be a source of germ cells and new primary follicles are formed by assembly of oocytes with nests of primitive granulosa cells in the ovarian cortex [Bukovsky et al., 1995]. The latter authors investigated the possibility that the oocytes and granulosa cells may be able to differentiate in cultures derived from adult human ovaries, scraped from the surface of ovaries. OSE cells cultured in the presence of an estrogenic stimulus differentiated into cells presenting with the phenotype of oocytes. Such cells exhibited germinal vesicle breakdown, expulsion of the polar body, and proteins characteristics for secondary oocytes. These in vitro studies uphold *in vivo* observations that in adult human ovaries, the OSE is a potential source of both oocytes and granulosa cells [Bukovsky et al., 2005].

Regarding follicular renewal in adult human females, reports provide some evidence that the OSE could be a source of germ cells, and new primary follicles are formed by assembly of oocytes with nests of primitive granulosa cells in the ovarian cortex

[Bukovsky et al., 1995; Bukovsky et al., 2004]. Components for the new primary follicles, primitive granulosa and germ cells, are proposed to differentiate *de novo* from mesenchymal progenitor cells residing in the ovarian TA. During differentiation into OSE cells the mesenchymal progenitor cells line either the ovarian surface or invaginated epithelial crypts. Mesenchymal progenitor cells would first contribute to the development of epithelial cells similar to granulosa cells, and these cells subsequently form epithelial nests descending into the deeper ovarian cortex. These cells may be a source of germ cells, which assemble together with nests of primitive granulosa cells to form primary follicles [Bukovsky et al., 2005]. Oogenesis may follow later.

The concept of cells from the OSE as the source of germline stem cells stands in contradiction to Johnson and colleagues [Johnson et al., 2005a], who favour cells from the BM and/or PB as the source of new germ cells that infiltrate the ovary to replenish the stock of primordial follicles. A lack of follicular renewal in aging ovaries may cause an accumulation of spontaneously arising or environmentally induced genetic alterations of oocytes, explaining why the oocytes in primary follicles among aging females have a higher incidence of genetic alterations [Bukovsky et al., 2005].

1.7 THE KINETICS OF PRIMORDIAL FOLLICLE DEPLETION IN YOUNG AND ADULT MICE

Following recent studies on germline stem cells and follicular renewal in the mouse ovary [Johnson et al. 2004, 2005a], Kerr and collaborators hypothesized that total, and particularly primordial, healthy follicle numbers should remain relatively constant, at

least for a significant part of adult reproductive life [Kerr et al., 2006]. Using unbiased stereological methods, immune-labeling of meiosis (germ cell nuclear antigen and ovarian cell proliferation) and electron microscopy, all healthy follicles in mouse ovaries were quantified at various time points after birth. After a marked depletion of follicles and oocytes during the first postnatal week, the mean number of primordial follicle per ovary did not decline significantly in mice and no significant decay in the total numbers of all healthy follicles was observed [Kerr et al., 2006]. Oocytes and primordial follicles were found either in the surface epithelium or in the periovarian space and migrated to these locations through the tunica albuginea [Kerr et al., 2006]. Byskov & Rasmussen [Byskov & Rasmussen, 1973] estimated that 5–10 % of oocytes or primordial follicles were associated with the OSE.

The concept, that new oocytes and follicles arising from surface epithelial cells of the postnatal mouse ovary was suggested in as early as 1917 by Kingery [Kingery 1917]. However, Kerr and collaborators present no evidence for conversion of surface epithelial cells into oocytes. In contrast, the group around Kerr suggests an alternative explanation: oocytes with or without associated stromal cells were exiting the ovary via the surface epithelium rather than arising from it [Kerr et al., 2006].

Although the proof of the existence of the ovarian germline stem cells in the postnatal and adult mouse ovary remains to be established, they suggested that primordial follicle numbers remain relatively constant in association with simultaneous, active folliculogenesis therefore the group support yet unknown mechanism for follicle renewal [Kerr et al., 2006].

1.8 OOGENESIS IN DROSOPHILA MELANOGASTER.

Many tissues including blood, skin, gut and germ cells are continuously maintained by tissue stem cells [Spradling et al, 2001; Watt et al, 2000]. Under certain conditions, however, other tissues can undergo repair using stem-cell-like progenitors generated by cell de-differentiation [Stocum, 2000; [Mikkola et al., 2002; Morrison et al., 2000; Prohaska et al., 2002; Pearson et al., 2003].

In the fruit fly *Drosophila melanogaster*, both spermatogenesis and oogenesis rely on germ-line stem cells. Similarities in the structural and molecular strategies used by the two sexes in germline stem cell maintenance and differentiation include the tight control that somatic support cells exert on every aspect of germline stem cell function and the similar molecular mechanisms for physical attachment, cell-cell signaling and gap-junction communication [Gilboa et al., 2004]. Some common principles underlying germline stem cell biology in the fly may be applied to other organisms.

According to common beliefs, the stem cell stage is unique in the life cycle of the germ cell. Recent findings suggest that the germline stem cells may not be as distinctive as we used to think. By definite circumstances in both males and females, a germline stem cell successor cells (the developing cyst) can revert and form germline stem cells again [Kai and Spradling, 2004]. It suggests that the somatic cells surrounding the germ cell greatly influence its developmental state, as they form a niche.

Kai and Spradling report that Drosophila gremlin stem cell successor cells convert into single stem cell-like cells. These de-differentiated cells can again develop into functional germline stem cells and support normal fertility [Kai and Spradling, 2004].

Results show that a germline stem cell successor cells represent a relatively good source of regenerative precursors that might help replenish germ cells after depletion by genotoxic chemicals, radiation or normal ageing. *Drosophila* cystocytes provide a system for studying de-differentiation and its potential as a source of functional stem cells [Kai and Spradling, 2004].

1.9 CASE REPORTS INDICATING THE POTENTIAL OF RENEWAL OF THE OVARIAN FOLLICLE POOL IN ADULT WOMEN

One of the most devastating effects of the current treatment of cancer is the damage of the reproductive system, which in young girls and women less than 40 years old is frequently leading to premature menopause and infertility [Meirow et al., 2001; Wenzel et al., 2005; Lee et al., 2006; Desmeules et al., 2006; Raz et al., 2002; Lopez et al., 2004]. This outcome seems to be, in large part, a result of cytotoxic effects of chemotherapy on the germ cells (e.g. the oocytes) in the ovaries. Thus, experimental trials aiming at sustaining fertility in female cancer survivors have been directed merely at preservation of existing oocytes [Meirow et al., 2001; Wenzel et al., 2005 ; Lee et al., 2006; Tilly et al.,, 2001; Morita et al., 2000; Paris et al., 2002].

However, although early menopause frequently occurs in female patients, suffering of cancer and treated withr chemotherapy (CT) and/or bone marrow (BM) transplantation (BMT), has been linked to an unexplained return of ovarian function and fertility in some survivors. In few women, treated with cytotoxic agents still during reproductive life and subsequently diagnosed with premature menopause, have been observed with an unexpected return of ovarian function and fertility after bone

marrow (BM) transplantation (BMT) [Salooja et al., 1994; Sanders et al., 1996; Salooja et al., 2001; Hershlag et al., 2002].

Mesenchymal stem cells (MSC) residing within the BM microenvironment are pluripotent adult stem cells, whose multipotency, easy isolation and culture as well as high *ex vivo* expansive potential make them attractive candidates for stem cell therapy [Vats et al., 2005]. Successful attempts of BM-derived MSC transplantation for repairing spinal, cardiac and skin injuries have been already reported [Orlic et al., 2001; Fu et al., 2006]. MSC have attracted interest for their possible use for both cell and gene therapies because of their capacity for self-renewal and multipotency [Prockop et al., 1997]. Transplantation of MSC directly into adult rat brain and heart reduces functional deficits resulting from stroke [Li et al., 2000] and acute obstruction of the heart muscle [Orlic et al., 2001], respectively. One of the mechanisms behind this is the integration of MSC into the tissue and replacement of damaged cells. In addition, the mediators secreted by MSC might be involved in the repair by preventing cell apoptosis and promoting functional recovery [Xu et al., 2007].

Premature ovarian failure (POF) as a consequence of childhood cancer treatment is considered permanent when present long after the initial therapy. POF in girls is a known complication of CT and radiation and has been estimated to occur in approximately 6 % of childhood cancer survivors [Chemaitilly et al. 2006].

However recovery of ovarian function after cancer treatment has been reported in single cases involving older children and adults.

The case report [Rahhal et al., 2008] describes a patient who received chemotherapy and radiation as leukaemia and growth hormone deficiency treatment. The patient was

exposed to total body irradiation, which is a major risk factor for the development of permanent ovarian failure [Chemaitilly et al., 2006; Sklar, 2005]. This treatment was followed by allogenic cord blood transplantation. Rahhal et al. report spontaneous recovery of ovarian function occurring 8 years after transplantation in the adolescent patient with history of cancer [Rahhal et al., 2008]. Rahhal et al suggest that recovery of ovarian function is possible after cancer treatment and after treatment with sex steroids [Rahhal et al., 2008].

As described before, new ovarian follicles may be generated in the mouse by germline stem cells supplied by the BM via the circulatory system [Johnson et al, 2004]. These authors have postulated clinical applications of their findings and have suggested that a blood transfusion alone might solve infertility through such a mechanism. However, these postulations have generated a harsh controversy and have ignited much opposition [Telfer et al, 2005, Eggan et al, 2006]. Notably, transplantation and parabiotic mouse models have failed to provide evidence that BM cells, or any other circulating cells, or even soluble factors, contribute to the formation of mature ovulated oocytes [Eggan et al, 2006].

Considering the above-mentioned results, one can question whether recovery of fertility might result from a repopulation of the ovary or by the circulating donor's germline stem cells. The existence of germline stem cells in human would bring many ethical, social and psychological implications for BM donors, receivers, and offspring. To clarify this issue, Veitia and collaborators examined a clinical situation of a woman who gave birth to a child after allogeneic BMT. Despite patient clinical background of chemotherapy and radiotherapy cancer treatment, she became pregnant and delivered a healthy child [Veitia et al., 2007]. The DNA from the mother

(patient), the daughter, and the donor were analyzed. The genetic relationship between the mother and the daughter clearly demonstrated no genetic inheritance from the donor. As such, Veitia and collaborators data provide clear evidence against the hypothesis from Johnson et al. [Johnson et al., 2004; Johnson et al., 2005] that BMT is responsible for fertility recovery by supplying GSCs [Veitia et al., 2007]. The recovery of fertility after BMT seems to better explained by the incomplete depletion of the ovarian follicle reserve.

Additional data have shown that, unlike after irradiation, chemotherapy does not completely sterilize female mice and allows a small population of residual endogenous oocytes and/or germ cells to survive to later restore ovulation [Eggan et al., 2006].

1.10 MESENCHYMAL STEM CELLS TRANSPLANTATION MAINTAIN DAMAGED TISSUE

Mesenchymal stem cell (MSC) transplantation can improve the structure and function of injured tissues. Fu and colleagues performed a study exploring the therapeutic potency of MSC transplantation in order to repair ovarian damage caused by CT in rats [Fu et al., 2008]. The group claims improvement of damaged ovarian function and reduced apoptosis of granulosa cells (GC) of the rats exposed to CT injection after MSC transplantation, probably by the mediators secreted by MSC [Fu et al., 2008]. They demonstrated that MSC secrete cytokines *in vitro*, thereby inhibiting CT-induced cell apoptosis protecting the GC against the cytotoxic chemotherapeutics.

MSC secrete significant amounts of VEGF, IGF-1 and HGF *in vitro*, which raises the possibility that MSC-derived cytokines may participate in follicular growth. VEGF is an angiogenic cytokine that promotes the proliferation of endothelial cells and formation of new vessels [Shin et al., 2006]. IGF-1, a growth hormone mediator, can stimulate GC proliferation, inhibit apoptosis and promote follicular antrum formation [Mao et al., 2004; Sirotkin et al., 2002]. HGF is a cytokine which is involved in promoting follicle maturation, cell growth required for folliculogenesis [Ito et al., 2001; Nilsson et al., 2001] and in suppressing apoptosis in GC and follicle cultures [Uzumcu et al., 2006].

After transplantation of MSC, MSC-derived cells were found in the ovarian tissue, but not within ovarian follicles. These results demonstrate that transplanted MSC may play an important role in supporting and promoting the ovarian microenvironment. However, it is unlikely that these MSC can differentiate into oocytes or GC [Fu et al., 2008].

Using a preclinical mouse model of CT-induced ovarian failure, Lee and collaborators observed a reversal of long-term infertility in CT-treated females by BMT that was influenced by several aspects. The most important were the time of the transplantation after CT, the amount of CT and the timing of mating initiation after BMT [Lee at al., 2007]. All of the offspring produced by CT-treated females and after BMT were derived from the recipient germline, despite the fact that the presence of donor BM–derived oocytes in their ovaries was suggested. Donor-derived oocytes were only observed in immature follicles up to the preantral stage of development but never observed in maturing antral or Graafian follicles from which ovulated eggs are derived.

BMT supports long-term fertility improvement in CT-treated females, but all offspring was derived from the recipient germline. Cell tracking showed that donor-derived oocytes were generated in ovaries of recipients after BMT. It is claimed that donor-derived immature oocytes detected in the ovaries of female mice after transplantation actually represent immune cells [Eggan et al., 2006]. Lee and collaborators suggest that BMT functions mainly by reactivating host oogenesis as a near-complete reversal of infertility in mice was achieved, when BMT was performed after CT at the time when the cytotoxic drugs were already removed from the body [Lee at al., 2007].

It is also possible that CT damages the ovaries such that engraftment or differentiation of germ cells fails to occur unless the gonadal microenvironment is repaired by the transplanted somatic cells or by factors released from the transplanted cells. This line of reasoning would be explained by recent studies of male germline stem-cell function in mice, in which the microenvironment was identified as principal cause of spermatogenic failure.

Adult bone-marrow-derived MSC in a favourable environment capable of differentiation along several tissue-forming cells such as bone, cartilage, fat, muscle, liver, kidney, heart, and even brain cells [Grove et al., 2004; Jiang et al., 2002]. Lue at al reported adult stem cell differentiation in the testis of busulfan-treated wild-type mice after bone marrow cells transplantation [Lue et al., 2007]. They state, a few weeks after transplantation the transplanted bone marrow cells were found in recipient testes [Lue et al., 2007]. However the bone marrow derived germ cells halted at the early spermatocyte stage without further differentiation into mature sperm. The mechanisms of donor-derived germ cells that failed to produce mature cells still

remain unknown. In addition, the possibility of fusion of donor cells with native germ cells cannot completely be excluded. Observation of Lue at al. suggests an essential role of recovering endogenous germ cells in the proper microenvironment [Lue et al., 2007].

Nayernia and collaborators demonstrated that somatic adult BMS cells could differentiate into male germ cells [Nayernia et al., 2004]. BMS cell-derived germ cells expressed all known molecular markers of primordial germ cells, as well as molecular markers of spermatogonial stem cells and spermatogonia [Nayernia et al., 2004]. They state that BMS cells are able to differentiate to early germ cells, PGCs and even spermatogonia in vitro and in vivo. However a similar developmental arrest in spermatogonia derived from male BM cells transplanted into adult mouse testes as in Lue at al work was reported [Nayernia et al., 2006; Lue et al., 2007].

Studies published recently are challenging the outlook on infertility and germ cell development. They present male and female gametes can be produced in vitro from embryonic or teratocarcinoma stem cells from established population of primordial germ cell-like cells after transplantation into the testes or ovaries or by extending cultures [Nayernia et al., 2004; Geijsen et al., 2004; Toyooka et al., 2003; Hubner et al., 2003].

Generally speaking, cell regeneration and replacement in the adult is dependent on tissue-specific stem cells. The specific stem cell in adult proliferates and differentiates into mature cell types that correspond to the tissue of origin and do not generate cell types of different lineages [Weissman, 2000]. However, more recent literature has

challenged this notion and demonstrated stem cells crossing tissue boundaries in response to a microenvironmental signaling [Wagers et al., 2004; Kucia et al., 2005].

As previously shown by Lue and colleagues, the resident neighboring cells in the recipient testis may control site-appropriate stem cell differentiation. This clinically relevant finding raises the possibility for treatment of infertility in men through the therapeutic use of stem cells [Lue et al., 2007].

1.11 CRITICAL ARGUMENTS AGAINST THE RENEWAL OF THE POOL OF OVARIAN FOLLICLES AFTER BIRTH

It seems highly unlikely that the human species evolved a mechanism, by which adult women are able to renew their germline cell pool. In effect, this creates a situation where eggs would then be placed at risk for cumulative damage from aging and external harm before being used to possibly generate offspring.

Recent data [Johnson et al., 2005a] not only reinforced this controversy but also surprisingly suggested that the BM is a potential source of oocyte-producing germ cells in adults. However, this study has been met with much skepticism. Doubts raised in commentaries on this work are largely based on inaccurate or incomplete assessments of experimental models and results [Johnson et al., 2005b].

Since the publication of two papers challenging the long-standing dogma that mammalian females are incapable of postnatal oocyte and follicle production (Johnson et al., 2004, 2005a), this topic has been the subject of at least 11 critical commentaries, that have voiced varying degrees of skepticism if not outright disbelief [Albertini, 2004; Gosden, 2004; Greenfeld and Flaws, 2004; Hoyer, 2004; Telfer,

2004; Ainsworth, 2005; Byskov et al., 2005; Gougeon, 2005; Powell, 2005; Telfer et al., 2005; Vogel, 2005].

One of the striking features of the mammalian ovary is the disappearance of oocytes through atresia, a process that begins early in life and proceeds until no more follicles are present in old age. In the mouse more than half the growing follicles will become atretic by day 21 after birth [Byskov, 1978]. Johnson found that atretic follicles only accumulate from day 30 onwards, and they assume that the number of 1,200 atretic follicles present on day 42 is so high that the oocyte pool would soon have disappeared provided new oocytes would not be formed instead. Thus, the pool of atretic follicles observed on day 30 by Johnson and coworkers [Johnson et al., 2004] most likely represents follicular remnants that have been degenerating previously [Byskov et al., 2005].

The primordial follicle depletion rate is usually estimated based upon the mathematical model given by Faddy et al. [Faddy et al., 1987], which predicts a day to day of loss of primordial follicles. Thus, the difference of existing follicles versus follicles depletion rate would represent the number of new follicles produced. It must be mentioned that the estimated loss of follicles [Faddy et al., 1987] is based on a different strain of mouse than the one used by Johnson and collaborators and the loss of ovarian follicles is known to differ greatly among various strains [Jones and Krohn, 1960; Faddy et al., 1983; Canning et al., 2003].

Johnson and collaborators [Johnson et al., 2004] further claim that functional germline stem cells reside in the surface epithelium of the adult mouse ovary and that they can give rise to new follicles. It has been shown previously that primordial

follicles may leave the ovary through the surface epithelium, particularly during early stages of development. In the prepubertal mouse primordial follicles accumulate in the bursal cavity surrounding the ovaries [Byskov and Rasmussen, 1973]. Thus, the large ovoid germ cells in the surface epithelium may also be primordial follicles instead of transitory stages of the first meiotic prophase [Byskov et al., 2005].

Oogenesis is the process that transforms the proliferative oogonium into an oocyte through meiosis, followed by folliculogenesis and follicular and oocyte maturation. The most crucial part in producing a functional oocyte is firstly, initiation and completion of the first meiotic prophase, and secondly, enclosure of the resulting diplotene oocyte in a follicle. Neither of these two events have been shown to take place in Johnson's study of the postnatal mouse ovary [Byskov et al., 2005].

Moreover, experiments have demonstrated that the ovarian surface epithelium is not needed for maintenance of follicle growth. The growing follicles and corpora lutea would still develop in the ovaries of rat, guinea-pig, cat, and opossum up to one year after destruction of the ovarian surface epithelium [Moore and Wang, 1947; Mandl et al., 1952; Mandl and Zuckerman, 1949]. Byskov and collaborators found no convincing evidence for a functioning "germinal" epithelium with new oocytes in prophase of meiosis [Byskov et al., 2005].

An interesting chimaeric ovarian model was created by combining fractions of wild-type and ubiquitous green fluorescent protein (GFP) transgenic ovaries [Johnson et al., 2004] and the authors hypothesized that new follicles were formed. Previous studies revealed that small follicles can be disaggregated and reformed spontaneously [Gosden, 1990], even when combining cell types of separate species [Eppig and

Wigglesworth, 2000]. Thus, it is suspected that the new observations reflect primordial ovarian plasticity and that chimaerism was a product of tissue injury after transplantation rather than being because of the generation of follicles *de novo* [Byskov et al., 2005].

To explore the idea on the extragonadal origin of germ cells in adult mammalian females, one may compare observations in animals with and without ovaries [Bukovsky, 2005b]. Johnson et al. [Johnson et al., 2005a] reported that the germ cell formation in BM disappears in ovariectomized mice. This observation may be considered as evidence for the ovarian origin of germ cells, which may enter the ovarian blood stream and, therefore, into the BM too [Bukovsky, 2005b].

Another important aspect is the presence of primitive granulosa cell clusters resembling fetal ovaries [Bukovsky, 2005a]. A re-colonization of adult human ovaries with new primary follicles would require the presence of such primitive granulosa cell nests. In other words, even transplantation of autologous germ cells may not be sufficient for follicular renewal in aging women, which lack nests of primitive granulosa cells in their ovaries [Bukovsky, 2004].

To investigate directly the capacity of naturally circulating peripheral blood cells to engraft in the ovary and contribute to oogenesis, Eggan and collaborators examined ovulated oocytes from adult female mice surgically joined by parabiosis [Eggan et al., 2006]. Parabiotic mice develop a common circulatory system and exhibit continuous, rapid exchange of cells and other circulating factors through the bloodstream [Bunster et al., 1933; Wright et al., 2001]. Thus, parabiosis allows direct tracking of genetically marked cells supplied continuously and at physiological levels through the circulation

and provides a powerful approach to determine whether blood-derived factors normally contribute to ovarian function or repair [Wright et al., 2001; Conboy et al., 2005; Sherwood et al., 2004; Wagers et al., 2002]. In contrast to the substantial chimerism evident in the peripheral blood of long-term parabionts, no chimerism of oocytes was observed in parabiotic mice [Eggan et al., 2006]. Occasionally cells observed associated with in the cumulus mass of ovulated oocytes originated from circulating blood cells [Eggan et al., 2006]. Thus, although circulating cells have the capacity to enter the ovary and to associate with ovulating oocytes, they maintain all haematopoietic features in this environment and did not contribute to the production of ovulated oocytes [Eggan et al., 2006].

Previous transplantation experiments have demonstrated that donor BM stem cells can undergo spontaneous fusion with differentiated host cells [Weimann et al. 2003; Alvarez-Dolado et al. 2003]. Haematopoietic stem cells and primordial germ cells develop in close proximity during embryogenesis and *in vitro* experiments have demonstrated that primordial germ cells can exhibit some of haematopoietic characteristics [Rich 1995]. It remained possible that damage to the bone marrow, ovary, or germ cells might be required to enable ovarian engraftment of circulating cells. Non-transgenic mice were pre-treated with CT [Johnson et al., 2005a; Shiromizu et al., 1984; Burkl et al., 1978; Mattison et al., 1981; Meirow et al., 2001] and then joined with untreated transgenic partners. However, in great contrast to the cross-engraftment evident in the haematopoietic lineages, no evidence for cross-engraftment of circulating oocyte precursors in parabiotic mice was found [Eggan et al., 2006].

A significant decline in the total numbers of oocytes ovulated by CT treated animals over time was observed, but CT did not cause complete depletion of the reserve of oocytes in the treated parabiotic mice. Furthermore, both maturing and mature follicles were observed in some histological sections, although CT-induced damage to the ovaries was widespread [Eggan et al., 2006].

The possibility that direct intravenous transplantation of bone marrow cells introduces cells into the blood circulation that are not normally present in the bloodstream but are capable of contributing to or stimulating oogenesis was also verified. As CT does not always lead to a complete depletion of the oocytes pool, animals sterilized by low-dose total body irradiation were also examined. Again, in contrast to the haematopoietic engraftment evident in mice transplanted with bone marrow, no evidence of bone marrow derived oocytes in parabiotic mice was found [Eggan et al., 2006]. In addition, no oocytes were found in the oviducts of animals pre-treated with irradiation, whether or not they received bone marrow transplants [Eggan et al., 2006].

1.12 THE EMINENT ROLE OF SURROUNDING SOMATIC STEM CELLS IN FOLLICULOGENESIS

The ovary contains a pool of inactive primordial follicles. Each follicle contains a small non-growing oocyte and a single layer of non-dividing cells encapsulated by the follicular basal lamina [Gougeon, 1996]. As part of an ongoing process, primordial follicles become active, and the oocyte starts to grow and its surrounding granulosa cells start to become mitotic. As the granulosa cells divide, the number of layers of granulosa cells (called the membrane granulosa or follicular epithelium) around the

oocyte increases, and the basal lamina expands [Rodgers et al., 1999; Rodgers et al., 2001]. Primordial follicles give rise to primary follicles which transform into preantral (secondary follicles) then antral follicles (tertiary follicles) and finally preovulatory and Graafian follicles, in a co-ordinated series of transitions regulated by hormones and local intraovarian factors [Gougeon, 1996; Gomez et al., 1999; Vigo et al., 2005].

Primordial follicles are not distributed uniformly in the ovary, but are predominantly located in the ovarian cortex. The ovarian cortex is covered by a layer of irregular shaped cells [Van Blerkom et al., 1979], commonly known as the ovarian "germinal" or surface epithelium (SE), which is attached to the TA. In functional human ovaries the SE is found in certain areas only, but in women with polycystic ovaries, the ovarian surface is completely covered with SE [Makabe et al., 1980]. These observations indicate that the SE-derived epithelial nests may represent primitive granulosa cells. They may either invade SE from adjacent structures and are extruded from the ovary [Motta, 1986].

In adult human females, mesenchymal cells in the ovarian tunica albuginea (TA) undergo a mesenchymal–epithelial transition into OSE cells, which may differentiate sequentially into primitive granulosa and germ cells. TA is a thick fibrous subepithelial layer with cells embedded in loose connective-tissue, which does not begin to form until the end of intrauterine life [Motta, 1986; Simkins, 1932]. These structures assemble in the deeper ovarian cortex and may form new follicles to replace earlier primary follicles undergoing atresia (Fig 1) [Bukovsky et al., 2005]. In other words, in adult human ovaries, surface epithelial cells derived from the tunica albuginea, have been reported to differentiate into granulosa cells and oocytes

forming new follicles [Bukovsky et al. 1995, 2004, Bukovsky 2005] but full proof of this observation is still lacking.

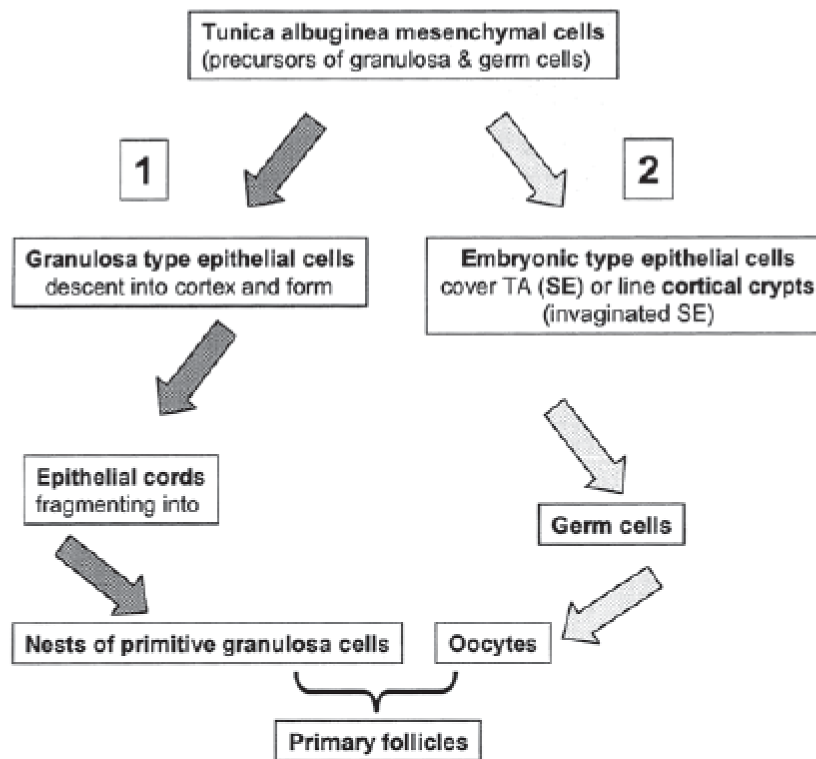

Figure 1. Working model of possible pathways for formation of primary follicles in adult human ovaries [Bukovsky et al., 2005].

The mammalian ovary produces mature oocytes capable of being fertilized and sustaining early embryonic development. Developmental competence of the oocyte correlates with follicular size, larger oocytes being more developmentally competent [Bachvarova et al., 1980; Berkholtz et al., 2006]. The development of an oocyte ultimately capable of undergoing fertilization and embryogenesis depends on appropriate signalling from surrounding ovarian granulosa cells (GCs) including follicle-stimulating hormone (FSH) [Thomas et al., 2007]

Conventional thinking considers the ovarian follicle as an isolated structure, distinct both in space and time, either destined for early degeneration through atresia or for growth to a mature, Graafian follicle, ovulation and formation of the corpus luteum. The cyclicity of follicular development, ovulation and luteal function are seen as discrete phenomena. In recent years, however, experimental evidence has shed some doubt on this conventional thinking. Most notably, it has been demonstrated that mature and fully grown mouse oocytes are able to influence the development of preantral follicles in mouse ovaries [Cecconi & Rossi, 2001], indicating interdepends of the cyclic events in the ovary. In addition to these early observations, other investigators were able to demonstrate that upon ovulation, the epithelioid granulosa cells redifferentiate into the mesenchymal cells of the corpus luteum [Rodgers et al., 1999].

Despite years of research little is still known of the cellular biology of developing follicles. This is unfortunate as there are some very interesting features of developing follicles, which have remained outside the scope of experimental research [Amsterdam et al., 1987; Hirshfield, 1991]. The membrana granulosa or follicular epithelium is more complex than most other epithelia for various reasons: at first it expands from a single to a multi-layered epithelium as the follicle grows. In the transition from a pre- to a post-antral follicle, the shape of GC change from non-dividing flattened appearance to dividing cubical appearance. The epithelium also expands laterally with time as the follicle enlarges. During the preovulatory phase the membrana granulosa becomes vascularised with capillaries sprouting from the surrounding theca interna. Finally, the granulosa becomes degraded upon ovulation and the remaining GCs are thought to differentiate into luteal cells. It is the faith of 99 % of all follicles to become atretic and apoptosis among the granulosa cells is one of

the first indicators of follicular atresia [Seifer et al., 1993; Rodgers et al., 1999; Berkholtz et al., 2006].

1.13 BEHAVIOUR OF HUMAN GRANULOSA CELLS DURING THEIR CULTURE *IN VITRO*

Human granulosa cells are recovered as waste during transvaginal ultrasound-guided oocyte collection in assisted reproduction technology (ART), either for in vitro fertilization (IVF) or for intracytoplasmic sperm injection (ICSI). The main characteristics of these differentiating cells are mitochondria clustering, increased number of lipid droplets, microvilli, and clustering granulosa cells forming lumen-like structures [O'Shea, 1987; Zhang et al., 2000]. The culture of granulosa cells *in vitro* will promote luteinization resulting in apoptosis after a few days.

Though GCs are deeply related with human ovarian function and various dysfunctions, little has been known because of their short life span *in vitro*. Most of studies on the ovarian functions have been provided by using subhuman primates and non-primate animals [Okamura et al., 2003] and result from short-term cultures *in vitro*. Existing immortalized human granulosa cell lines, obtained from developing follicles or ovarian carcinomas, showed little steroid hormone biosynthesis and/or limited detectable expression of the genes characteristics for GCs markers [Lie et al., 1996; Hosokawa et al., 1998; Zhang et al., 2000; Nishi et al., 2001; Okamura et al., 2003]. Immortalized human GC lines are useful for study follicular and oocyte maturation in vitro, however, those lines are not physiological as most of them were established from a primary human GC tumor or were established by transfection of luteinizing GC.

It has been postulated that during follicular growth granulosa cells arise from a population of stem cells [Lavranos et al., 1996; Rodgers et al., 1995a; Rodgers et al., 1996]. It is predicted that some or all of the early granulosa cells surrounding the oocytes of primordial follicles are committed stem cells, although inactive ones. This model encompasses stem cells, transiting amplifying cells (replicating and specializing), and differentiating cells. The progeny of stem cells, the transit amplifying cells, can further replicate during follicular growth a limited number of times before they differentiate into specialized cells as well [Rodgers et al., 2001]. It is clear that all GCs have not the equal potential to divide [Rodgers et al., 1999]. However, the question is whether the various granulose cell layers actually contain different cell types, or merely the same cells of different ages or stages of development. In any event, studies in the mouse have suggested that basal and antral GCs can be derived from the same progenitor cells [Boland et al., 1994]. Therefore, the cells that gave rise to them must have been pluripotent. In addition, studies by Honda and collaborators [Honda et al., 2007] provide evidence for the presence of stem cells in the neonatal mouse ovary – the thecal stem cells.

If indeed GCs arise from stem cells and if stem cells exist within the follicle, then the *in vitro* culture of such cells should be possible. Moreover, cells that are stem cells have the capacity to differentiate into others cell lines.

2 AIMS OF THE THESIS

The first part of this study has been performed to confirm the presence of pluripotent or multipotent stem cell populations among granulosa cells collected from mature human ovarian follicles. This work includes attempts to promote the growth of GCs over prolonged time periods *in vitro*. Previous studies in our laboratory have demonstrated that this is not possible with culture media which contain FSH and androgens [Zhang et al., 2000]. We identify the specific markers for mesenchymal stem cells and mature GCs and differentiate luteinizing GCs into other cell types of the mesenchymal lineage.

In the second part we demonstrate that granulosa cells can indeed be maintained *in vitro* thereby preserving their main characteristics, including the FSH receptor and steroidogenesis.

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3 THE MULTIPOTENCY OF LUTEINIZING GRANULOSA CELLS COLLECTED FROM MATURE OVARIAN FOLLICLES

Katarzyna Kossowska-Tomaszczuk [1,2]

Christian De Geyter [1,2,4]

Maria De Geyter [1]

Ivan Martin [2]

Wolfgang Holzgreve [1]

Arnaud Scherberich [2,3]

Hong Zhang [2,3]

Woman´s Hospital [1] and Department of Research [2], University of Basel, Spitalstrasse 21, CH-4031 Basel, Switzerland. [3] are acting as joint senior authors.

[4] *To whom correspondence should be sent:*
Prof. Christian De Geyter
University Hospital of Basel
Spitalstrasse 21, CH-4031 Basel
Switzerland
www.ivf-basel.ch

3.1 ABSTRACT

Graafian ovarian follicles consist of follicular fluid, one single mature oocyte and several hundred thousands of granulosa cells (GC). Until now, luteinizing GCs are considered to be terminally differentiated, destined to undergo death after ovulation. Present concepts of luteal function, endocrine regulation of early pregnancy and the recruitment of new ovarian follicles are all based on the cyclical renewal of the entire population of GC. We demonstrate that luteinizing GC isolated from the ovarian follicles of infertile patients and sorted with flow cytometry based upon the presence of their specific marker, the follicle stimulating hormone receptor (FSHR) can be maintained in culture over prolonged periods of time in the presence of the leukemia-inhibiting factor (LIF). Under those conditions the markers of GC function such as FSHR and aromatase gradually disappeared. POU5F1, a typical stem cell marker, was expressed throughout the culture, but not germ line cell markers such as nanog, vasa and stellar. Mesenchymal lineage markers such as CD29, CD44, CD90, CD105, CD117 and CD166, but not CD73, were expressed by substantial subpopulations of GC. The multipotency of a subset of GC was established by in vitro differentiation into other cell types, otherwise not present within ovarian follicles, such as neurons, chondrocytes and osteoblasts. Follicle-derived stem cells were also able to survive when transplanted into the back of immuno-incompetent mice, in vivo generating tissues of mesenchymal origin. The unexpected findings of multipotency of cells with prolonged lifespan originating from ovarian follicles are likely to have a significant impact on evolving theories in ovarian pathophysiology, particularly with reference to the ovarian endometriosis and ovarian cancer.

3.2 INTRODUCTION

3.2.1 THE GROWTH OF FOLLICLE

Having been laid down in fetal ovaries, the primordial follicles remain quiescent for decades. Once their development starts, they give rise to primary follicles, which are characterized by a slow growth of the enclosed oocyte and by low GC proliferation rates [Gougeon et al., 1996]. After their transformation into primary follicles, both the oocyte´s growth and the proliferation of the granulosa gain momentum culminating in the rapid growth of the antral follicle, finally resulting in the development of the mature Graafian follicle destined for ovulation. Several hundreds of thousands of GC exert a multitude of specialized functions encompassing the function of the follicle, such as producing large amounts of estradiol, adapting its FSH- and luteinizing hormone-receptivity to the endocrine milieu, nursing the oocyte and communicating both with the enclosed oocyte and the surrounding thecal cells. The signalling leading to ovulation results in luteinization of the tissue. Luteinized GC are considered to be terminally differentiated, being replaced in the midluteal phase of the menstrual cycle by small, luteinized cells originating from the surrounding theca [Niswender et al., 2000].

3.2.2 STEM CELLS

Both the rapid proliferation of the GC within the growing follicle and the exertion of such a large variety of specialized functions can only be thought of by accepting the notion, that the population of GC in a healthy follicle is not uniform, but rather consists of subpopulations of differentiated and less differentiated cells, the latter being more capable of mitosis. A similar situation is encountered in other rapidly

proliferating tissues with specialized functions, such as the bone marrow. The bone marrow contains a variety of specialized haematopoietic cells, such as myelocytes, reticulocytes or megakaryocytes, but also undifferentiated cells, which possess many of the characteristics contributed to stem cells. Within the particular anatomical environment of the bone marrow, stem cells are first capable of proliferation, then of differentiation, thereby steadily replacing ageing and apoptotic haematopoietic cells [http://stemcells.nih.gov/]. Other examples are found throughout the body, such as the intestines, the brain, the placenta and the testis [Young et al., 2005]. However, in contrast to many other tissue types, cells with stem cell properties have not yet been described within ovarian follicles.

3.2.3 OVERVIEW

The present communication is the first to describe, how luteinizing GC can be cultured over prolonged time periods under conditions similar to those known to support the survival of adult stem cells. We characterized a subpopulation of these cells, either shortly after their collection or after prolonged periods of culture in vitro, and demonstrated that these cells under appropriate conditions both in vitro and in vivo can be differentiated into other cell types, normally not encountered in the ovary. In this way, for the first time we were able to establish the multipotency of a subpopulation of cells collected from the antrum of human ovarian follicles.

3.3 MATERIAL AND METHODS

3.3.1 COLLECTING OF LUTEINIZING GC

Luteinizing GCs were collected by transvaginal ultrasound-guided aspiration from infertile patients treated with controlled ovarian hyperstimulation for assisted reproduction. Patients were treated with various exogenous gonadotropin including human menopausal gonadotropin (HMG, Menopur, Ferring, Switzerland; Merional, IBSA, Switzerland), and recombinant FSH (Gonal F, Serono, Switzerland; Puregon, Organon, Switzerland) followed by 10'000IU of human chorionic gonadotropin (HCG, Pregnyl, Organon). After removal of the cumulus oophorus-oocyte-complexes (COC), the freshly collected follicular aspirates were centrifuged for 5 min, 111g. GCs were separated from other cells by density gradient centrifugation on 5ml Ficoll PLUS (Amersham Bioscences, Sweden) for 20 min, 391g. GC were clearly visible in the interphase layer, isolated by pipetting, washed twice in 10ml Dulbecco's modified Eagle's (DMEM) culture medium and centrifuged again at 111g for 5 min for final collection of the cells [Zhang et al., 2000]. The purified cells were placed in freezing medium (fetal calf serum, FCS with 10% (v/v) dimethylsulphoxide (DMSO) and stored at –80ºC until flow cytometry (FACS, Fluorescence Activated Cell Sorter). In order to reduce inter-patient variability, each experiment was performed with mixed populations of GC collected from at least 8 patients after an informed consent obtained from each patient. This study was approved by our local Ethical Committee.

3.3.2 CELL CULTURE

GC were cultured in DMEM containing a high concentration of glucose (4500mg/L, Gibco, Switzerland), supplemented with 15% (v/v) fetal calf serum (Gibco),

penicillin/streptomycin (50µg/ml), L-glutamine (3mmol/l), β-mercaptoethanol (10mM stock solution in DMEM), recombinant FSH (100ng/ml or 3 x 10^{-4} IU/ml, Gonal F; Serono) and 1000 IU/ml of leukaemia inhibing factor [Gough et al., 1988] (LIF, Chemicon International, USA). As cells were highly sensitive to trypsin, a cell scraper was used for passages. Identical culture conditions were used for the incubation of bone marrow stromal cells in order to check for a potential contamination with fibroblasts.

3.3.3 IDENTIFICATION OF THE LUTEINIZING GC USING FACS AND SORTING

GCs were identified by the presence of FSHR and subsequently sorted using FACS. GC identification and sorting was performed by a dual labelling technique, where GCs were identified as CD3-negative cells, distinguishing them from CD3-positive leukocytes (anti-CD3-APC monoclonal mouse antibodies – Becton Dickinson) [De Neubourg et al., 1998]. GCs were kept frozen at –80°C and were thawed on the day of performing FACS. The first polyclonal goat antibody, raised against a peptide mapping near N-terminus of the FSHR of human origin (Santa-Cruz Biotechnology), was added for 30 min, kept on ice in the dark. The second donkey anti-goat IgG antibody labelled with fluorescence isothiocyanate (FITC) (Santa-Cruz Biotechnology) was used incubating for 30 min on ice in the dark. Isotype controls were used. Isolated populations of FSHR-positive cells, considered to be pure GC, were used for prolonged culture. As GCs were cultured either immediately after their aspiration from ovarian follicles or after thawing, their viability was tested using propidium iodide (pI) exclusion or calcein tests (Live/Dead Kit, Invitrogen).

3.3.4 FLUORESCENCE-ACTIVATED CELL SORTING ANALYSIS

Cell suspensions were incubated for 30 min at 4°C with fluorochrome-conjugated antibodies against the indicated protein or an isotype control. All antibodies were purchased from Becton Dickinson except the one against CD105 (Serotec) and FSHR (Santa-Cruz Biotechnology). Cells were washed, resuspended in PBS and analyzed with FACSCalibur (Becton Dickinson).

3.3.5 RT-PCR

Total RNA was extracted from GC using a RNeasy Total RNA kit from Qiagen (Germany). The quantity of RNA was measured by optical density at A260 nm (ND-1000 Spectrophotometer, NanoDrop Technologies, USA). Total RNA (1µg) was reverse transcribed into single strand cDNA using the cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Primers were synthesized by Microsynth, Switzerland: FSHR F (forward) 5'TGGGCTGGATTTTTGCTTTTG, R (reversed) 5'CCTTGGATGGGTGTTGTGGAC (annealing temperature 55°C, DNA product size 529 bp); Aromatase F5'CAAGTGGCTGAGGCAT, R5'GAGAATAGTCGGTGAA (55°C, 429 bp); POU5F1 (OCT-4) [Abdel-Rahman et al., 1995]; stellar [Ezeh et al., 2005]; vasa [Ezeh et al., 2005]; nanog [Ezeh et al., 2005]; LIFR [Abir et al., 2004]; nestin [Scintu et al., 2006]; neurofilament (NF) [Scintu et al., 2006]; B-3-tubulin [Dozier et al., 2003]. cDNA amplification primers for POU5F1, FSHR and LIF-receptor (LIFR) were designed to span introns as to eliminate genomic DNA contamination. The β-actin PCR product was used as internal control (Rapid Scan). The single strand cDNA was subjected to 35 cycles of PCR amplification using one of the primer sets. The amplified products were separated on 1 or 2% agarose gels. The RT-PCR products were analyzed by DNA sequencing

(ABI, PE Applied Biosystems, USA), mRNA from bone marrow was used as a positive control for POU5F1 [Pochampally et al., 2004].

3.3.6 IMMUNOHISTOCHEMISTRY

For the morphological examination of freshly collected or frozen/thawed GC were fixed in 1% paraformaldehyde overnight at 4°C, stained with haematoxylin/eosin (H&E) and observed microscopically at various magnifications (Leitz, Dialux 20, Germany). The presence of FSHR and POU5F1 in GC was demonstrated with immunohistochemistry using an antibody against human FSHR (Santa-Cruz Biotechnology) and POU5F1 (Abcam), respectively, following standard protocols. The secondary antibody against FSHR consisted of FITC-labelled donkey anti-goat antibodies (Santa-Cruz Biotechnology). The secondary antibodies against POU5F1 consisted of biotin-conjugated rabbit anti-goat antibodies (DAKO, Denmark A/S) or donkey anti-rabbit TexRed. Stainings for immunohistochemistry were performed by incubation with the ABC-alkaline phosphatase complex kit (Dako, Glostrup, Denmark), counterstained with H&E and mounted. As positive controls for the detection of POU5F1 sections of mouse ovaries were used.

3.3.7 DIFFERENTIATION *IN VITRO*

The multilineage differentiation capacity of the sorted luteinizing GC was evaluated by their differentiation into cell types, normally not encountered within the antrum of ovarian follicles, such as neuronal cells, osteoblasts and chondrocytes. Freshly collected and sorted GC were cultured in neuro-inductive medium for 10 days. For differentation of GC after long-term culture in vitro, GC were first incubated in medium supplemented with LIF for two weeks, then three weeks in one of the three

specific differentiation media described below. After five weeks, the pellets were harvested for histological examination and gene expression analysis.

Differentiation towards the neurogenic lineage was induced by DMEM supplemented with 10 %FCS and 30μmol/L transretinoic acid (Sigma) [Portmann-Lanz et al., 2006]. Differentiation towards the chondrogenic lineage was induced in DMEM culture medium supplemented with 10% FCS, ITS-1 (Insulin, Transferrin, Selenium; Sigma), 0.1mM ascorbic acid 2-phosphate, 10ng/ml TGFβ1 and 10^{-7}M dexamethasone [Barbero et al., 2003]**.** Osteogenic differentiation was induced in DMEM culture medium supplemented with 10%FCS, 0.1mM ascorbic acid 2-phosphate, 10^{-2}M β-glycerophosphate and 10^{-8}M dexamethasone [Barbero et al., 2003]. Neuro-differentiation was performed in monolayers. Chondro-differentiation was performed in three-dimensional (3D) cell cultures [Barbero et al., 2003], osteo-differentiation in both. For the 3D-cell culture approximately $3.5x10^5$ cells were cultured in pellets in conical microtubes (Sarstedt) on an orbital shaker. The media were changed three times weekly. For histological examination pellets were fixed in 4% formalin overnight at 4°C, paraffin embedded and sectioned (7um thickness). The sections collected from the osteogenic culture medium and the respective controls were stained with H&E or incubated for 10 minutes with alizarin red, washed extensively with water and observed microscopically (Leitz, Dialux 20, Germany). An alternative procedure consisted of staining with an antibody against bone sialoprotein (anti-BSP, Immundiagnostik AG, Germany). Decalcification of osteo-differentiated pellets was performed with Osteodec (Bio-Optica, Italy). The sections after chondroinduction were stained with Safranin-O or Alcian Blue.

3.3.8 QUANTITATIVE REAL-TIME PCR

Primers for real-time PCR were synthesized by Microsynth: COLL1, COLL2, OC [Barbero et al., 2003], OP F5' CTC AGG CCA GTT GCA GCC, R5' CAA AAG CAA ATC ACT GCA ATT CTC or synthesized by Roche: BSP [Barbero et al., 2003], SOX9 F5'CCCGCACTTGCACAACG, R5'TCCACGAAGGGCCGCT. Power SYBR Green PCR Master Mix (AB Applied Biosystems) for real-time PCR and TaqMan GAPDH Control Reagent (PE Applied Biosystems) was used as internal control. cDNA was subjected to 40 cycles of amplification using ABI PRISM 7000 Sequence Detector System (AB Applied Biosystems). Expression of the different genes was presented as percentage of expression of GAPDH, a house-keeping gene, by using the formula: $1/2^{\Delta Ct}$. Where ΔCt=gene–GAPDH, ΔCtq=control gene–GAPDH, ΔΔCt=ΔCt-ΔCtq and Delta-Delta CT Method, fold change, was presented using the formula: $2^{-\Delta\Delta Ct}$.

3.3.9 DIFFERENTIATION *IN VIVO*

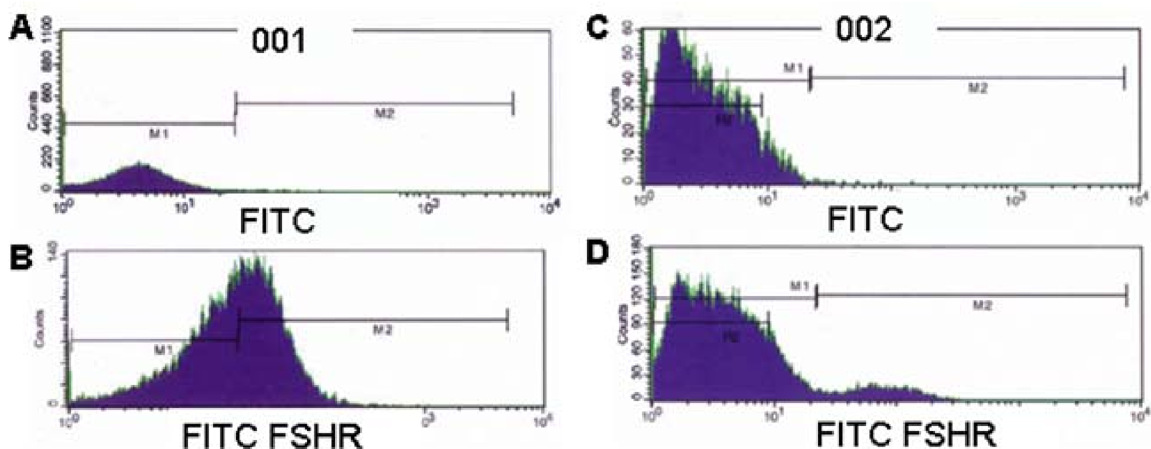
The multilineage differentation capacity of GC was assessed by implantation into the back of immuno-incompetent mice. For that purpose GC were cultured *in vitro* for three weeks in 3D and transplanted in nude mice (CD-1 nu/nu, 1-month old; Charles River Laboratories, Wilmington, MA, http://www.criver.com) in accordance with institutional guidelines. Four to eight weeks after implantation, the mice were sacrificed. The constructs were harvested and fixed overnight in 1% paraformaldehyde, paraffin embedded and sectioned. Sections were then stained by H&E and observed microscopically. Immunohistochemistry for BSP was performed with a BSP-biotin conjugated antibody (Cedarlane labs) followed by incubation with ABC-alkaline phosphatase complex (Dako, Glostrup, Denmark), counterstained with

H&E and mounted. For immunohistofluorescence of BSP, polyclonal rabbit-anti-human BSP antibodies from Alexis Biochemicals with secondary goat anti-rabbit PE antibodies from Becton Dickinson were used. To distinguish human from murine cells immunohistofluorescence was carried out with anti-human monoclonal HLA-ABC-biotin conjugated (Cedarlane Laboratories Ltd) antibody with avidin-FITC secondary antibodies from Becton Dickinson. Some sections were also stained with Safranin O to assess the formation of cartilage.

3.4 RESULTS

3.4.1 PURE POPULATION OF GC

The cellular content of follicular fluid aspirated during oocyte collection for assisted reproduction consisted of a mixture of luteinizing GC, both single and in clumps, erythrocytes and large epithelial cells, probably also arising from the vaginal epithelium. Most of the erythrocytes were excluded during the Ficoll density gradient purification. With FACS a subpopulation of FSHR bearing cells from the follicular aspirates was consistently identified and separated from any contaminating cells. As illustrated in supplemental Figure 1 (Fig.1), the relative number of cells expressing FSHR among the entire population of cells in the unsorted follicular aspirates ranged between 7% and 50%.



Histogram statistics
A. Isotype control
M1 – 95%
M2 – 0%
B. FSHR
M1 – 48%
M2 – 50%

Histogram statistics
C. Isotype control
M1 – 91%
M2 – 0%
D. FSHR
M1 – 88%
M2 – 7%

Figure 1. FACS analysis for FSHR (**B and D**) and isotype control (**A and C**) for two IVF patients labeled 001 (**A and B**) and 002 (**C and D**). M1 – cells negative for FSHR, M2 – cells positive for FSHR. As presented, population of GC from single patient is very different. Therefore all experiments were performed with mixed population of GC from different patients.

This broad range corresponds to the individual characteristics of infertile women, from whom the GC were collected, and to the technical variabilities of transvaginal, ultrasound-guided aspiration of ovarian follicles. Pooling of GC from different patients was used to overcome this variability. After sorting and adhesion to the culture dish all cells expressed the FSHR, as demonstrated both by cytofluorimetry and immunocytochemistry (Fig.2A, B, C). After thawing and sorting, approximately 40% of GC survived after one day in culture (Fig.3). The sorted cells were characterized as luteinizing GC through their expression of both FSHR and aromatase (Fig.6A). These purified luteinizing GC were then used for prolonged culture.

3.4.2 LIF EFFECT ON GC CULTURE

In order to evaluate the effect of LIF on the prolonged survival of GC in vitro, sorted luteinizing GC were split into two groups and cultured separately. One group was cultured in DMEM medium supplemented with LIF, while another group without LIF. The expression of LIFR in sorted luteinizing GC was first confirmed (Fig.4). The luteinizing GC cultured without LIF consistently died within two weeks (Fig.2D), whereas those cultured in medium supplemented with LIF remained viable for up to four months and could be passaged (Fig.2E, F). After 7 days with LIF, GC retained their morphology, constructed intercellular connections and became strongly attached to the culture dish (Fig.5). In contrast, GC cultured in the absence of LIF became folded after 7 days (green arrow) and lost intercellular connections (red arrow).

Moreover, expression of FSHR remained present for at least 7 days in cells cultured with LIF, whereas it was mostly lost after 7 days in the absence of LIF (Fig.5).

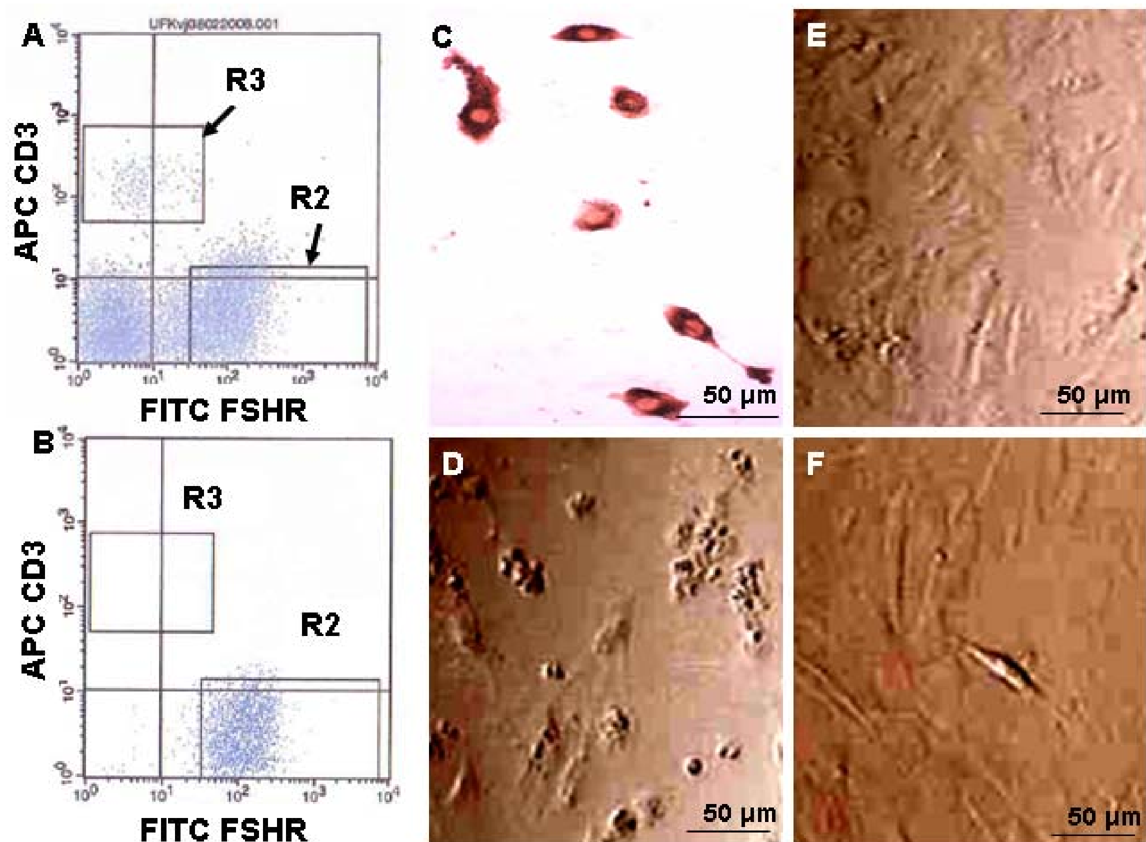


Figure 2. Purification and long-term culture of luteinizing GC. FSHR in GC (**A, B, C**). (**A**) FACS/sorting results of freshly collected GC. (**B**) Pure population of GC after FACS/re-sorting of already sorted cells. Cells: (**R2**) FSHR-positive, (**R3**) CD3-positive. (**C**) Immunocytochemistry of GC for FSHR after FSHR sorting. GC cultured in medium: (**D**) after 5 days without LIF, (**E**) after 5 days in the presence of LIF, (**F**) after 1 month in the presence of LIF.

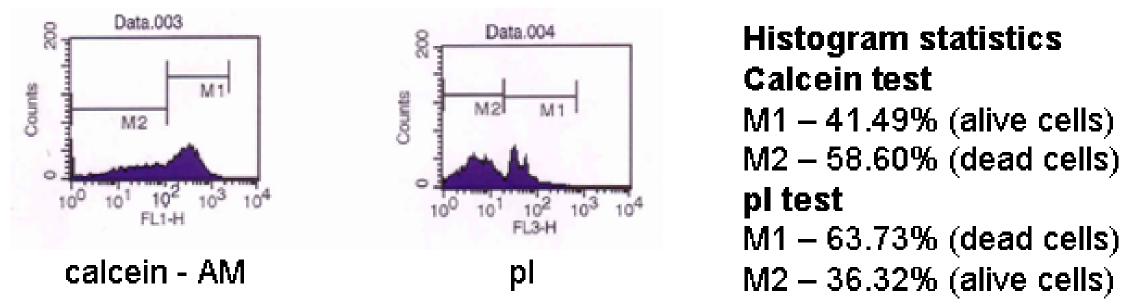


Figure 3. Viability test for frozen/thawed GC positive for FSHR. For experiments mixture of GC were used. There was no difference observed in experiments with only frozen/thawed or mixed population of GC.

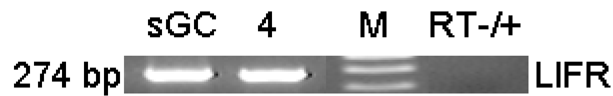


Figure 4. Characterization of luteinizing GC, sorted with FACS and cultured in medium supplemented with LIF. Expression of LIFR.

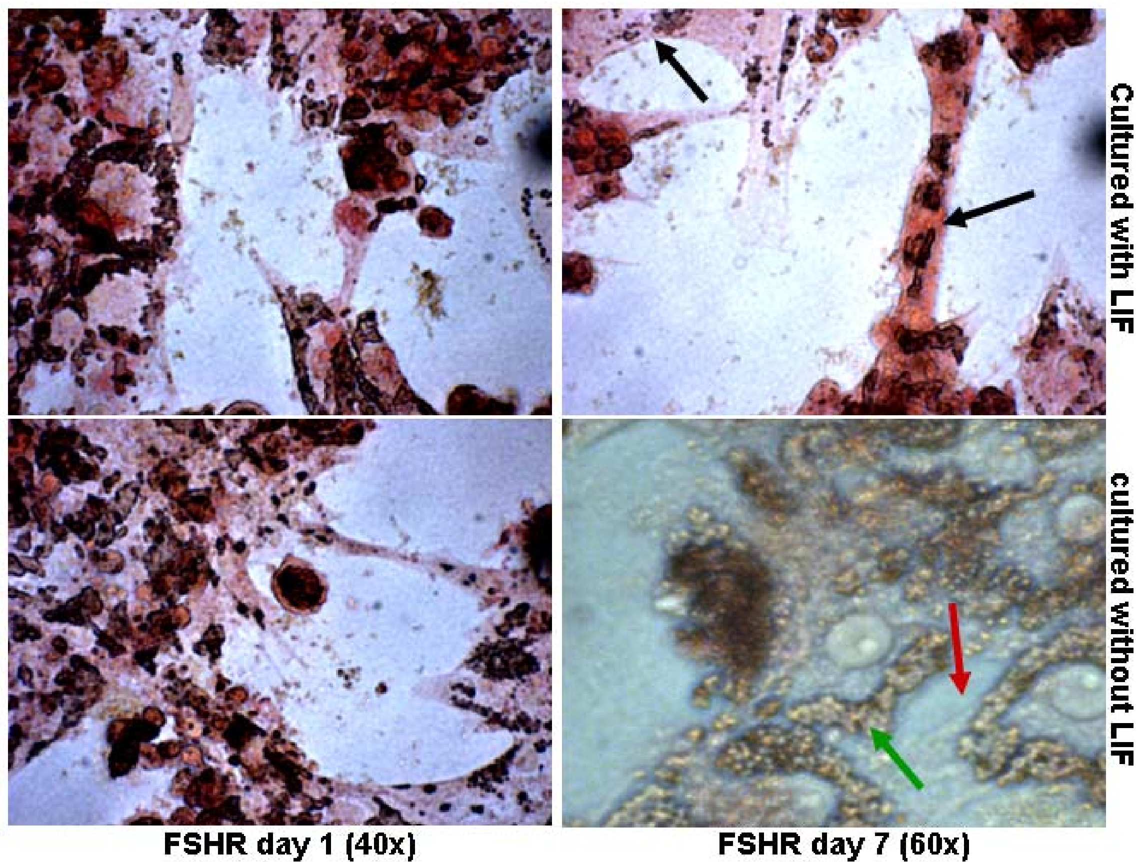


Figure 5. Survival of GC. GC cultured with LIF kept their morphology and were strongly attached to the dish. Cells were big and spread in a bigger area (**black arrow**). Cells keep contact to each others. GC cultured without LIF after 7 days are starting to fold the cytoplasm (**green arrow**) and therefore break the contact with other cells (**red arrow**).

Sorted GC upon FSHR show 100% positivity of FSHR marker after ICC when cultured in monolayer (day 2 of culture with LIF and without LIF). After day 7 of culture in monolayer GC keep the FSHR in about 40% cells when cultured with LIF. When cultured without LIF only about 10% of cells present weak staining for FSHR.

3.4.3 GC PHENOTYPE

The expression of FSHR, aromatase and POU5F1 (POU domain, class 5, homeobox 1) was then examined on sorted luteinizing GC cultured in the presence of LIF at

various time intervals (after 7, 21, 28 and 56 days) and compared with freshly collected sorted and unsorted luteinizing GC (Fig.6A). After approximately 7 days the luteinizing GC progressively lost their ability to express FSHR, after 8 weeks also that of aromatase. POU5F1 was expressed in the freshly collected luteinizing GC and remained expressed in the luteinizing GC throughout their culture in medium supplemented with LIF (Fig.6A).

The expression of POU5F1 was confirmed by immunocytochemistry in the nucleus of some GC attached to unmarked GC (Fig.6B). The same immunostaining for POU5F1 in both oocytes and GC inside antral follicles of mouse ovaries was used as a positive control (Fig.7). Double stainings for FSHR and POU5F1 were performed in GC after 1, 3, 5, 7 and 9 days in culture to examine whether the FSHR-expressing GC were co-expressing POU5F1 and to exclude any contamination of extrafollicular stem cells.

Throughout culture in the presence of LIF, 1-3% of GC expressing FSHR also expressed POU5F1 (Fig.8 and Fig.9). Additionally, the potential overgrowth of the GC culture by contaminating fibroblasts or other cells was excluded by the observation that no cells survived in the absence of LIF. Furthermore, in another set of experiments bone marrow stromal cells were cultured in the same medium either supplemented or not supplemented with LIF. Under those conditions the bone marrow stromal cells remained viable over prolonged time periods in both media and expressed neither FSHR nor POU5F1 (Fig.10).

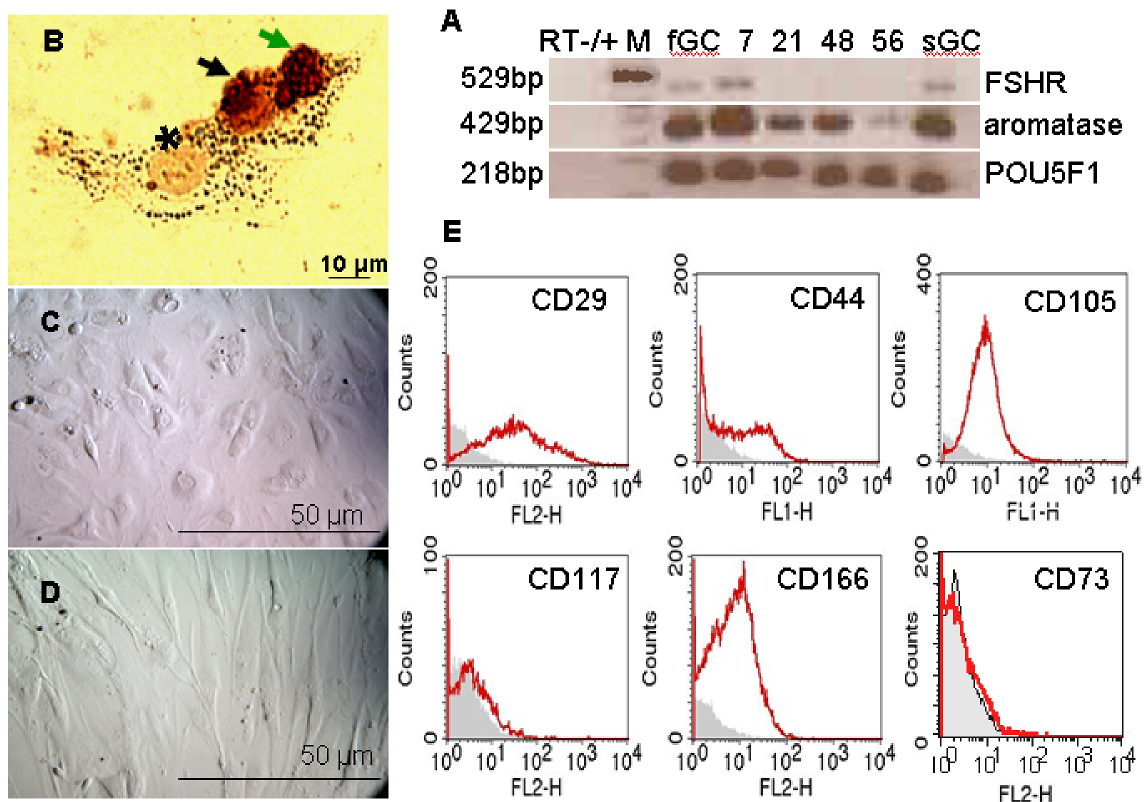


Figure 6. Characterization of luteinizing GC. Characterization of luteinizing GC, sorted with FACS and cultured in medium supplemented with LIF. (**A**) RT-PCR analysis shows progressive loss of FSHR/aromatase expression during prolonged culture, but not of POU5F1 (OCT4). RT+/-: negative control, M: marker, fGC-freshly collected GC, sGC-sorted GC, cultured GC after: 1-1w (week), 3-3w, etc. (**B**) POU5F1 staining of GC. Three GC stained for POU5F1 are visible. Two GC are fibroblast-type cells (**asterisk and black arrow**). One GC is epidermal-type cell and did not attach to the culture dish (**green arrow**). One GC presents the negative nucleus staining (**asterisk**), one with positive nucleus staining (**black arrow**). In both cells cytoplasm is negative for POU5F1 staining (**asterisk and black arrow**). (**C**) Epithelial-like and (**D**) fibroblast-like morphology of GC after 10 days culture. (**E**) Immunophenotyping results for GC-derived multipotent cells. The following markers of mesenchymal stem cells were detected: CD29, CD44, CD105, CD117, CD166 whereas CD73 was negative. The red line indicates the respective markers, the grey shaded area the isotype control.

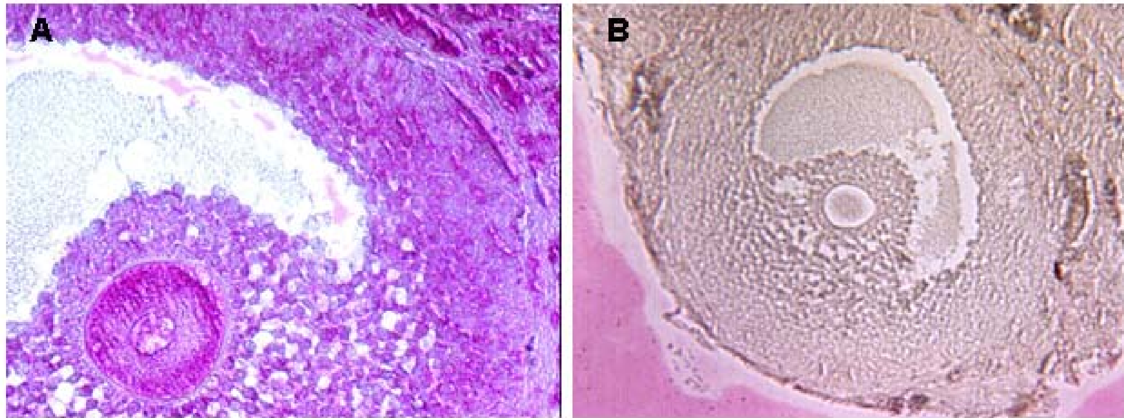


Figure 7. (**A**) Oct4 (POU5F1) immunohistochemistry in sections of mouse ovaries (magnification 10x). (**B**) negative control.

As visualized with light microscopy, cultured luteinizing GC exhibited two distinct morphologies: epithelial (between 5% and 35% of all cells) or fibroblastic (Fig.6C, D). The epithelial-like cells disappeared after about 3 weeks in culture, whereas the remaining cells retained their fibroblastic morphology.

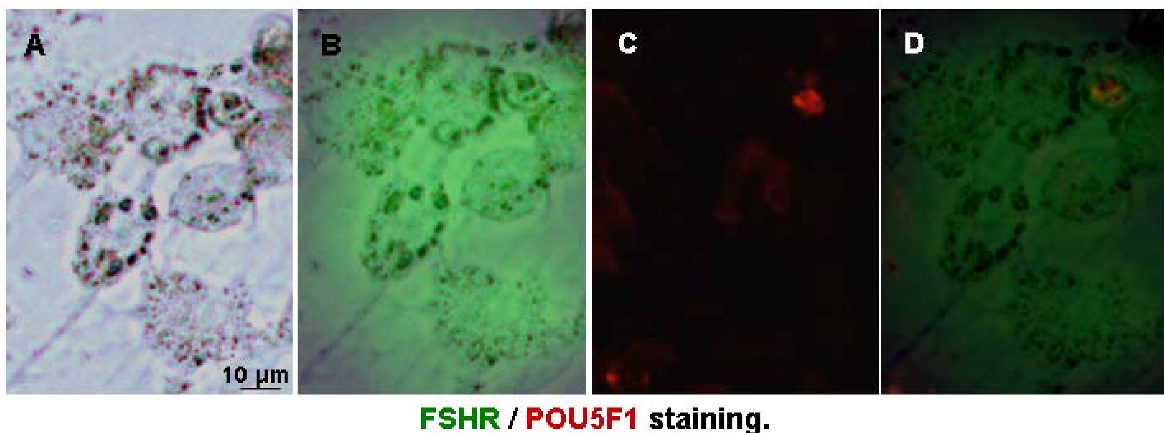


Figure 8. Immunocytofluorescence for GC cultured in monolayer. (**A**) Light microscopy image. (**B**) FSHR-FITC staining. (**C**) POU5F1-TexRed staining. (**D**)

Double staining with FSHR-FITC and POU5F1-TexRed presenting FSHR GC positive for POU5F1.

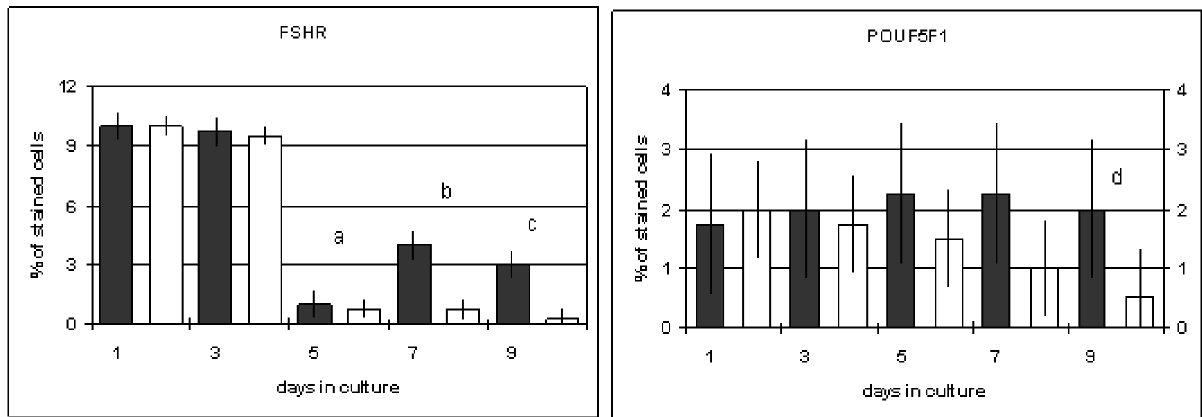


Figure 9. The relative number of stained cells (%) for FSHR and POUF5F1 during their observation in culture medium either supplemented with LIF (black columns) or without LIF (empty columns). The heighth of each column represents the median with the 95 % confidence interval. Using Kruskal-Wallis statistically significant differences were found: a, b and c denote a statistically significant difference $p<0.0001$ at day 5, 7 and 9, respectively, as compared with day 1 and 2, resp., equally for cells cultured in the presence of LIF and without LIF; d denotes a statistically significant difference between the relative number of cells stained between culture with and without LIF.

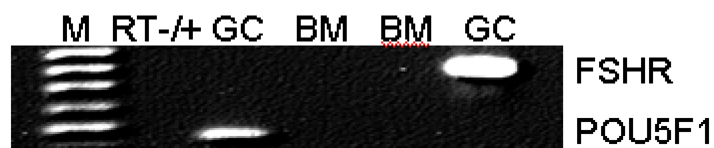


Figure 10. RT-PCR for bone marrow stromal cells (BM)

3.4.4 PLURIPOTENCY OF GC

As the transcription factor POU5F1 remained expressed throughout the prolonged culture, we examined other markers of pluripotency, characteristic for germ cells, such as nanog, stellar and vasa (Fig.11). All specific markers of germ cells, however, were negative. All PCR products yielded the expected fragment sizes. There was no contamination of genomic DNA in any of the samples tested and all negative controls (RT±) processed without reverse transcriptase yielded no amplification product.

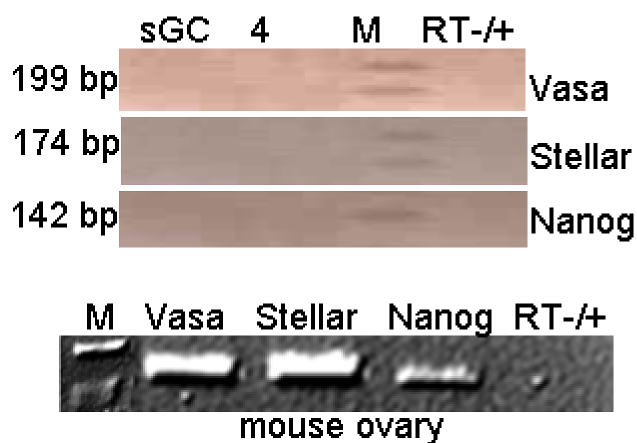


Figure 11. Characterization of luteinizing GCs, sorted with FACS and cultured in medium supplemented with LIF. The germ cells markers vasa, stellar and nanog were not expressed in GCs. RT-PCR of mouse ovary as a positive control for Vasa, Stellar and Nanog.

3.4.5 MESENCHYMAL STEM CELLS MARKERS

As GC originates from the mesoderm, we subsequently examined the mesenchymal cells characteristic of the freshly collected GC using various markers of mesenchymal stem cells (MSC). Cells were positive for markers: CD29, CD44, CD90 CD105,

CD117 and CD166, but not CD73 (Fig.6E). CD117 was positive in only 4.5% (+/- 3%) of GC (mean +/- standard deviation, 10 different donors). The typical marker for hematopoietic cells, CD45, was present only in freshly isolated GC probably due to a contamination with blood cells, although CD34 was not expressed (data not shown).

3.4.6 NEURODIFFERENTIATION

The multipotency of a subpopulation of cells in the follicular aspirates was assessed. Neuronal markers for neurodifferentiation of freshly isolated GC were thus examined. Two neuronal markers, nestin and β-3-tubulin, were weakly expressed in freshly collected GC, whereas another, neurofilament, was not found to be expressed in freshly collected GC (Fig.12). Subsequently, the multilineage differentiation capacity of GC was assessed after prolonged culture. The cells were differentiated in vitro to neuronal, osteoblastic and chondrogenic lineages respectively under conditions known to direct the differentiation of MSC. A clonal analysis of GC was attempted, but failed probably due to the deleterious effect of trypsin and the increased general sensitivity of passaged GC. The capacity of luteinizing GC to undergo neurogenic differentiation after prolonged culture in medium supplemented with LIF was examined as well. After five days GC cultured as monolayers in medium containing retinoic acid developed neuron-like structures. After 8 days of culture, approximately 15 % of all cells displayed the distinct morphology suggestive of neurons (Fig.13A,B). Various neuronal markers, such as nestin, neurofilament and β-3-tubulin, were found to be expressed in GC cultured in retinoic acid-enriched medium but not in the control medium supplemented with LIF (Fig.13C). All experiments were performed in triplicate. Brain tissue was used as a positive control and the expression of all markers was confirmed by sequencing (data not shown).

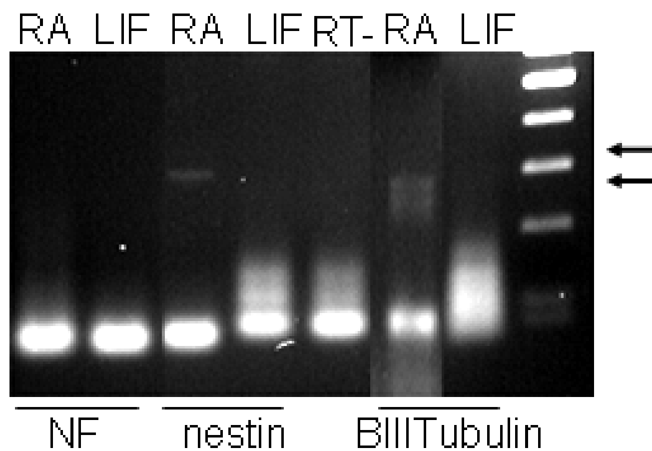


Figure 12. RT-PCR for neurodifferentiation markers of freshly isolated GC presents only 2 genes (nestin and BIIITubulin - arrows) weakly expressed, where the NF - neurofilament gene is not expressed. RA – medium with retinoid acid; LIF – medium with LIF; RT- – negative control.

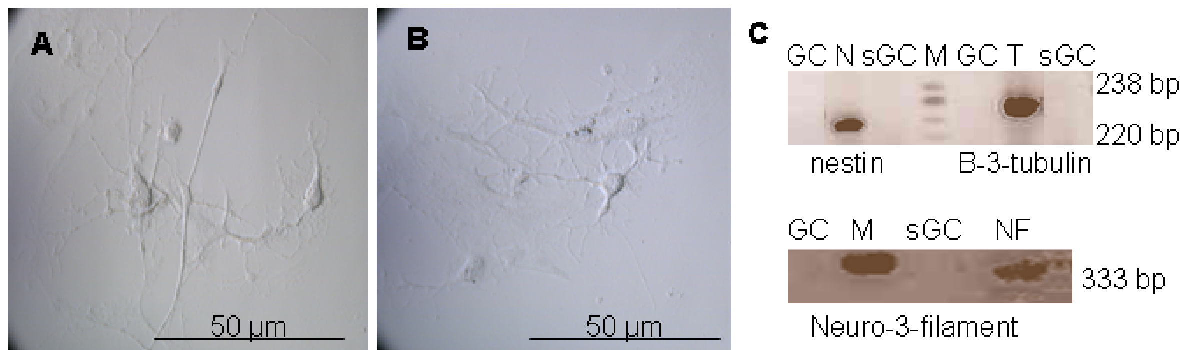


Figure 13. Neurogenic differentiation of GC. Neurogenic differentiation of GC after prolonged culture in medium supplemented with LIF. (**A, B**) Neuron-like morphology after neurogenic induction of GC. (**C**) RT-PCR results of GC-neural induction showing expression of the neuronal markers, nestin (N), B-3-tubulin (T) and neuro-3-filament (NF). GC: GC cultured in control medium supplemented with LIF, sGC: sorted GC.

3.4.7 THE OSTEOBLASTIC DIFFERENTIATION

The osteoblastic differentiation potential of cultured luteinizing GC was examined by alizarin red and BSP-staining and gene expression of various osteoblastic markers, such as BSP, osteocalcin (OC) and osteopontin (OP). As GCs cultured as monolayers in osteoinductive medium exhibited typical changes in their cellular morphology (Fig.14), but were too sensitive and became detached from the culture plate, a 3D-culture system was introduced. Under those conditions and in the presence of an osteo-inductive medium, previously luteinizing GC were stained positively with alizarin red and marked with anti-BSP antibodies, whereas the same cells cultured in medium supplemented with LIF or sections of mouse ovaries remained negative (Fig.15). The matrix of osteo-differentiated cell pellets was demonstrated to be mineralized, as documented by rapid dissolution of crystallized structures by treatment with an acidic decalcification buffer, Osteodec® (data not shown). Real-time PCR showed that expression of BSP was increased 14-fold, OP 66-fold and OC 3-fold in osteo-differentiated tissue pellets, when compared to control cells cultured with medium supplemented with LIF (Fig.14B). When compared with bone marrow-derived cells (BMSC) cultured as a monolayer in a similar osteoinductive medium during the same time [Frank et al., 2002], BSP was found to be expressed 8 times more in BMSC than in osteo-differentiated GC pellets, but both OP and OC were expressed more in the GC pellets (5 and 1.5-fold respectively).

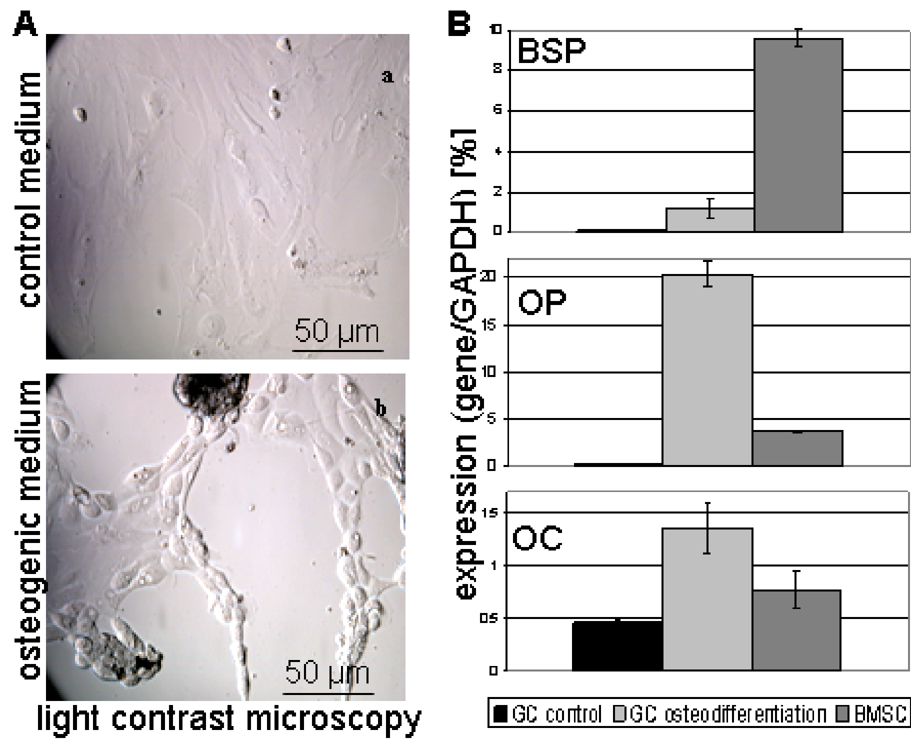


Figure 14. Osteogenic differentiation of GC. GC cultured in monolayers (**A**) in control medium exhibiting fibroblast-like cells and in osteo-inductive medium after 5 days, showing epithelial-like morphology of cells. (**B**) Real time PCR results for BSP, OP and OC expression in control GC, GC after osteodifferentiation and in bone marrow stem cells (BMSC).

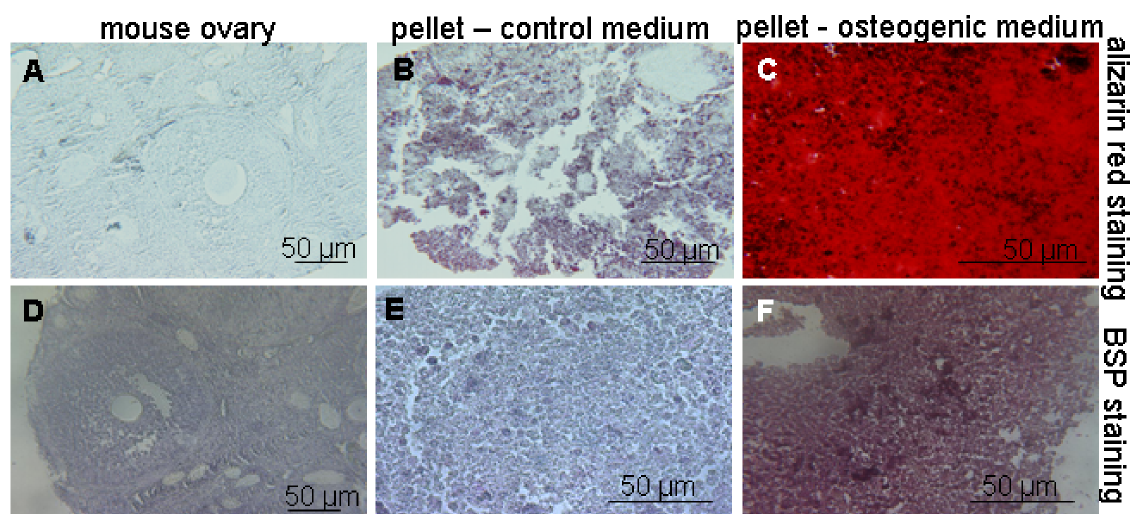


Figure 15. Osteogenic differentiation of GC. Osteogenic differentiation of GC after prolonged culture in medium supplemented with LIF. (**A, B, C**) Stainings for alizarin red and (**D, E, F**) BSP for 3D-cultured luteinizing GC and sections of mouse ovaries.

3.4.8 THE CHONDROGENIC DIFFERENTIATION

The chondrogenic differentiation potential of luteinizing GC cultured over prolonged periods of time was demonstrated by the presence of glycosaminoglycan (GAG) in GC pellets cultured in 3D in chondro-inductive medium using Safranin-O-staining (Fig.16A, B). After chondrogenic differentiation, the tissue sections were weakly positive for Safranin-O-staining, whereas GC cultured in medium supplemented with LIF were negative. Sections of mouse ovary were also negative for GAG-staining (data not shown). With real-time PCR the expression of various genes specific for chondrogenic differentiation was upregulated in GC cultured in chondrogenic differentiation medium, when compared to GC cultured with control medium with LIF: collagen-1 (Coll1) 4.5-fold, collagen-2 (Coll2) 6.5-fold and Sox9 12.5-fold (Fig.16C). These values were also compared with expanded primary chondrocytes cultured as a monolayer in the same chondrogenic medium during the same period [Barbero et al., 2003]. Expression of Coll2 and Sox9 was higher in chondrocytes (2.5-fold and 1.9-fold respectively), but that of Coll1 was higher in chondro-induced GC (1.6-fold).

3.4.9 IN VIVO DIFFERENTIATION

The capacity of GC cultured in medium supplemented with LIF to survive and differentiate *in vivo* into other, distinct tissue types was examined through subcutaneous transplantation into the back of immuno-incompetent, nude mice. The implants were harvested either four weeks or eight weeks after transplantation. After

eight weeks the implanted cell pellets appeared to be more integrated within the murine tissue than after 4 weeks (Fig.17A, B) and expression of BSP was detected (Fig.17C, D, F). Those cells were always surrounded by murine cells also showing expression of BSP (Fig.17E, F). The distinct origin of both cell types was tested by HLA-ABC-staining, which is specific for human tissue. Eight weeks after transplantation some GAG deposition was also detected as demonstrated through the Safranin-0-staining (Fig.17G, H, I).

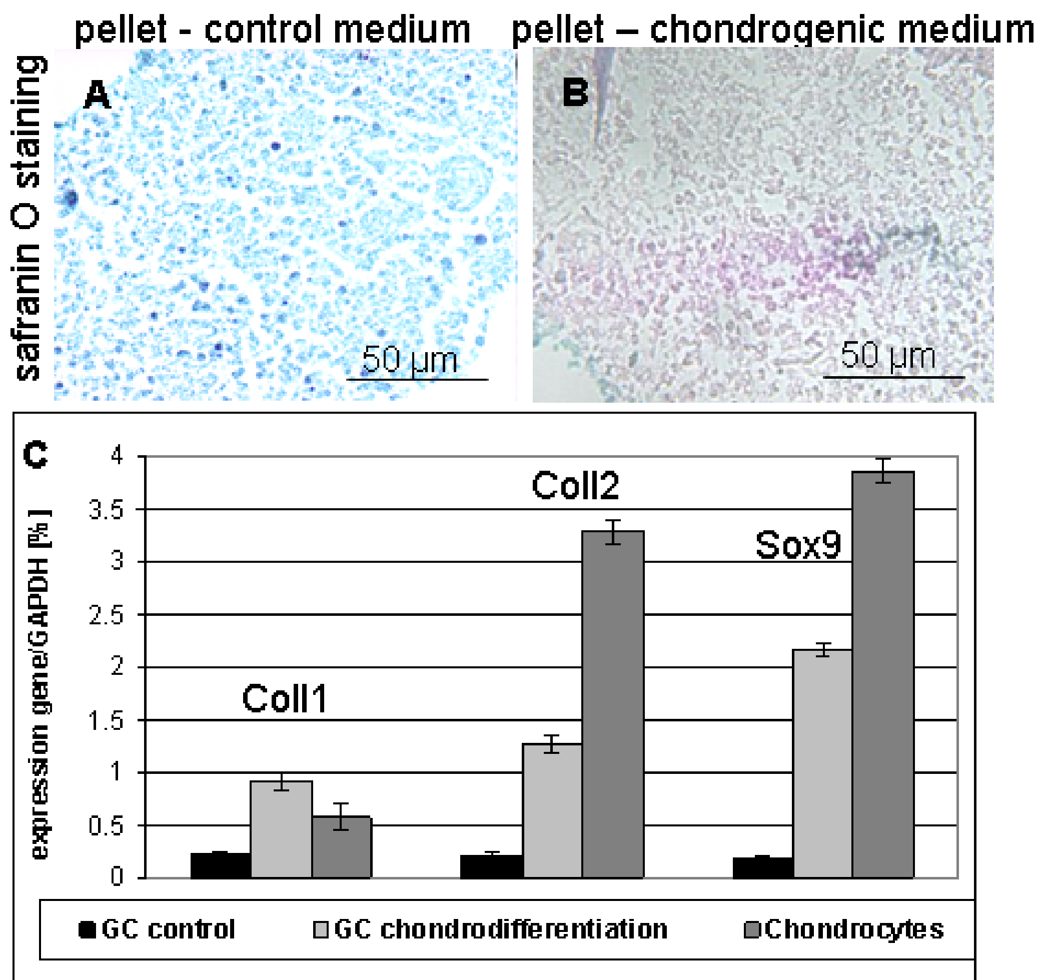


Figure 16. Chondrogenic differentiation of GC. Chondrogenic differentiation of GC after prolonged culture in medium supplemented with LIF. (**A**) Safranin-O staining in control GC and (**B**) in GC after chondro-induction. Real time PCR results (**C**) for expression of COLL1, COLL2, Sox9 in control GC in regular culture medium, GC after chondroinduction and in chondrocytes.

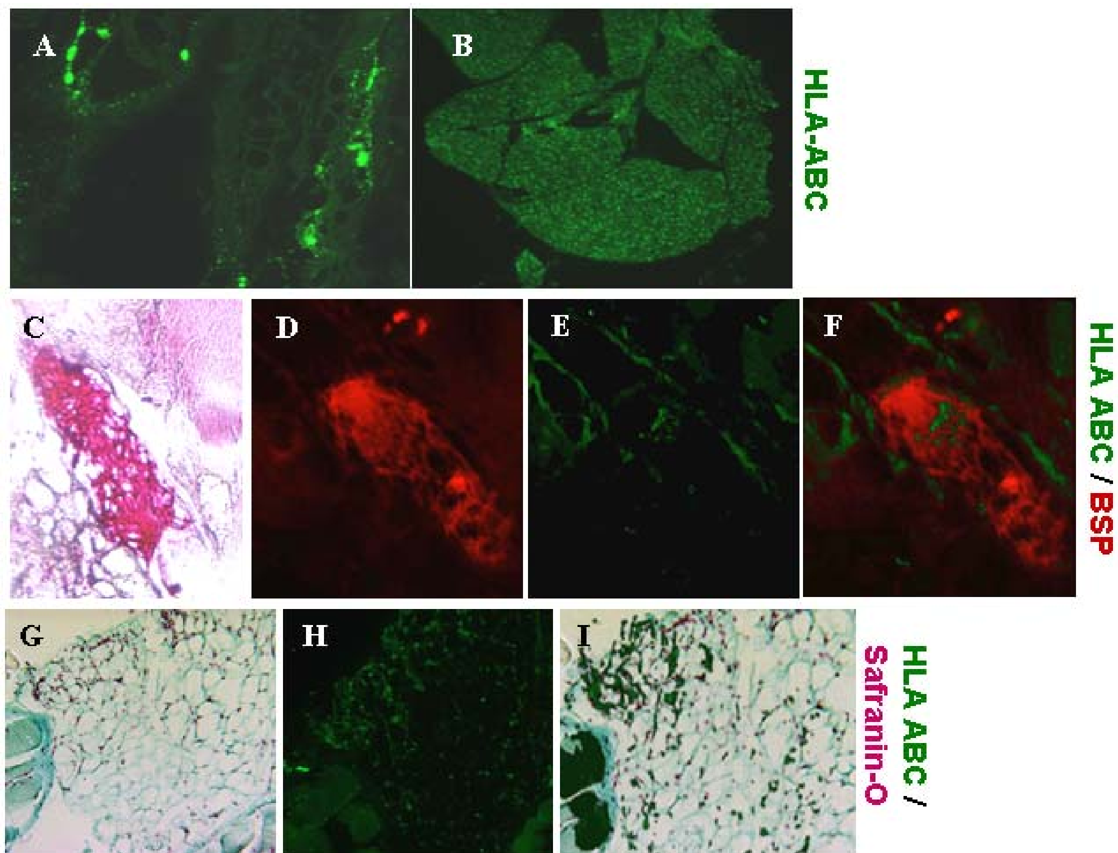


Figure 17. *In vivo* differentiation of GC. HLA-ABC (green) staining of pellets containing GC, (**A**) 8 weeks after implantation at 20x magnification and (**B**) 4 weeks after implantation, at 10x magnification. Staining for BSP using immunohistochemistry, (**C**) 8 weeks after transplantation (red) at 20x magnification, (**D**) BSP using immunohistofluorescence (red) at 20x magnification, and (**E**) for HLA-ABC (green) at 20x magnification. (**F**) Double staining for BSP and HLA-ABC at 20x magnification. (**G**) Staining for Safranin-O, 8 weeks after transplantation (red)

at 10x magnification and (**H**) HLA-ABC 10x. (**I**) Double staining for Safranin-O and HLA-ABC, magnification 10x.

3.5 DISCUSSION

3.5.1 LIF IMPACT ON CULTURE

Up to now, luteinizing GCs are considered to be terminally differentiated, unavoidably becoming apoptotic a few days after ovulation. Indeed, prolonged culture in vitro of GC collected from preovulatory follicles has not been possible beyond 10 days. Instead, various researchers have attempted to use GC from granulosa tumors [Zhang et al., 2000; Nishi et al., 2001] or immortalized GC [Tajima et al., 2002] in order to construct GC lines suitable for research purposes. We were now able to culture luteinizing GC, collected from infertile women treated with controlled ovarian hyperstimulation for assisted reproduction, over prolonged time periods. The variable fertility status of single patients certainly impacts on GC function. Therefore, in all experiments the samples of several patients were pooled in order to reduce this potential confounding factor. The crucial difference between our present approach and earlier trials [Zhang et al., 2000] was the use of LIF, a cytokine commonly used in culture media supporting the development and growth of stem cells. LIF promoted the long-term survival of luteinizing GC, whereas in the absence of LIF these cells invariably became apoptotic. LIF is a glycoprotein with a remarkable range of biological actions in different tissues, such as long-term maintenance of mouse, but not human embryonic stem cells [Daheron et al., 2004]. In a number of tissues LIF has been shown to be important for stem cell self-renewal, such as the brain [Bauer et al., 2006], the gut [Kalabis et al., 2003] and bone marrow [Jiang et al., 2002].

LIF has been detected both in fetal and adult human ovaries [Abir et al., 2004] and may be involved in the transition of primordial to primary follicles [Nilsson et al., 2002]. LIF is present in the follicular fluid and its secretion can be enhanced by

human chorionic gonadotropin [Arici et al., 2997; Coskun et al., 1998]. LIF receptor activity has been detected in oocytes and preimplantation human embryos [Van Eijk et al., 1996], suggesting a role of genital tract LIF in the process of follicular development and implantation [Arici et al., 1995].

3.5.2 GC PHENOTYPE

Although LIF permitted the prolonged survival of luteinizing GC, they progressively lost their major characteristics, such as the FSHR and aromatase. The overgrowth of the luteinizing GC by a subpopulation of other cells such as fibroblasts was excluded by the extraction of a pure population of GC with FACS based on the FSHR. Apart perhaps from the oocyte [Meduri et al., 2002], GC are the only cell type in the female body possessing the FSHR. Both with flow cytometry and immunocytochemistry we demonstrated that all experiments were performed with a highly homogeneous and almost pure population of GC. The sorted GC continued to possess all typical characteristics such as aromatase and FSHR over a period of at least ten days. As follicles mature, the amount of mRNA for FSHR is known to decrease, whereas that of aromatase increases [Slomczynska et al., 2001]. The presence of FSHR in sorted GC was confirmed by immunocytochemistry.

The two different morphologies of GC, epithelial and fibroblastic, found to be present in the medium during initial culture, correspond to the different intrafollicular locations, from which the GC were removed during transvaginal ultrasound-guided follicular aspiration. There is evidence that GC originating from the close to the basal membrane are columnar, whereas those originating from the middle layer are rounded and those originating from the central part of the follicle, close to oocyte, are flattened [Rodgers et al., 2001]. Some authors argue that the elongated GC may have lost

aromatase activity, cytoplasmic changes compatible with luteinization [Gutierrez et al., 1997]. When cultured in monolayers, GCs invariably become luteinized and convert their epithelial morphology into a fibroblastic one, explaining why the latter morphology became dominant during prolonged culture.

3.5.3 STEM CELLS PROPERTIES OF GC

The progressive loss of all characteristics of GC during prolonged culture and the continued expression of POU5F1 in luteinizing GC gave rise to the hypothesis that some follicular cells might exhibit stem cell properties. POU5F1, also known as OCT-4A, is a transcription factor and one of the two isoforms produced by the OCT-4 gene, which is considered as a main regulator of differentiation and self-renewal [Pan et al., 2002; Cauffman et al., 2006; Liedtke et al., 2007; Zangrossi et al., 2007; Kotoula et al., 2008]. The expression of POU5F1 has not been demonstrated in granulosa before. Using immunostaining we confirmed the presence of POU5F1 in the nucleus of human GC and in mouse ovaries.

To confirm the multipotency of GC, we first examined typical mesenchymal markers of multipotent MSC. With the exception of CD73, subpopulations of GC expressed those markers, thereby adding to the notion that during prolonged culture in the presence of LIF these cells possess many but not all attributes of the MSC lineage. We then provided evidence of the multipotency of a GC subpopulation by demonstrating their differentiation potential when cultured with specific neuroinductive, chondroinductive and osteoinductive culture media.

3.5.4 CHONDRODIFFERENTIATION

Chondroinduction was demonstrated by real-time PCR and detection of GAG, a method commonly used for the detection of cartilage matrix [Barbero et al., 2003]. Safranin-O-staining was used, because the Alcian Blue staining, commonly applied for GAG-staining, also stained Call-Exner bodies in ovarian tissue, as described previously [Van Wezel et al., 1999]. Safranin-O-staining was weak, probably due to the fact that some GC died early during the chondrogenic differentiation. Expression patterns of cartilage-related genes during chondrogenic differentiation in GC and expanded primary chondrocytes control were similar. The COLL1 and COLL2 genes are expressed during cartilage development. COLL1 is expressed by cells first entering differentiation where COLL2 is expressed in differentiated cells [Bonaventure et al., 1994]. SOX9 is a key regulator of chondrogenesis [Jenkins et al., 2005]. The difference in expression levels of the three genes could suggest that GC, under the conditions described, undergo very early chondrogenic differentiation.

3.5.5 OSTEOBLASTIC DIFFERENTIATION

Osteoblastic differentiation was demonstrated by alizarin-red-staining, a dye assessing the presence of calcium in mineralized matrices. BSP-staining was also performed and confirmed the osteoblastic differentiation of GC. BSP and OP are prominent components of bone extracellular matrix. They are expressed by differentiated osteoblastic cells and serve as indicators of osteoblastic differentiation of BMSC [Barbero et al., 2003]. After 3 weeks of osteoblastic differentiation, the expression of BSP messenger RNA exhibited a 14-fold increase and OP messenger RNA a 66-fold increase as compared to GC cultured in LIF medium. OC messenger RNA showed a limited 3-fold increase in differentiated GC. This low expression level is explained by

OC starting to be expressed later during the osteoblastic differentiation process, between days 16-30 of culture, resulting in a maximal though limited expression at day 21.

3.5.6 NEURODIFFERENTIATION

The differentiation potential of GC into the neuronal lineage was less pronounced among freshly isolated GC than after prolonged culture. Neuro-induction of freshly collected GC induced the expression of only two neuronal markers, nestin and β-3-tubulin, but not neurofilament. Obviously, the size of the subpopulation of GC with multipotent stem-cell characteristics is smaller shortly after follicular aspiration than after prolonged culture.

3.5.7 DIFFERENTIATION POTENTIAL OF GC

As some MSC markers were not uniformly present in the sorted GC and although we can not entirely exclude the overgrowth of the sorted GC by contaminating mesenchymal stem cells admixed from other tissues and expanding during prolonged culture, we hypothesize the presence of several subpopulations of GC in preovulatory follicles, each expressing different MSC markers according to their degree of differentiation. In many organs, adult tissues typically contain various cell populations, including multipotent stem, progenitor cells and terminally differentiated cells [Hochedlinger et al., 2006].

For the first time, prolonged culture of luteinizing GC in medium supplemented with LIF allows the selection of less differentiated GC, which exhibited a certain degree of plasticity, as they could be differentiated in vitro into three distinct lineages: neuronal, chondrocytic and osteoblastic, all normally not found in healthy ovarian follicles.

Both the survival of GC after prolonged culture in the presence of LIF and their ability to differentiate into cells of the mesodermal lineage was also confirmed in vivo. The transplanted and differentiated human cells were surrounded by specific mouse cells differentiated into the same direction, all within mesenchymal lineage.

3.5.8 OOGENESIS – RECENT DEBATE

Recently, much controversy has arisen concerning the possible presence of stem cells in the germinal layer of the mouse ovary and their potential for replacing germ cells postnatally [Johnson et al., 2004]. The ovarian surface epithelium of adult human females as well as bone marrow MSC was reported to be a source of germ cells [Bukovsky et al., 2005]. However, the possibility that the luteinizing GC cultured in the presence of LIF were germ cells was ruled out by the lack of expression of nanog, vasa and stellar.

3.6 CONCLUSIONS

The high concentration of LIF in the follicular fluid of mature follicles and the presence of LIFR in GC both suggest that our findings physiologically relevant. Multipotent stem cells in ovarian follicles may be involved in the early origin of some forms of ovarian cancer as well as to the origin of ovarian endometriosis, which is considered to arise from undifferentiated, metaplastic cells in the ovary. This hypothesis is supported by the abundant secretion of LIF by endometrial cells [Arici et al., 1995]. Evidence for the presence of stem cells in the ovarian follicle was provided recently by the identification of stem cells from the thecal layer in the neonatal mouse ovary [Honda et al., 2007]. A model of how GC arises from a population of stem cells has been discussed previously [Rodgers et al., 1999]. Here, we demonstrate the presence of multipotent follicular cells, characterized as GC, which survive in the presence of LIF.

3.7 ACKNOWLEDGEMENTS

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4 A THREE-DIMENSIONAL CONSTRUCT MADE OF COLLAGEN TYPE I ALLOWS PROLONGED CULTURE OF HUMAN GRANULOSA CELLS IN VITRO WITHOUT LOSS OF THEIR MAIN CHARACTERISTICS

Katarzyna Kossowska-Tomaszczuk [1,2]

Pawel Pelczar [3]

Sinan Güven [2]

Christian De Geyter [1,2]

Arnaud Scherberich [2]

Woman´s Hospital [1]

Department Biomedicine [2]

University Hospital of Basel, Switzerland.

Institute of Laboratory Animals [3]

University Hospital of Zurich, Switzerland

[4] Whom correspondance should be sent to:

Prof. Christian De Geyter, University Hospital of Basel, Spitalstrasse 12, CH-4031 Basel, Switzerland

4.1 ABSTRACT

The most common cell type in preovulatory ovarian follicles are luteinizing granulosa cells (GCs), which are considered as terminally differentiated undergoing cell death only few days after ovulation. We recently demonstrated that GCs cultured as monolayers remain viable *in vitro* over prolonged time periods when the culture medium is supplemented with the leukaemia inhibiting factor (LIF). However, under those conditions, GCs rapidly lose their main characteristics, such as follicle-stimulating hormone receptor (FSHR) and cytochrome P450 aromatase. Here, we describe a three-dimensional (3D) pellet culture system containing type I collagen, which together with LIF allowed not only the survival and growth of primary human GCs, but supported a significant subpopulation of GCs to maintain their characteristics for prolonged time periods. GCs progressively organized into spherical follicle-like structures with patches of cells expressing the FSHR surrounded by cells producing collagen type IV, reminiscent of the basal membrane. In those structure Call-Exner bodies were observed. The steroidogenic capacity the GCs after prolonged culture in 3D was further demonstrated by the presence of both P450-aromatase and 3β-hydroxysteroid dehydrogenase (3β-HSD). After transplantation into the ovaries of immunoincompetent mice the GCs became concentrated within follicles and the prolonged expression of the FSHR was confirmed. This studies document that it is possible to culture human GC collected from preovulatory follicles over prolonged time periods while retaining their functional characteristics.

4.2 INTRODUCTION

4.2.1 GRANULOSA CELLS IN THE FOLLICLE

Particularly during the final stages of follicular growth, which culminates in the release of a mature oocyte during ovulation, GCs are at the centre of ovarian function. On the one hand, they form a functional syncitium surrounding and nursing the oocyte, on the other they produce the bulk of steroids, which are secreted into blood circulation to orchestrate the various organs of the female body for successful ovulation, the oocyte`s fertilization and subsequent implantation of the embryo. During the final stages of antral follicle growth, the proliferation, differentiation and function of GCs are initially controlled by the follicle-stimulating hormone (FSH) alone, later by both FSH and luteinizing hormone (LH). FSH targets its receptor (FSHR) and induces the maturation of ovarian follicles through proliferation of GCs, induction of the LH-receptor (LHR) and formation of a functional syncitium [Buccione et al., 1990; Dias, 2002; Themmen et al., 2000; Drummond, 2006]. Functional GCs express 3β-hydroxysteroid dehydrogenase (3β-HSD) [Wang et al., 1995; Richards, 1994], an enzyme which plays an important role in synthesis of various steroid hormones, particularly progesterone [Frindik, 2008], whereas cytochrome P450 aromatase is required for the production of estradiol.

4.2.2 PREVIOUS STUDIES

Research focusing on GC function, particularly in the human, has been severely hampered by the impossibility to culture these cells over prolonged time periods due to the spontaneous luteinization of GCs followed by spontaneous cell death within few days [Channing et al., 1975; Channing et al., 1978; Luck, 1990]. We previously

demonstrated that human luteinizing GCs can be maintained *in vitro* over prolonged time periods in culture medium supplemented with LIF and that a subpopulation exhibits multipotent stem cell-like properties [Kossowska-Tomaszczuk et al., 2009]. However, even in the presence of LIF GCs cultured in monolayers gradually lost their characteristics such as aromatase and FSHR. Similarly, during spontaneous luteinization of GCs *in vitro*, the initially globular GCs progressively loose their ability to express aromatase, FSHR but also collagen type IV (Coll IV) [Zhao et al, 1996].

4.2.3 EXTRA-CELLULAR MATRIX

Coll IV-fibers are the principal component of the follicular basal membrane which marks both a structural and functional divide between GCs and internal theca cells [Amsterdam et al., 1975]. Coll IV affects the morphology of the parietal GCs, but also influences metabolic processes and binds several growth factors [McArthur et al., 2000]. It has indeed been demonstrated that the ECM of the ovary is composed of a variety of molecules such as collagens type I and type IV, laminin and fibronectin [Hynes, 1992; Berkholtz et al., 2006; Irving-Rodgers et al., 2006] and that these are involved in a multitude of functional processes, including steroidogenesis and luteinization [Ben-Ze'ev et al., 1986; Yamada et al., 1999; Wang et al., 2000]. Cellular shape is influenced by various components of the ECM and differences in cell shape in turn influence important cellular processes such as metabolism, attachment and migration of cells [Folkman et al., 1978].

4.2.4 THREE-DIMENSIONAL CULTURE

In addition to the specific effects of the various components of the ECM, the latter allows the cells to grow in a 3D-environment, which has been shown to be essential

for sustaining the morphology of ovarian follicles, including cell–cell and cell–matrix interactions [Kreeger et al., 2003; Gomes et al., 1999], thereby promoting follicular growth and cell proliferation. In a 2D culture system, murine follicles fail to maintain their *in vivo*-like architecture [Cortvrindt et al., 1996; Berkholtz et al., 2006; West et al., 2007] and typically fail to grow [Abir et al., 2001]. The short-term beneficial effects of culturing GC in a 3D environment have been demonstrated previously [Carnegie et al., 1988; Wang et al., 2000], but not the long-term effects. As it has now become possible to culture human GC over prolonged time periods in the presence of LIF [Kossowska-Tomaszczuk et al., 2009], we conceived a set of experiments, to demonstrate that a significant subpopulation of human luteinizing GCs collected from mature ovarian follicles are able to maintain their functional characteristics over prolonged time period, when they are cultured in a 3D-matrix made of collagen type I, and that they can become integrated into newly developing follicles after GC transplantation into the ovaries of immuno-incompetent mice.

4.3 Material and Methods

4.3.1 Collection of luteinizing GC

Luteinizing GCs were collected by transvaginal ultrasound-guided aspiration from infertile patients treated with controlled ovarian hyperstimulation for IVF or intracytoplasmic sperm injection (ICSI). For this purpose patients were treated with various gonadotropin preparations including either human menopausal gonadotropins (HMG, Menopur, Ferring, Switzerland; or Merional, IBSA, Switzerland) or recombinant FSH (Gonal F, Merck-Serono, Switzerland, or Puregon, Organon, Switzerland) followed by 10.000 IU of human chorionic gonadotrophin (HCG, Pregnyl, Organon). After removal of the cumulus oophorus-oocyte complexes (COC), the freshly collected follicular aspirates were centrifuged for 5 min at 800 rpm. GCs were separated from other cells by density gradient centrifugation on Ficoll PLUS (Amersham Bioscences, Sweden) for 20 min, 1500 rpm. Cells in the interphase layer, isolated by pipetting, were washed twice with Dulbecco's modified Eagle's (DMEM) culture medium and centrifuged again at 800 rpm for 5 min for final collection of the cells [Zhang et al., 2000]. The purified cells were placed in freezing medium (FCS with 10 % (v/v) dimethylsulphoxide (DMSO) and stored at –80 ºC until culture. All experiments outlined above were presented to and approved by the Ethics Committee of Basel, Switzerland, and patients signed an informed consent.

4.3.2 Cell culture

All the experiments were performed in triplicate with pooled cells from different patients in order to reduce inter-individual differences between single patients. Using DMEM-high glucose (4500 mg/L glucose, Gibco, Switzerland), 15 % (v/v) fetal calf

serum (FCS, Gibco) human GCs were cultured either as monolayers or in 3D. The culture medium was also supplemented with penicillin/streptomycin (50 µg/ml), L-glutamine (3 mmol/l), β-mercaptoethanol (10 mM stock solution in DMEM), recombinant human FSH (100 ng/ml or 3 x 10^{-4} IU/ml, Gonal F; Serono), recombinant human LH (200 ng/ml, Luveris, Serono) and 1000 IU/ml of the leukaemia-inhibing factor (LIF, Chemicon International, USA). For the 3D cell culture, approximately 3.5×10^5 GCs were mixed with 2,5 mg/ml type I collagen of rat origin (BD Biosciences), incubated for 15 min in 37 °C, and later centrifuged for 3 min, at 1200 rpm, to form 3D pellets. Pellets were then cultured in conical microtubes (Sarstedt) set on an orbital shaker.

4.3.3 Clonogenic cultures of GCs and Fluorescence-Activated Cell Sorting

GCs were identified and sorted by the presence of FSHR (Santa-Cruz Biotechnology) [Kossowska-Tomaszczuk et al., 2009] with FACSCalibur (Becton Dickinson). Single FSHR-bearing GCs were placed separately one by one in the wells of a 96-well plate. After one week of culture, small cellular clones were observed using an inverted microscope. At day 17, alkaline phosphates (AP) staining was performed, following the instructions provided by the manufacturer, (AP kit, Sigma-Aldrich).

4.3.4 RT-PCR

Total RNA was extracted from pooled GCs using RNeasy Total RNA kit from Qiagen (Germany). The quantity of RNA was measured by optical density at A260 nm (ND-1000 Spectrophotometer, NanoDrop Technologies, USA). Total RNA (1 µg) was reverse transcribed into single strand cDNA using the cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Primers were synthesized by

Microsynth, Switzerland: FSHR F (forward) 5'TGGGCTGGATTTTGCTTTTG, R (reversed) 5'CCTTGGATGGGTGTTGTGGAC (annealing temperature 55 °C, DNA product size 529 bp); Aromatase F5'CAAGTGGCTGAGGCAT, R5'GAGAATAGTCGGTGAA (55 °C, 429 bp). The cDNA amplification primers for OCT-4, FSHR and the LIF-receptor (LIFR) were designed to span introns to avoid genomic DNA contamination. The β-actin PCR product was used as internal control (Rapid Scan). The single strand cDNA was subjected to 35 cycles of PCR amplification using one of the primers sets. The amplified products were separated on 1 or 2 % agarose gels. The RT-PCR products were analyzed by DNA sequencing (ABI, PE Applied Biosystems, USA).

4.3.5 IMMUNOHISTOCHEMISTRY AND IMMUNOHISTOFLUORESCENCE

To evaluate the characteristics of GCs after prolonged culture, the pellets were harvested for histological examination and gene expression analyses. For histological examination pellets were fixed overnight in 1 % paraformaldehyde at 4 °C, paraffin-embedded and sectioned in 7 μm thick slices. Sections were stained with haematoxylin/eosin (H&E) and observed microscopically. For the demonstration of FSHR, LHR, Coll IV, 3β-HSD and Ki-67 either immunohistochemistry or immunohistofluorescence were performed using antibodies against human FSHR (Santa-Cruz Biotechnology), against human LHR (Santa-Cruz Biotechnology), against human Coll IV (DAKO, Denmark A/S), against 3β-HSD (Santa-Cruz Biotechnology) and against Ki-67 (Abcam), following the instructions provided by each manufacturer. Secondary antibody against FSHR were donkey anti-goat FITC (Santa-Cruz Biotechnology) or rabbit anti-goat biotin-conjugated (DAKO, Denmark A/S) antibodies. For LHR anti-rabbit mouse PE (Santa-Cruz Biotechnology)

secondary antibodies were used and for Coll IV goat anti-mouse biotin-conjugated (DAKO, Denmark A/S) secondary antibodies were used. Staining for immunohistochemistry were followed by incubation with ABC-alkaline phosphatase complex kit (Dako, Glostrup, Denmark), counterstained with hematoxylin and mounted. Some sections were also stained with Alcian Blue to identify Call Exner–like bodies [van Wezel et al., 1999].

4.3.6 IMMUNOASSAY FOR IN VITRO DETERMINATION OF ESTRADIOL AND PROGESTERONE

Secretion of estradiol and progesterone by GCs was measured in the culture supernatants by using the Elecsys Estradiol II and Progesterone II assays (Roche Diagnostics). The experiment was repeated four times. The results were presented either in pmol/L, for real values, for estradiol and progesterone. For calculation purpose the conversion factors were given (Roche Diagnostics):

pmol/L x 0.273 = pg/mL (ng/L) or nmol/L x 0.314 = ng/mL (μg/L)

pg/mL x 3.67 = pmol/L or ng/mL x 3.18 = nmol/L

4.3.7 TRANSPLANTATION OF GCS INTO THE OVARIES OF IMMUNE-INCOMPETENT MICE

The *in vivo* transplantation of cultured cells into the mouse ovary has never been successfully performed before, though some attempts were presented [Honda et al., 2007]. The oocyte nursing functionality of human GCs cultured over prolonged time periods in 3D in the presence of LIF was assessed after transplantation into the ovaries of immuno-incompetent mice (e.g. nude mice). For that purpose GCs were first cultured *in vitro* for three weeks under the 3D conditions described above. The

pellets were then transplanted unilaterally into the right ovary of 8 nude mice (Harlan, NL) in accordance with institutional guidelines. The cells were transplanted under the bursa within the capsula of the mouse ovary. GC transplantation was always performed in the right ovary, the left one remained unoperated. Four to five weeks after implantation, the mice were sacrificed, the constructs harvested, fixed overnight in 1 % paraformaldehyde, paraffin-embedded and sectioned. Sections were then stained with H&E. To distinguish human (donor) from murine (recipient) cells, immunohistofluorescence was carried out with anti-human monoclonal HLA-ABC-biotin-conjugated antibodies (Cedarlane Laboratories Ltd) with avidin-FITC secondary antibodies from Becton Dickinson. Additionally, chromogenic *in situ* hybridization (CISH, Zytovision kit) for the detection of Alu sequences was carried out. The Alu family consists of repetitive elements of DNA, characteristic of primate genomes [Roy-Engel et al., 2001].

4.3.8 ASSESSMENT OF GC'S PHENOTYPE AFTER ECTOPIC TRANSPLANTATION INTO IMMUNE-INCOMPETENT MICE

As no specific anti-human FSHR antibodies were found, that could be used to differentiate human from mouse GCs, the continued presence of the GC phenotype after prolonged cultured in 3D was evaluated through ectopic, extra-ovarian transplantation in immuno-incompetent mice. For that purpose, GCs were first cultured *in vitro* for three weeks under 3D conditions as described above. Pellets containing the GC were then transplanted into the back of nude mice (CD-1 nu/nu, 1-month old; Charles River Laboratories, Wilmington, MA, http://www.criver.com) in accordance with institutional guidelines. Four and eight weeks after implantation, the mice were sacrificed, the constructs harvested and fixed overnight in 1 %

paraformaldehyde, paraffin-embedded and sectioned. Sections were then stained with H&E or with antibodies against FSHR.

4.4 RESULTS

4.4.1 CULTURE IN MONOLAYER

When selected out off a large cohort of GCs based on the FSHR and culture as a single cell in one singe well, GCs were able to undergo clonogenic growth resulting in multicellular colonies (Figure 1A) staining for AP (Figure 1B). When GCs were cultured as monolayers (2D), cells adhered to the bottom of the well, spreaded and adopted a fibroblast-like shape (Figure 1C) while loosing characteristics typical of GCs. Indeed, the expression of the FSHR became undetectable after 17 days in culture in 2D (Figure 1D). However, when GCs were cultured as 3D pellets together with type I collagen, they maintained their round-spherical shape with clumping of cells (Figure 1E) and the FSHR remained expressed (Figure 1D). Cytochrome P450 aromatase was expressed in both culture systems (Figure 1D). The presence of FSHR in GCs cultured in 3D with collagen type I was also demonstrated at the protein level after 21 days, as visualized by immunocytochemistry using an anti-FSHR polyclonal antibody (Figure 2 and 3).

4.4.2 THREE-DIMENSIONAL CULTURE

In the 3D pellets patches of GCs were stained with FSHR, suggestive of the spatial organization of granulosa relative to the basal membrane, similar as in ovarian antral follicles (Figure 2G). In order to further substantiate this, the organized structures were stained with antibodies against Coll IV (Figure 2H), a typical marker of the basal membrane in mature ovarian follicles [Timpl et al., 1986; Rodgers et al. 1999].

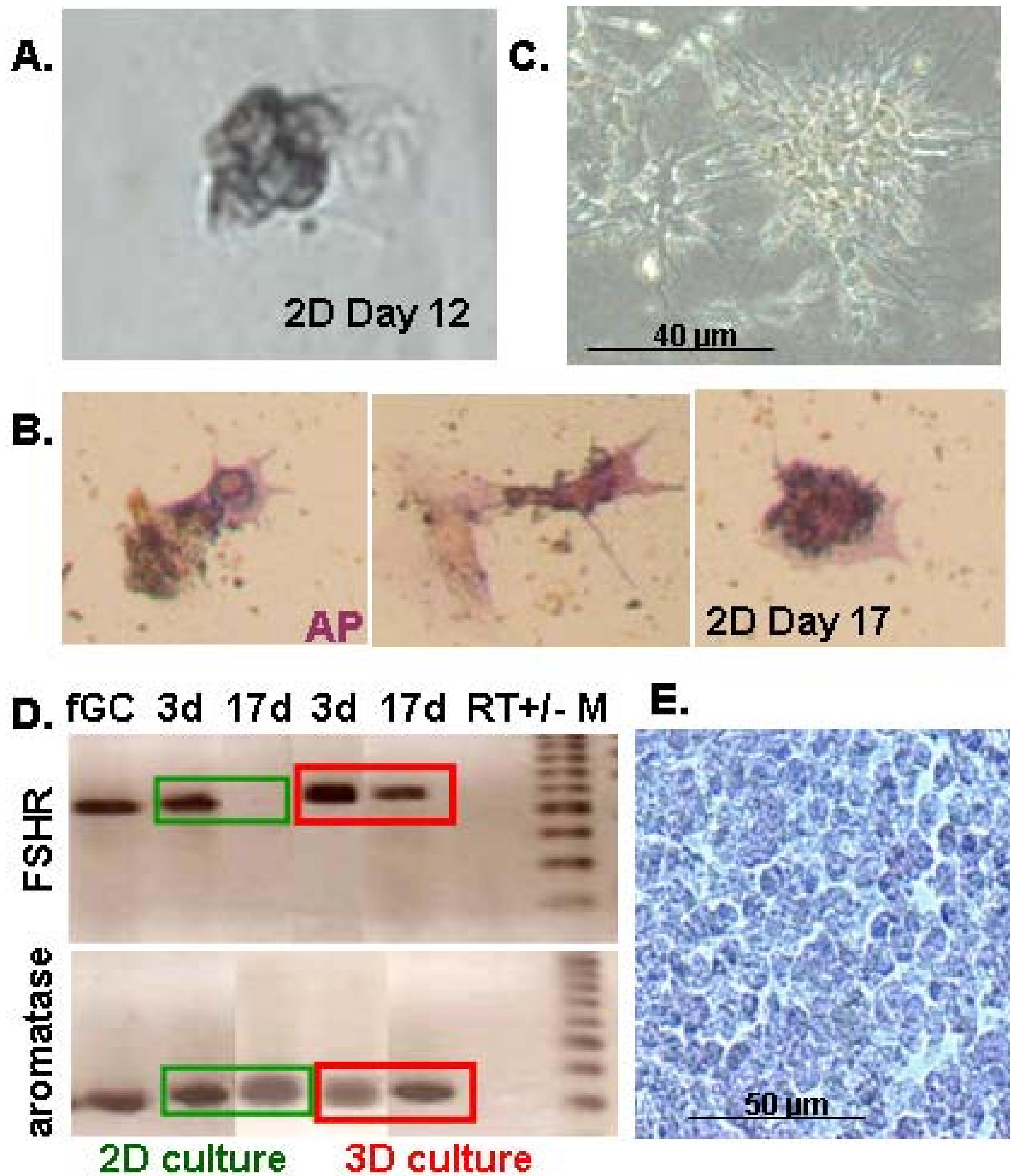


Figure 1. Clonogenic proliferation of GC collected from mature ovarian follicles of infertile women treated with assisted reproductive technology. (A) Clonogenic proliferation of one single GC cultured for 12 days in a single well in LIF supplemented medium. (B) Staining of clones of GCs for AP. (C) Flattened appearance of GCs during prolonged culture in 2D in the presence of collagen type I. (D) RT-PCR of various markers typical for GC function, FSHR and P450-aromatase, in freshly collected GC (fGC), after short (3 days) and prolonged (17 days) culture in

the presence of collagen type I in either 2D or 3D. RT +/- signifies RT-PCR control. M signifies DNA marker. (E) Rounded appearance of GCs cultured in 3D in the presence of collagen type I.

Expression and organization of FSHR-expressing cells and collagen type IV in 3D pellets were analyzed by immunocytochemistry (Figure 2). After two days of culture, Coll IV was expressed by GCs randomly distributed throughout the pellet. At day 7, spherical structures composed of several GCs were observed. At day 21, organized structures of GCs expressing FSHR and containing laminar structures with collagen type IV were visible, some of which of large scale (several hundreds of micrometers), suggestive of the organization of a genuine ovarian follicle [Irving-Rodgers 2005]. Collagen type IV was more expressed in the outer layers of the organized structures, whereas staining for FSHR-positive GCs, was mostly visible in the inner part of the structures (Figure 2G, 2H). The number of GCs patches in the 3D pellets significantly increased with time (Figure 3A, 3B, 3C).

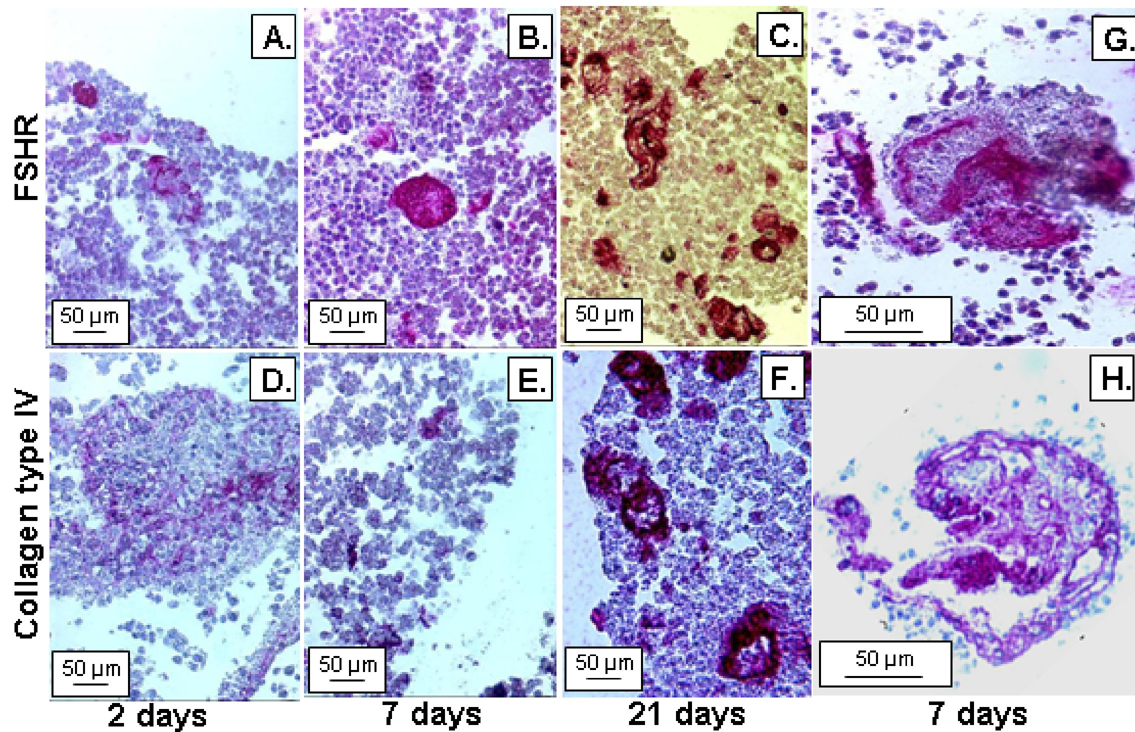


Figure 2. Immunocytochemistry for FSHR (A,B,C) and collagen type IV (D,E,F) in GCs cultured in 3D together with collagen type I during various time intervals. (G,H) Patches of GCs cultured in the presence of collagen type I, as stained with immunocytochemistry for the FSHR (G) and Coll IV (H). Cells with the FSHR are more abundant in the center of each patch, whereas Coll IV is more abundant in cells at the periphery of each patch.

4.4.3 Presence of FSH and LH receptors

Using immunocytofluorescence, we also demonstrated the presence of LHR in patches of GCs growing in 3D (Figure 3). The expression of LHR required concomitant expression of FSHR. We encountered two distinct staining patterns of LHR and FSHR. The first group was negative for LHR but positive for FSHR (Figure 3D, 3E, 3F). The second group was positive for both LHR and FSHR (Figure 3G, 3H and 3I). We were not able to precisely quantify the proportion of cells with the FSHR alone and those with both receptors within the 3D-pellets, because the patches had

different sizes and because it was not possible to distinguish single cells inside the patches with a sufficient degree of accuracy. All negative controls, performed without first antibodies, presented minimal background staining (data not shown).

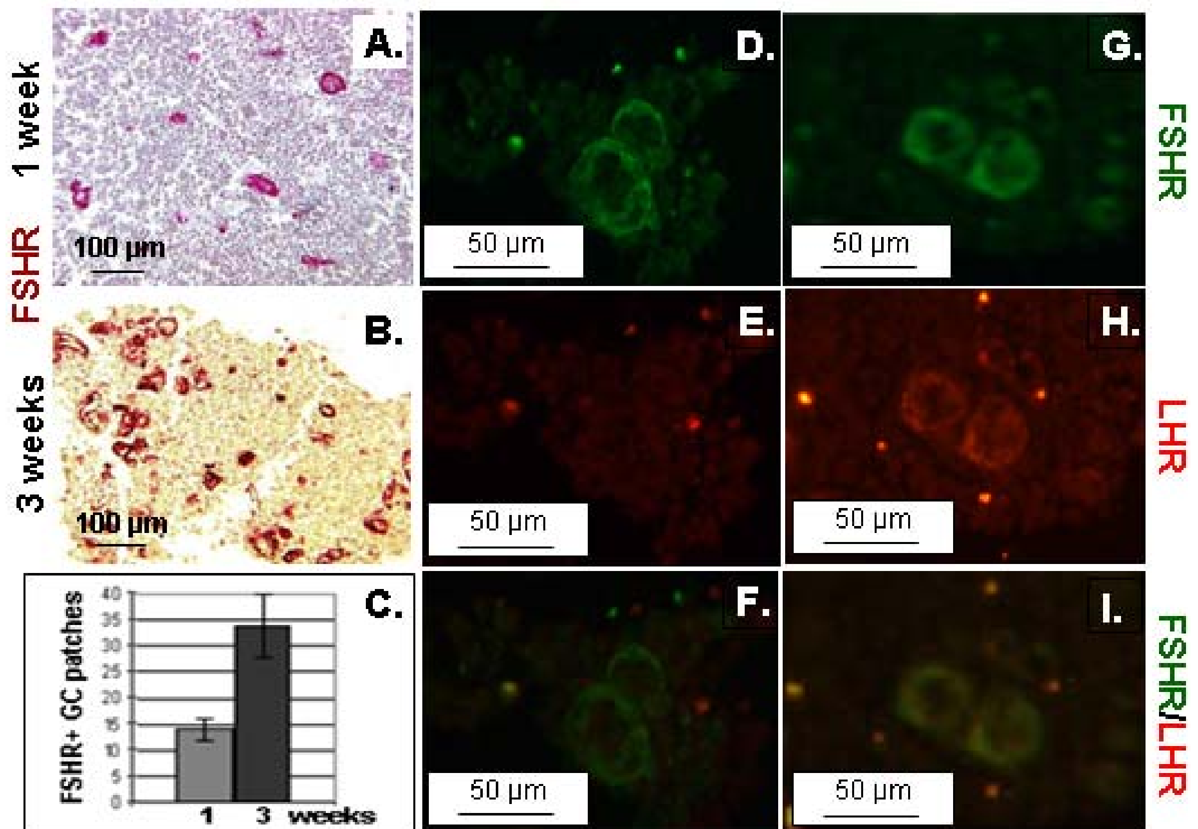


Figure 3. FSHR-immunocytochemistry in GCs cultured in 3D together with collagen type I after 1 week (A) and after 3 weeks (B). (C) The number of patches positive for FSHR was counted after one week (grey column) and after three weeks (black column) in culture. Both FSHR (D,E.F) and LHR (G,H,I) were visualized with immunocytofluorescence in GC after three weeks in 3D culture in the presence of collagen type I.

4.4.4 PROLIFERATION STATUS

Moreover, when observing the 3D pellets at higher magnification, spherical clumps of GCs surrounding a central cavity which recall a rosette-like structure, became visible within the patches stained for FSHR (Figure 4A-E). The connective tissue surrounding these rosettes contained Coll IV, whereas their central cavity was stained by Alcian Blue, exhibiting a staining pattern typical of Call-Exner bodies which are also present in ovarian follicles [van Wezel et al., 1999]. The proliferation status of GC, another marker of GC functionality [Bullwinkel et al., 2006], was verified using Ki-67 staining (Figure 4E). Around half of the cells in the follicle-like structures were positive for Ki-67. Few cells staining for Ki-67 were also found outside the patches of cells staining for FSHR, but most were found in their close vicinity.

4.4.5 ENDOCRINE FUNCTION

The endocrine function, characterized by the steroidogenic capacity of GCs cultured in 3D was also examined. The concentrations of progesterone and estradiol were measured in the supernatant medium after various time intervals (Figure 5A and 5B). These measurements were performed in 4 independent experiments. When compared to GCs cultured in 3D in the presence of collagen type I, significantly lower concentrations of progesterone were measured in the supernatant medium of GCs cultured in 3D together with collagen type ($p<0.01$), whereas the concentrations of estradiol were similar. In addition to aromatase, characteristic of GC function, the presence of another steroidogenic enzyme, namely 3β-HSD, was tested by immunohistochemistry (Figure 5C). Most of the cells positively staining for 3β-HSD were found inside the patches of cells staining for FSHR.

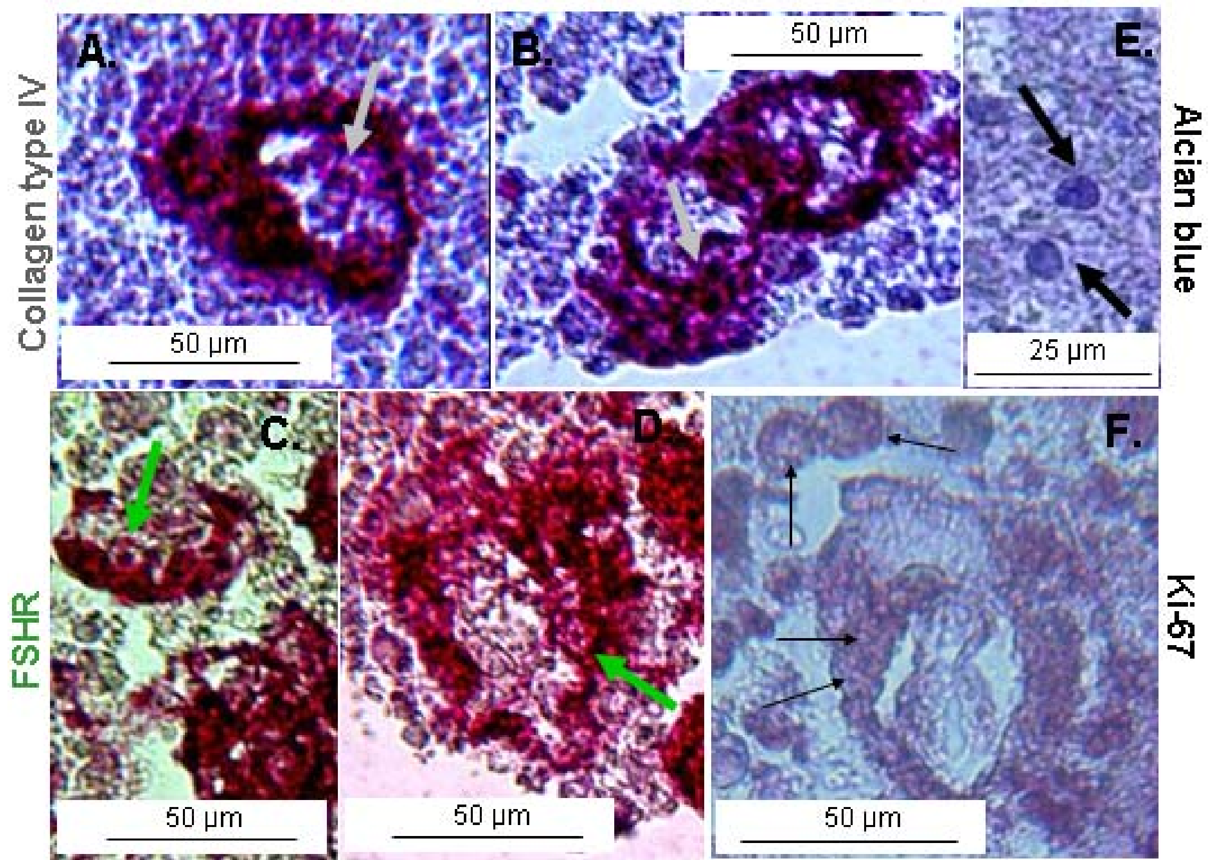
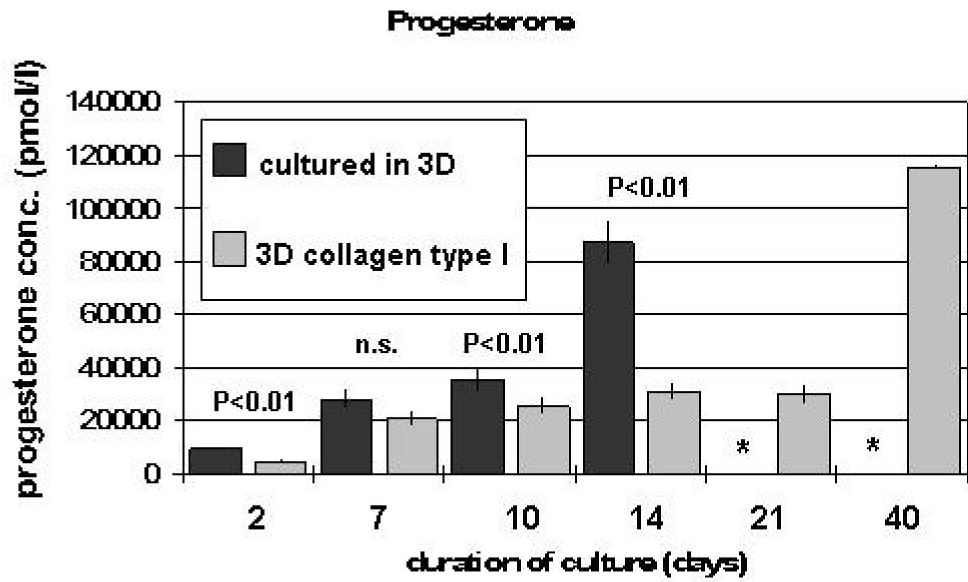
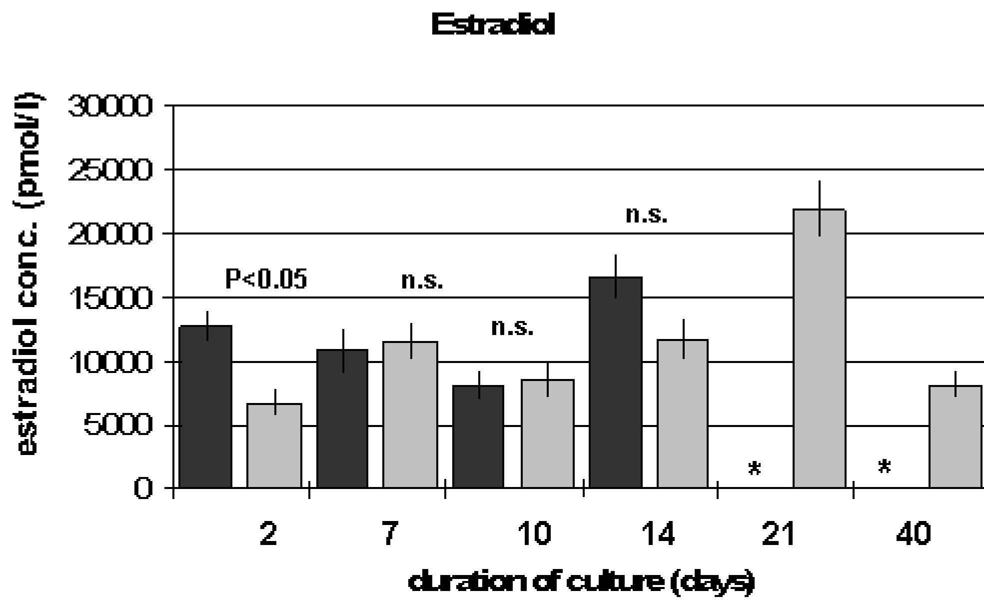


Figure 4. GCs cultured in 3D in the presence of collagen type I progressively form follicle-like structures, which also include rosette-like structures similar to Call-Exner bodies, as stained with Coll IV (A,B, grey arrows), Alcian blue (E, black arrows) and also containing cells with FSHR (C,D, green arrows). (F) Proliferation of GCs cultured in 3D in the presence of collagen type I was demonstrated with Ki-67 stainings. GCs marked with Ki-67 staining are arranged in patches of cells reminiscent of ovarian follicles (F, black arrows).


Progesterone
progesterone conc. (pmol/l)
140000
120000
100000
80000
60000
40000
20000
0
cultured in 3D
3D collagen type I
P<0.01
n.s.
P<0.01
P<0.01
*
*
2
7
10
14
21
40
duration of culture (days)


Estradiol
estradiol conc. (pmol/l)
30000
25000
20000
15000
10000
5000
0
P<0.05
n.s.
n.s.
n.s.
*
*
2
7
10
14
21
40
duration of culture (days)

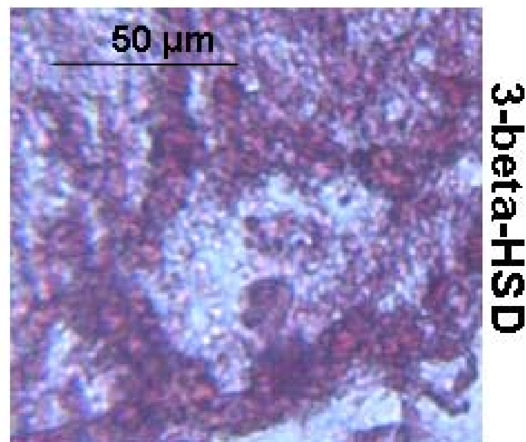

50 µm
3-beta-HSD

Figure 5. The concentrations of estradiol and progesterone were measured in the supernatant medium of GCs cultured in 3D either together with collagen type I or without collagen type I after various time periods in culture. Whereas at all time points the concentrations of estradiol were not significantly different among both groups, the concentrations of progesterone were significantly lower at various time points ($P<0.01$, Mann Whitney-*U* test), indicating a lesser degree of spontaneous luteinization in the presence of collagen type I. The asterisks indicate lacking values, which were caused by the disintegration of the pellets after three weeks in culture in the absence of collagen type I. The presence of the key steroidogenic enzyme 3β-HSD involved in the production of progesterone was demonstrated with immunocytochemistry in patches of GCs forming follicle-like structures.

4.4.6 FOLLICLE GROWTH

Additional proof of the 3D-system's utility and relevance arose from its ability to promote the development of early-stage antral follicles (Figure 6A and B). Previous anecdotal observational studies have demonstrated that follicular aspirates of infertile patients treated with IVF or ICSI may contain primordial follicles, usually not identified during routine workup of the aspirates for oocyte collection [Heng et al., 2005]. Accidentally, three primary ovarian with multiple layers of GCs, each with an enclosed oocyte, were occasionally detected in the 3D-pellets after approximately 14 days of culture (Figure 6C).

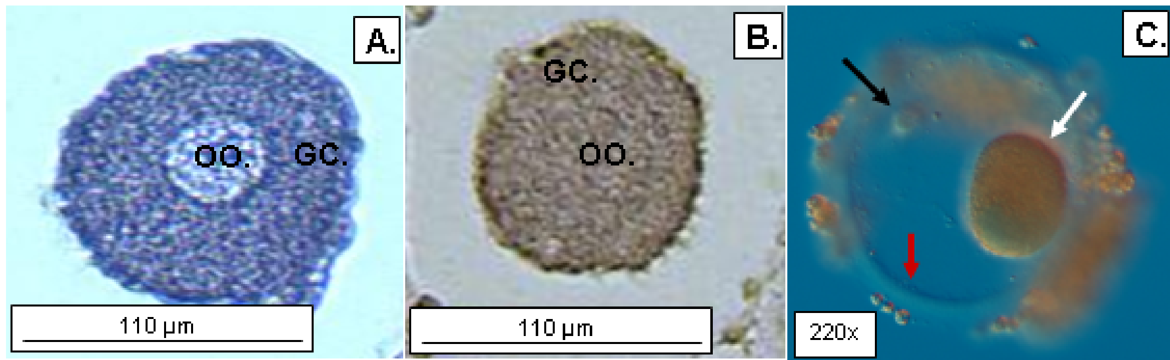


Figure 6. (A and B) Multilayered follicles founded within 3D cultured GC after more than 2 weeks (OO – oocyte; GC – granulosa cells). (C) Post-matured oocyte founded within 3D cultured GC after more than 3 weeks (black arrow – first polar body; red arrow – zone pellucida; white arrow - cytoplasm).

4.4.7 TRANSPLANTATION OF HUMAN GRANULOSA CELLS

In order to further examine their functionality 3D-pellets with human GCs were transplanted in the ovaries of eight immuno-incompetent mice. Four mice were sacrificed 4 weeks after transplantation, whereas another four mice were sacrificed after 8 weeks. In each animal the contralateral, non-operated ovary was used as a control. To evaluate possible differences between operated and non-operated ovaries, H&E staining of whole ovaries was performed (Figure 7A and B). The presence of human cells within ovarian tissue was then established using two specific stainings: CISH for Alu sequences (Figure 7C) and immunohistofluorescence for HLA-ABC (Figure 7D). With both methods the presence of cells of human origin was confirmed almost exclusively within the boundaries of mouse antral follicles.

4.4.8 MAINTENANCE OF FSHR IN VIVO

In order to demonstrate the maintenance of human GC phenotype after *in vivo* transplantation, cultured GC pellets were implanted into the back (ectopically) of

nude mice. After 4 and after 8 weeks of implantation, the FSHR was still found to be present within the explanted tissue (Figure 7E and F), as observed both by immunohistochemistry and immunohistofluorescence.

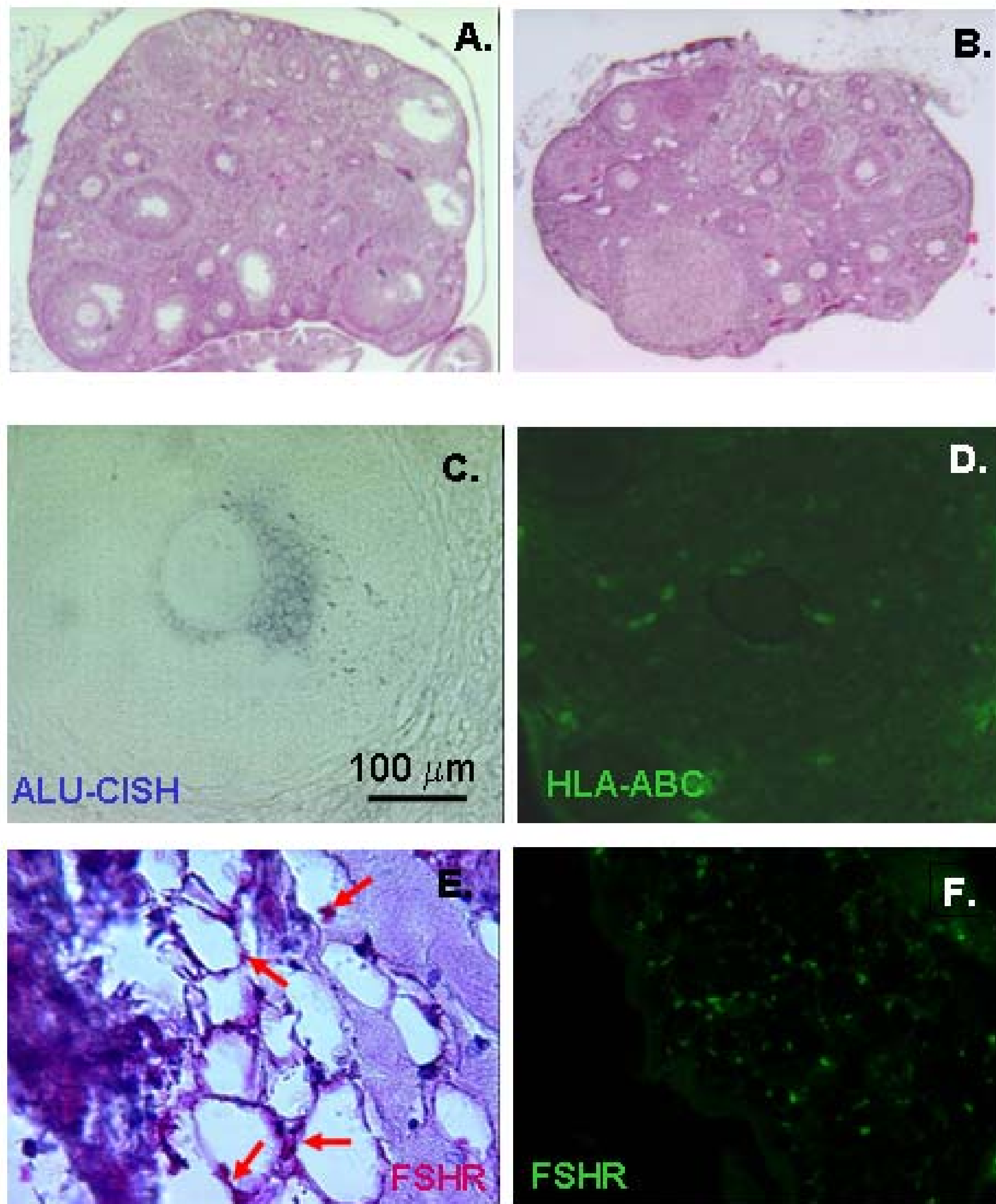


Figure 7. GCs cultured for three weeks in 3D in the presence of collagen type I were transplanted into the right ovaries of immunoincompetent mice. The contralateral ovary was left unoperated for control purposes. The animals were sacrified 4 or 8 weeks after transplantation. (A,B) no differences were found in gross morphology

between the operated and the non-operated ovaries. (C) with alu sequences and (D) with HLA-ABC staining human cells were mostly detected within the boundaries of mouse follicles. After transplantation in the back of immunoincompetent mice FSHR-bearing cells were still present eight weeks later, as visualized with immunocytochemistry (E, red arrows) or with immunocytofluorescence (F, green dots).

4.5 DISCUSSION

4.5.1 PREVIOUS STUDIES

Within few days in culture, rat, ovine and human GCs plated on culture dishes invariably undergo spontaneous luteinization followed by apoptosis within few days [Aharoni et al., 1997; Hwang et al., 2000; Huet et al., 2001]. Previous research in our laboratory has demonstrated that among GCs collected from infertile women treated with assisted reproduction a subpopulation with multipotent stem cells can be identified, which can be cultured over prolonged time periods *in vitro* in the presence of LIF [Kossowska-Tomaszczuk et al., 2009]. In the present communication, these findings are supported by the clonogenic growth of single GCs to 3D colonies, staining positively for AP, when they are cultured in the presence of LIF. However, although in the presence of LIF some GCs could be maintained in culture, they progressively lost all their major characteristics, such as P450-aromatase and FSHR [Kossowska-Tomaszczuk et al., 2009].

4.5.2 THREE-DIMENSIONAL CULTURE

As the beneficial role of 3D-cultures on the endocrine properties of cells was already documented in previous studies [Carnegie et al., 1988; Gomes et al., 1999; Berkholtz et al., 2006; Aten et al., 1995; Vigo et al., 2005], we decided to improve our culture system by incubating GCs in 3D together with LIF instead of monolayers. Alginate hydrogels, a widely used substitute of the ECM in tissue engineering and characterized by optimal biomechanical properties, have been used to promote the development of mouse ovarian follicles *in vitro* and both oocyte maturation and life offspring has already been achieved with this method [Xu et al., 2007; West et al., 2007]. However, alginate hydrogels are manufactured from brown algae and consist

of a polymeric scaffold of polysaccharides, therefore, being unphysiological for human ovarian tissue. An alternative solution was offered by collagen type I, which is a normal constituent of ovarian tissue. We therefore evaluated the effectiveness of a 3D-culture system based on collagen type I in a medium supplemented with LIF on the maintenance of the properties of GCs during prolonged culture. Initially, control GCs were cultured as 3D-pellets without collagen type I. Unfortunately, under those conditions the pellets progressively disaggregated within 1 to 2 weeks. Therefore, GCs cultured as monolayers in the absence of collagen type I were used as controls.

4.5.3 GRANULOSA CELLS PHENOTYPE

The 3D-culture system of GCs coated with collagen type I not only extended cellular survival *in vitro* but also allowed the GCs to maintain many of their morphological and functional characteristics. Aggregated, 3D-cultured GCs retained key features such as FSHR, LHR and P450-aromatase, which otherwise progressively disappeared during culture as monolayers. The demonstration of both FSHR and Coll IV in GCs cultured in 3D over a period of 3 weeks was highly reproducible and suggests that this culture system mimics physiological ovarian follicular development. Similar experiences were made earlier when whole ovarian follicles were cultured *in vitro* [Ben-Ze'ev et al., 1986; Ben-Rafael et al., 1988; Amsterdam et al., 1989; Mauchamp et al., 1998; Richardson et al., 2000; Berkholtz et al., 2006]. In a 3D-culture system with intact murine follicles collagen type I promoted an increase in size of two-layered follicles but had no effect on multilayered follicles [Berkholtz et al., 2006]. Collagen type I and collagen type IV have been shown to stimulate the formation of preantral follicles. In the 3D model presented here, Coll IV was found to surround patches of GCs containing the FSHR. This is in accordance with the observation that

in early antral follicles Coll IV is localized specifically in the basal membrane [Amsterdam et al., 1975], whereas in pre-ovulatory follicles Coll IV is also detected in more central layers of the granulosa [Yamada et al., 1999]. The basal membrane influences GC proliferation and differentiation [Andersen et al., 1976; Amsterdam et al., 1989; van Wezel et al., 1998; Richardson et al., 1992; Luck, 1994] and our observation confirms previous results [Irving-Rodgers et al., 2006] by demonstrating that Coll IV, a major component of the basal membrane and of the ECM, is produced by the GCs themselves.

4.5.4 EXTRA-CELLULAR MATRIX

Collagen fibers are the most abundant protein constituents of the ECM and various subtypes of collagens have been demonstrated in both the animal and human ovary [Hynes, 1992; Irving-Rodgers et al., 2005; Berkholtz et al., 2006; Irving-Rodgers et al., 2006]. There are approximately 28 types, that have been described, but the first four types are the most common:

Collagen type I	the most abundant collagen type, present in scar tissue
Collagen type II	the main component of cartilage
Collagen type III	component of early granulation, produced by fibroblasts
Collagen type IV	main component of the basal membrane.

Collagens have been demonstrated to be in various compartments of the ovary of many species, such as the rat and the human. Whereas collagen type IV has been shown to be present in the basal membrane, separating the theca interna and the

granulosa, collagen type I and type III are present in the theca externa and type I in the granulosa cell layer [Lind et al., 2006]. Together with laminin, another important component of the basal membrane, collagen interacts with its neighbouring granulosa cells via integrins expressed on the membrane of GCs [Le Bellego et al., 2002; West et al., 2007].

4.5.5 PRESENCE OF FSH AND LH RECEPTORS

During follicular development *in vivo*, the FSHR density rises progressively until a few days before ovulation, whereas the LHR becomes expressed only towards the final stages of follicular development. Therefore, expression of LHR is thought to be a hallmark of the later stages of GC differentiation [O'Shaughnessy et al., 1997]. We demonstrated both at the mRNA level and at the protein level the presence of the LHR in a subset of GCs cultured in 3D but not in 2D.

4.5.6 PROLIFERATION STATUS

The granulosa of ovarian preantral and antral follicles in many species including the human contains Call–Exner bodies. Call-Exner bodies consist of round globular sets of granulosa cells (called rosette), containing chains of Coll IV [Rodgers et al., 1998], surrounding a small cavity with a fluid, reminiscent of follicular fluid. Call-Exner bodies are found only in healthy follicles [Rodgers et al., 1999; Van Wezel et al., 1999] and their presence seems to correlate to GC's proliferation and differentiation [Assoian, 1997; Correia et al., 1998; Lee et al., 1996]. Call-Exner bodies can be demonstrated in follicular sections either with Alcian Blue staining or with specific antibodies against collagen IV [van Wezel et al., 1999]. Using these two methods structures similar to Call-Exner bodies were observed within patches of GCs, cultured in 3D but not in 2D. The presence of Call-Exner bodies in ovarian follicles has been

used as an index of granulosa cell proliferation [Miller et al., 1997; Gomes et al., 1999]. Using Ki-67 [Bullwinkel et al., 2006; Schonk et al., 1989] we were able to demonstrate that cell proliferation occurs in 3D-cultured GCs [Scholzen et al., 2000]. Interestingly, during prolonged culture most proliferating cells progressively became organized in structures reminiscent of ovarian follicles with cells staining for the FSHR and the LHR and surrounded by Coll IV [Rodgers et al., 1999; Rodgers et al., 2001; Vigo et al., 2005; Gomez et al., 1999].

4.5.7 ENDOCRINE FUNCTION

Steroidogenesis was then evaluated as another important feature of GCs. In all experiments both recombinant FSH and LH were supplemented to the culture medium, in order to maintain and stimulate steroidogenesis. Through the presence of the cytochrome P450-aromatase, intact GCs are able to produce significant amounts of estradiol [Okamura et al., 2003], which, among other characteristics, reflects their functional health [Rodgers et al., 2001]. The concentrations of estradiol were similar in the supernatant medium of GCs cultured in 3D in the presence of collagen type I as compared to GCs cultured as pellets without collagen type I (Figure 5). In contrast, the concentration of progesterone was significantly lower in the supernatant of GCs cultured in 3D together with collagen type I when compared to GCs cultured in pellets without collagen type I ($p<0.01$), suggesting that spontaneous luteinization is less pronounced in the presence of collagen type I. In addition to the secretion of progesterone and estradiol and to the P450-aromatase expression, the presence of the 3β-HSD, a key enzyme in steroidogenesis, involved primarily in the synthesis of progesterone, was observed in 3D-grown GCs as well, [Frindik, 2008; Fanjul et

al.,1984] and only functional GC are able to express this enzyme. [Wang et al., 1995; Richards, 1994].

4.5.8 FOLLICLE GROWTH

Follicular aspirates are potentially an abundant source of immature ovarian follicles [Wu et al., 1998]. Attempts to culture primordial follicles in vitro, to increase the yield of viable mature oocytes for fertility treatments have not yet met with success [Heng et al., 2005]. Taking all these findings together, we suggest that this newly described 3D culture system based on collagen type I and LIF display a development which is reminiscent to surrogate follicle-like structures. It remains to be demonstrated, whether these can be further developed to sustain the growth and the maturation of oocytes.

4.5.9 GRANULOSA CELLS FUNCTIONALITY

In order to further establish the robustness of functionality of GCs after prolonged 3D-culture in the presence of collagen type I, human GCs were transplanted into the ovaries of immunoincompetent mice. The human GCs were differentiated from the surrounding mouse ovarian tissue both with HLA-ABC-immunostaining and with primate-specific Alu sequences [Roy-Engel et al., 2001]. Human GCs were found to be located predominantly within the boundaries of mouse follicles and to a much lesser extent between individual mouse follicles [Hatano et al., 1999]. The human cells were grouped in patches suggestive of localized proliferation. Transplanted cells migrated towards the center of follicles and were also found in close proximity of mouse oocytes, where they retained the typical epithelial morphology of GCs surrounding an oocyte. As no specific anti-human FSHR-antibody was available to differentiate human FSHR from mouse FSHR, pellets with 3D-cultured GCs were

also transplanted ectopically into the back of immunoincompetent mice. With this approach we additionally demonstrated the continuing expression of FSHR in GCs coated with collagen type I up to 8 weeks after their transplantation into this environment.

4.6 CONCLUSIONS

Taking all these findings together, we have demonstrated that subpopulations of GCs collected from mature ovarian follicles can be cultured over prolonged time periods in a 3D-system consisting of collagen type I and that under those conditions these cells are able to proliferate into organized patches of cells, reminiscent of ovarian follicles. In female mammals, physiological production of oocytes with high developmental competence crucially relies upon the controlled growth and differentiation of the GCs in the surrounding ovarian follicle. For decades, the inability to culture these GCs over prolonged time periods has greatly contributed to the current lack of knowledge about their functionality. It is now possible to culture human GCs, otherwise thought to enter apoptosis within days after their removal from the follicle, over prolonged time periods thereby retaining many aspects of their function. Although maintenance of human oocytes in the presence of 3D-cultured GCs has not yet been performed, the present data provide the basic system which may now be further developed for the maturation in vitro of oocytes both in human and animal reproductive medicine. The possibility for long-term culture of subpopulations of GCs is likely to have considerable impact on various aspects of research into ovarian physiology, high throughput screening of new medications targeting ovarian function and ovarian endometriosis.

4.7 ACKNOWLEDGEMENTS

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5 Discussion and Conclusions

5.1 Somatic Stem Cells in the Ovary

In contrast to the ongoing controversy with regard to the possibility of ongoing renewal of oogenesis in the ovary and the possible existence of adult germ stem cells, the existence of somatic stem cells in the ovary has not been hypothesized for a long time.

Granulosa cells (GCs) cultured *in vitro* invariably cease to proliferate already after two to three passages. Similar results have been obtained when culturing thecal cells. Recently, it has been shown that the four growth factors, bFGF, EGF, LIF, and IGF1 exhibited significant enhancing effects on colony growth of thecal cells, leading to the detection of stem cells in the thecal layer of the ovary [Honda et al., 2007].

In vivo, the follicular basal membrane leads to the entrapment in the follicular fluid containing large molecules (e.g., growth factors) synthesized both by granulosa cells and oocytes. The molecular mass cut-off of the follicular barrier is calculated to be 100 to 500 kd [Shalgi et al., 1973]. The basal membrane may also restrict the diffusion of growth factors into the blood circulation. This isolating property of the basal membrane surrounding the follicle allows it, in part, to determine the micro-environment, in which granulosa cells and the oocyte may thrive [Irving-Rodgers et al., 2006].

Growth factors - such as LIF - may be one of those factors influencing estradiol biosynthesis in the ovarian follicle. It has been demonstrated previously that LIF is involved in ovulation, as the concentration of LIF in the follicular fluid has been

shown to rise steadily after the LH surge or after administration of HCG [Arici et al., 1995; Coskun et al., 1998].

Functional properties of stem cells in general include pluripotency, mitosis without differentiation, when confined to their niche, and by their ability to proliferate when isolated out off their niche [Potten et al., 1990]. The extracellular matrix is crucial for maintaining the pluripotency of stem cells through contact inhibition. Lack of contact inhibition occurs *in vivo* during growth of a follicle [Rodgers et al., 1995a] particularly during the preantral stage of follicular development, when granulosa cells are in close physical contact to the extracellular matrix. [Rodgers et al., 2001].

The hypothesis for the presence of stem cells in the granulosa was first postulated, when it was demonstrated that the granulosa possesses some marked similarities to other epithelia in the body [Rodgers et al., 1999; van Wezel et al., 1998]. The granulosa of ovarian follicles resides on a basal membrane and the morphology of the cells highly differs in various regions of the granulosa. These authors concluded that there must be populations among the granulose cells containing less differentiated cells and, at a distance from these, populations with highly differentiated cells (Figure 1).

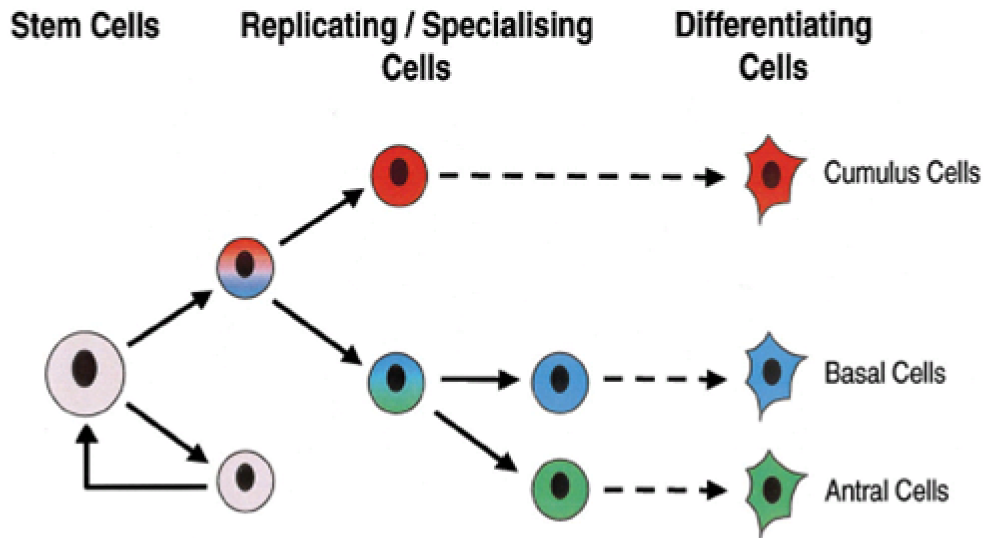

Figure. 1. A model of replication and differentiation of the granulosa and cumulus cells that occurs during follicular development. The model encompasses stem cells, transit amplifying cells (replicating:specializing), and differentiating cells. It thus allows for different factors, such as matrix, cellular contacts or growth factors, to act separately on each lineage and at each stage of development [Rodgers et al., 1999]

Additional support for the presence of cells with stem cell characteristics in the granulosa was provided by nuclear transfer from somatic cells to oocytes in cloning, particularly in cattle [Wells et al., 1999]. It is known that embryonic development via nuclear transfer is more successful, when the donor nucleus is taken from a less differentiated cell, i.e. granulosa. The high success of cloning using the nuclei of granulosa cells in cattle may well be due to the stem cell character of some of the granulosa cells [Rodgers et al., 2001].

Additional evidence for the existence of stem cells in granulosa comes from their ability to express telomerase. Telomerase activity is inactive in normal somatic cells [Chiu et al., 1997] and the length of the telomere-repeat sequence decreases during every cell division. In contrast, telomerase activity is more expressed in tissues with

high numbers of stem cells [Kim et al., 1994; Wright et al., 1996]. In the ovary, the telomerase-RNA was found in the granulosa of growing follicles, but not in primordial follicles [Rodgers et al., 2001].

Another recent study in the *Drosophila* model [Kai et al., 2004] provided convincing evidence that somatic progenitors (nurse cells) of germ-line stem cells had the ability to revert back to the stem-cell stage. This introduces the possibility that within the mammalian ovarian follicle, similar somatic progenies of germ-line stem cells may also possess a greater intrinsic ability to revert back into functional stem cells. If this is the case, then a favoured candidate would be the cumulus/granulosa of ovarian follicles, since such cells are the homologues of nurse cells found within *Drosophila* ovary [Heng et al., 2005]. Hochedlinger and coworkers postulate that the epigenetic changes that direct terminal differentiation and permanent exit from the cell cycle are reversible [Hochedlinger et al., 2006].

The present thesis provides first line evidence for the presence of stem cells in the granulosa, as we observed that cells isolated from mature human ovarian follicles expressed the stem cell marker Oct-4. POU5F1 (Oct-4) is known to be expressed in a few cells found in the basal layer of human skin epidermis [Tai et al., 2005]. As GCs represent one of the most dynamic epithelia in the body, this could be as well true for GCs and explain its stable expression of Oct-4.

As mentioned, most adult tissues contain a heterogeneous population of cells with a hierarchy of multipotent stem cells, progenitor cells and terminally differentiated cells. [Hochedlinger et al., 2006]. We hypothesized that within preovulatory follicle there are several subpopulations of GCs with distinct characteristics. GCs expressing

both FSHR and LHR, which we denominated luteinized GCs, will enter apoptosis during prolonged culture *in vitro*. GCs, positive for FSHR but not for LHR, may either become dedifferentiated to the progenitor GCs or differentiate to luteinized GCs (Figure 2).

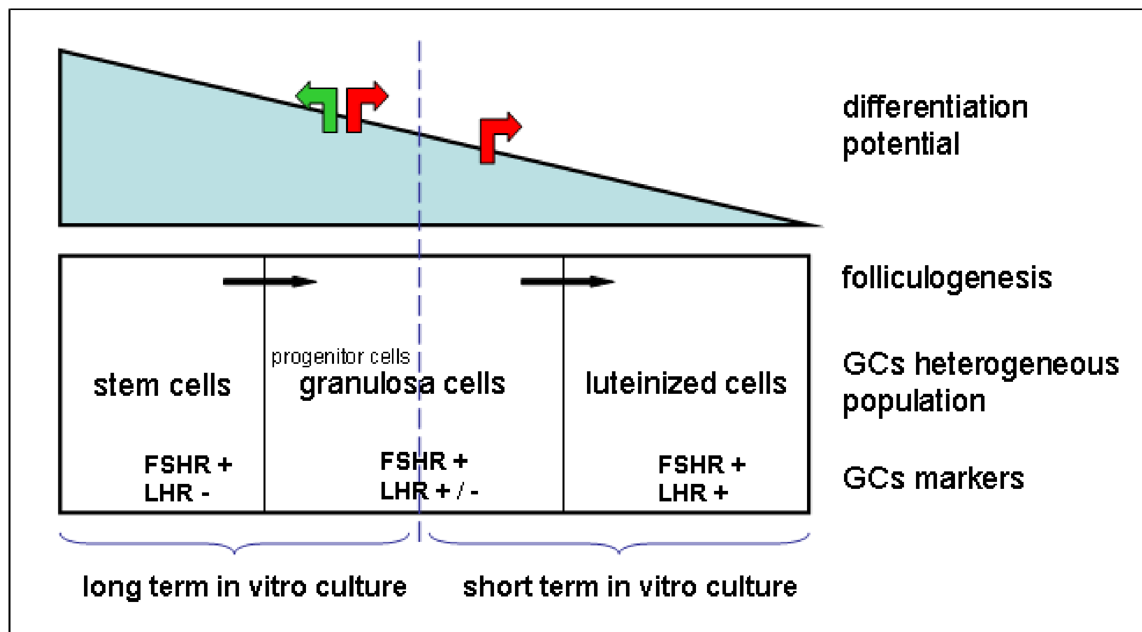

Figure 2. Hypothesis presenting several subpopulations of GCs within preovulatory follicle. GCs stem cells: positive only for FSHR. Progenitor GCs: positive for both FSHR and moderately for LHR markers. Luteinized GCs: positive for FSHR and LHR markers.

The study by Van Deerling and coworkers suggests that the cohort of granulosa cells in a human preovulatory follicle is derived from a clonal expansion of a small number (3 cells) of ovarian stem cells [Van Deerlin et al., 1997].

The presence of a subpopulation of GCs with multipotent stem cell characteristics explains why GCs, taken from preovulatory follicles and cultured under the appropriate conditions, can survive over prolonged time periods, and can be differentiated into other tissue types, otherwise not present in the ovary. In addition, when cultured in 3D in an extracellular matrix similar to the ovary, the GCs seem to retain most of their characteristics including the FSHR and steroidogenesis.

The unexpected finding of cells displaying multipotency and having a prolonged lifespan, extracted from mature ovarian follicles, is likely to have a significant impact on evolving theories in ovarian physiology, particularly with reference to the pathogenesis of ovarian endometriosis and ovarian cancer. Multipotent stem cells in ovarian follicles may be involved in the early origin of some forms of ovarian cancer as well as to the origin of ovarian endometriosis, which is considered to arise from undifferentiated, hitherto labeled as metaplastic cells in the ovary (Figure 3).

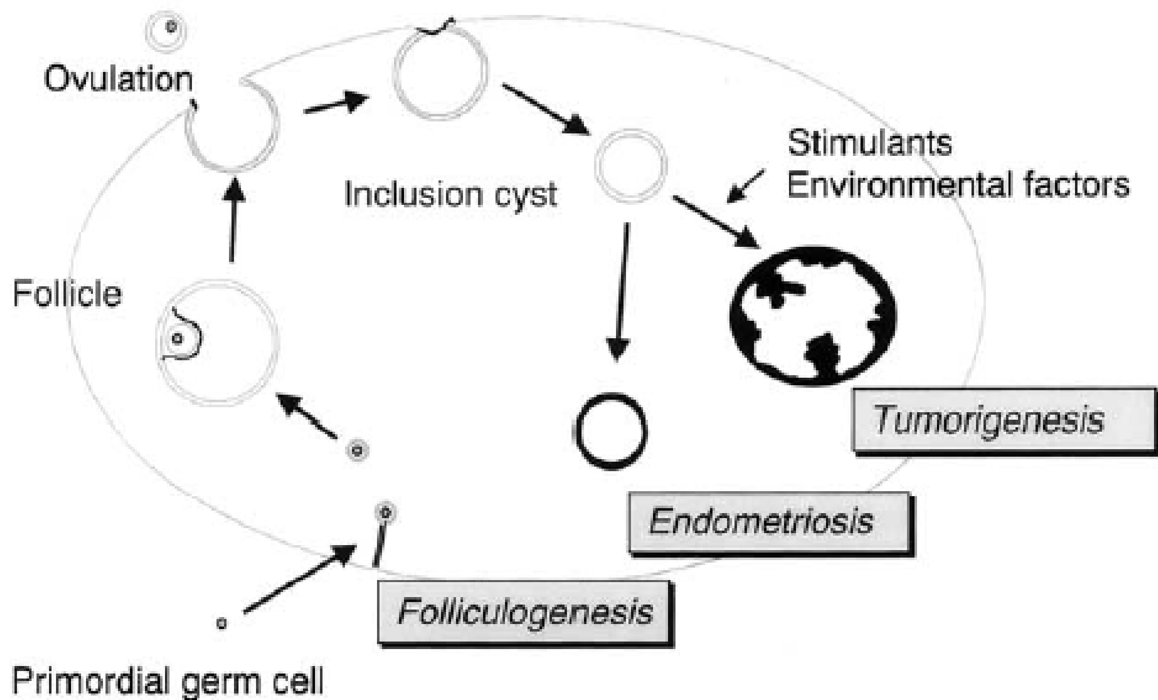

Figure 3. Discovery of the multipotent granulosa stem cells strengthen evolving theories in ovarian physiology [Okumara et al., 2003].

In female mammals, the normal and physiological production of good quality gametes relies upon the highly controlled growth and differentiation of the surrounding ovarian follicle. GC proliferation is maintained throughout folliculogenesis, providing not only a specialized micro-environment but also nutrients for oocytes growth. *In vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes is now an intriguing challenge in human and veterinary reproductive biotechnology. The discovery of GC multipotency and the use of granulosa stem cells in the newly-developed 3D *in vitro* culture system presented here may provide a promising technical tool for IVM but also for drug targeting purposes, as it provides an environment in which GCs preserve their functional properties. The reorganization of 3D-cultured granulosa stem cells into *in vivo* follicles could suggest as well the possibility of treatments against infertility by transplantation of 3D-expanded GCs which might be able to help to

construct healthy follicles. Such GCs transplantation into the ovary could as well restore the proper ovarian endocrine function in women suffering of premature ovarian failure or in women afflicting by a malignant disease, who can only be treated with chemotherapy leading to premature menopause.

The outlook for the studies would be the co-culture of granulosa stem cells with oocytes. Those important studies would provide not only the information about oocyte nursing function, biology function, of GC in the newly-developed 3D *in vitro* culture system. But also open the opportunities for other new research as the biology and production of follicular fluid.

5.2 GERM-LINE STEM CELLS IN THE OVARY

For decades, scientists have thought that female mammals are born with a lifetime supply of oocytes in the ovary, irreversibly destined to decline after birth. However, in recent years a significant controversy with regard to the potential replenishing effects of cells from the bone marrow and blood on ovarian follicular renewal has been stirred up. Although these claims have been met with harsh skepticism, if they prove to be true, the current understanding of the female reproductive system must be revisited. Although these observations and allusions have been limited to the mouse system only, they have opened new discussions about the potential consequences of bone marrow transplantation and even blood donation to the replenishment of the female genital system in general. Still, these findings have not been replicated in other research laboratories so far and the proof that oogenesis can be renewed after birth from cells originating in the bone marrow is still lacking.

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Katarzyna Tomaszczuk-Kossowska

Date of Birth: 17th January 1979
Place of Birth: Gdansk
Nationality: Polish
E-mail: kct@nardis.co.uk

Professional experience

07.2007 – present Affable GS GmbH, Switzerland
Independent Consultant, Medical and Biotechnology Project Leader

02.2005 – 06.2007 University Woman's Hospital Basel, Switzerland. Project leader.
Shared clinical and scientific position.

1. Embryologist and Geneticist. Reproduction Medicine.
2. Identification of multipotency among luteinizing granulosa cells cultured over prolonged time periods.
3. The new 3D culturing system for luteinizing granulosa cells. Remaining of granulosa cells characteristics after prolonged cell culture.
4. Transcriptional response of ovarian granulosa cells to follicle-stimulating hormone in normal woman and in woman with incipient ovarian failure with use of FACS and Affymetrix gene chip mRNA expression arrays. Winning Swiss National Fund .

10.2005 - 10.2006 Oxford University, The Wellcome Trust Centre for Human Genetics, United Kingdom and University Hospital for Children Basel, Switzerland

Preimplantation genetic diagnostics of laser-assisted polar body biopsy. Fluorescence in situ hybridization on polar bodies with the use of probes for detection of chromosomal translocations.

01.2004 – 01.2005 Research position at the University of Zurich, Switzerland

Generation of mammalian embryonic stem cells with the use of molecular genetic tools for RNA and DNA, bioinformatics, transgenic techniques for embryonic stem cells and assisted reproduction techniques

10.1998 - 07.2003 M.Sc. Biotechnology; University of Gdansk and Medical University of Gdansk Intercollegiate Faculty of Biotechnology, Department of Molecular Virology

Overproduction of Histidine-tagged Vhs Protein of Bovine Herpesvirus 1 using BAC-TO-BAC Baculoviruses Expression System. Protein analysis, native and SDS PAGE with immunological methods and bioinformatics. Column chromatography and obtaining polyclonal serum

10.2000 - 05.2001 Copernicus Hospital in Gdansk, Intern at the Department of Bacteriology and the Blood Bank

10.1999 - 05.2001 Medical University of Gdansk; Intern at the Department of Pediatric Surgery

Skin immunology and skin transplantation after burnings

Education

02.2005 – 04.2009 Ph.D. studies. University Woman's Hospital Basel, Switzerland, Gynaecological Endocrinology and Reproduction Medicine

02.2005 – 06.2007	Medical Reproduction Specialisation. University Woman's Hospital Basel, Switzerland
02.2003 - 12.2003	German Language School, Federation of German Minority in Gdansk, Poland
10.1998 - 07.2003	M.Sc. Biotechnology. University of Gdansk and Medical University of Gdansk, Poland, Intercollegiate Faculty of Biotechnology
01.2002 - 06.2002	Warsaw Agricultural University, Biotechnology of Gametes and Embryos and *In vitro* Fertilization
07.2001	Summer School of Biotechnology in Laczyno (Poland) Courses: Gene Therapy, Botany, Legislative and Social Aspects of Biotechnology

Publications and Academic Achievements

2009	PNAS (in progress). A three-dimensional construct made of Collagen type I allows prolonged culture of human granulosa cells *in vitro* without loss of their characteristics. Kossowska-Tomaszczuk K, Pelczar P, Guven S, De Geyter C, Scherberich A.
2009	Stem Cells. The multipotency of luteinizing granulosa cells collected from mature ovarian follicles. Kossowska-Tomaszczuk K, De Geyter C, De Geyter M, Martin I, Holzgreve W, Scherberich A, Zhang H. 2009; 27:210-219.
2008	2nd Prize in the 5th Women's Health Congress 2008 (SGRM)
2008	US, European and Australian patent filing in progress. Procedure for the culture of functional human primary ovarian granulosa cells in tridimensional systems. Kossowska-Tomaszczuk K, De Geyter C and Scherberich A.
2007	Molecular Human Reproduction. FSH stimulates the expression of the ADAMTS-16 protease in mature human ovarian follicles. Gao S, De Geyter C, Kossowska K, Zhang H. 2007; 13(7):465-471.
2002	President Award - Scholarship for academic achievements
2002, 2003	University of Gdansk Rector's Award for academic achievements
2001, 2002, 2003	Intercollegiate Faculty of Biotechnology Scholarship for academic achievements

Certificates

2005, 2006	Medical Reproduction Biology Workshop, Bern, Switzerland
2004	Targeted mutagenesis of the mouse, LTK Module 9, University of Zurich
2004	Introductory Course in Laboratory Animal Science, LTK Module 1, University of Zurich

Foreign Languages

Polish	Native language fluency
English	Fluent knowledge
German	Very good knowledge
French	Basic knowledge

Personal Interests/Hobbies

Sports (dancing, sailing, skiing, tennis), traveling, choreography, interior decorations, guitar playing