Reproductive System and Sexual Disorders: Current Research

Varras , Reprod Sys Sexual Disorders 2012, 1:4
DOI: 10.4172/2161-038X.1000e106

Editorial Open Access

Clinical Significance of Expression of Stem Cell Markers in Human Ovarian Luteinized Granulosa Cells during Assisted Reproduction Technologies

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Abstract

The adult human ovary is composed of various cell types. Preovulatory follicles contain two distinct types of granulosa cells that arise during folliculogenesis as the cell populations segregate upon formation of the fluid-filled follicular antrum. The granulosa cells line in the follicle wall, reside very close to the basement membrane and are essential for estrogen production and follicular rupture. The cumulus cells are closely connected to the oocyte through a gap junction network and are associated with the oocyte development. Paracrine interactions between ovarian somatic cells and germ cells are critical for normal follicular development and oocytes also promote granulosa cell proliferation and differentiation. There are strong evidences that a subpopulation of granulosa ovarian cells have pluripotent and self-renewal capabilities. The presence of stem cell markers in the ovarian luteinized granulosa cells of women undergoing assisted reproduction technologies is an important subject for studies in terms of their possible regenerative role and theirs possible prognostic significance for the infertile citizens. Expression of the stem cell marker Oct-4 in human ovarian luteinized granulosa cells from women undergoing IVF or ICSI indicates the presence of stem cells in these cells. The absence of DAZL gene expression in these cells indicates that the stem cells found in granulosa cells cannot be differentiated in germ cells. Also, a clinical significance for the number of oocytes retrieved and the expression levels of Oct-4 in human luteinized ovarian granulosa cells is possible to exist. More specifically, the expression of Oct-4 mRNA in granulosa cells appears to play an important role in the regulation of follicular growth during assisted reproduction technologies.

Keywords: Oct-4; Stem cells; Markers; Ovary; Granulosa cells

Abbreviations: ART: Assisted Reproduction Technology; OCT-4: Octamer-Binding Transcription Factor 4; DAZL: Deleted In Azoospermia-Like; IVF: *In vitro* Fertilization; ICSI: Intracytoplasmic Sperm Injection; GDF-9: Growth Differentiation Factor 9; BMP-15: Bone Morphogenetic Protein 15; FSH: Follicle-Stimulating Hormone; ESCs: Embryonic Stem Cells; VSELs: Very Small Embryonic-Like Stem Cells; SSEA: Stage Specific Embryonic Antigen; RRM: RNA Recognition Motif; LIF: Leukemia-Inhibiting Factor; PCR: Polymerase Chain Reaction; CMOCs: Cumulus–Mature Oocyte Complexes; DOR: Diminished Ovarian Reserve; ET: Embryo Transfer; LH: Luteinizing Hormone; IGF1: Insulin Growth Factor; IGF2: Insulin Growth Factor 2

The adult human ovary is composed of various cell types. Preovulatory follicles contain two distinct types of granulosa cells that arise during folliculogenesis as the cell populations segregate upon formation of the fluid-filled follicular antrum. The granulosa cells line the follicle wall, reside very close to the basement membrane and are essential for estrogen production and follicular rupture. The cumulus cells are closely connected to the oocyte through a gap junction network and are associated with the oocyte development [1,2]. Paracrine interactions between ovarian somatic cells and germ cells are critical for normal follicular development and oocytes also promote granulosa cell proliferation and differentiation [3,4]. It has been found that mouse follicle lacking the granulosa cell oocyte junction protein Connexin37 have defects in meiotic maturation [5]. In rodents, oocyte-secreted GDF-9 and BMP-15 promote proliferation of granulosa cells from small antral follicles and BMP-15 inhibits FSH-stimulated progesterone production [6]. Evidence also indicates a close association between the expression of LOX in mural granulosa cells and the developmental competence of oocytes [4].

Octamer-Binding Transcription Factor-4 (OCT-4, also known as POU5F1) is a member of Pit-Oct-Unc (POU) transcription factor family and is known to play a precise role in the maintenance of self-renewal and pluripotency in Embryonic Stem Cells (ESCs) [7-9]. OCT4 is expressed in unfertilized oocytes, the Inner Cell Mass (ICM) of the blastocyst, embryonic stem (ES) cells and germ-cell tumors [9]. Oct-4 works with other transcription factors (e.g. FOXD3, SOX2, STAT3) in a cooperative fashion to regulate many genes via the consensus motif ATGCAAAT [10-13]. Oct-4 gene contains the Proximal Enhancer (PE) and the distal enhancer (DE) that are important for Oct-4 cell type-specific expression, and the four conserved domains CR1-CR4, that are important for Oct-4 basal expression [14]. Oct-4 expression is restricted to pluripotent cells, while the loss of OCT-4 expression may be associated with loss of pluripotentiality [12]. OCT-4 is involved in the self-renewal of undifferentiated embryonic stem cells and is therefore used as a marker of Embryonic Stem Cells (ESCs) [7,15]. In addition, it has been found that a minor population of Very Small Embryonic-Like Stem Cells (VSELs) with pluripotent potential and positive for Oct-4, Stage Specific Embryonic Antigen-(SSEA)-3/4 (human), Sca-1 and NANOG are present in the bone marrow, cord blood, epidermis, heart, pancreas, testis, bronchial epithelium and ovaries [9,16-18]. These results suggest that isolation Oct-4 positive VSELs may serve as a good source of pluripotent stem cells in adult tissues and have a potential application in regenerative medicine [9,18].

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Received December 17, 2012; **Accepted** December 18, 2012; **Published** December 24, 2012

Citation: Varras M (2012) Clinical Significance of Expression of Stem Cell Markers in Human Ovarian Luteinized Granulosa Cells during Assisted Reproduction Technologies. Reprod Sys Sexual Disorders 1:e106. doi:10.4172/2161-038X.1000e106

The protein DAZL (DAZ-like) is RNA binding protein which is a member of the DAZL (deleted in azoospermia like) family which also includes BOULE and DAZ. The genes of the DAZL family encode proteins with a highly conserved RNA-binding motif (RNA Recognition Motif, RRM) and a unique DAZ repeat of 24 amino acids. These proteins are believed to function in the posttranscriptional regulation of messenger RNA (mRNA) expression [19,20]. The proteins of the DAZL family are located to nucleus and cytoplasm of the fetal germ cells [21]. In males, DAZL is expressed during spermatogenesis in gonocytes, spermatogonia and primary spermatocytes. During meiosis, DAZL is translocated from the nucleus of the spermatogonia into the cytoplasm of secondary spermatocytes, spermatids and spermatozoa [22-25]. During oogenesis, human DAZL is expressed in the cytoplasm of oogonia and in developing follicular oocytes in fetal and adult ovaries [26-30]. In human males, decreased DAZL expression has been reported in testes which produce little or no sperm [31] and in human females may be associated with primary amenorrhea or premature ovarian failure [26,32]. DAZL has also been reported to be expressed in human and mouse granulosa cells [24,25], human theca interna cells [27] and in the granulosa-luteal cells of human corpus lutea [33,34], but this remains controversial [35]. In addition, DAZL and Oct-4 gene expression has been found in human amniotic fluid cells suggesting the potential of these cells as a multipotent cell course for regenerative somatic cell therapy [21].

In adult human ovaries, the ovarian surface epithelium is a source of germ cells. Bukovsky et al. [36] reported that granulosa cell nests migrate through the dense ovarian stroma to the deep cortex to contribute to the follicular renewal [36]. Granulosa cell nests were not observed in climacteric ovaries and in women with Premature Ovarian Failure (POF) or in postmenopausal ovaries [37,38]. Virant-Klun et al. found stem cells in the adult human ovaries that develop into oocyte-like and parthenote-like structures [39]. Also Parte et al. detected pluripotent gene transcripts of Oct-4, NANOG, Sox-2, TERT and STAT-3 in ovarian surface epithelium, while germ cell markers like c-Kit, DAZL, GDF-9, VASA and ZP4 were localized in oocyte-like structures [40]. Stem cells in the ovarian surface epithelium are an important subject for further studies in terms of their possible regenerative role (potential oogenesis *in vitro* and differentiation into different types of somatic cells for regenerative medicine) and therapeutics in patients with aggressive ovarian epithelial cancers [41]. Kossowska-Tomaszczuk et al. [42] demonstrated the presence of multipotent granulosa cells, which survived in the presence of Leukemia-Inhibiting Factor (LIF) [43]. The researchers in order to get past the fact that the status of infertility of individual patients might affect the function of granulosa cells, pooled granulosa cells from all the different studied patients and found expression of POU5F1 CD29, CD44, CD90, CD105, CD117, and CD166 by substantial subpopulations of granulosa cells in the sum of cells through the culture. Prolonged culture of luteinizing GCs in medium supplemented with LIF allowed the selection of less differentiated granulosa cells, 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. Moreover, follicle-derived stem cells were able to survive when transplanted into the backs of immunoincompetent mice, *in vivo* generating tissues of mesenchymal origin [42]. These observations were supported by our findings, in which we showed the expression of Oct-4 in luteinized granulosa cells of women who underwent IVF or ICSI using quantitative real time PCR in almost half of the cases [43].It is strongly supported that the follicular granulosa cells consist of a subpopulation with pluripotent and self-renewal capabilities [42,43]. Absence of Oct-4 gene expression in the other half of cases means probably the end of the productive journey of these cells, towards oocytes. We also showed absence of DAZL gene expression, which possibly suggests the existence of stem cells not originated from primordial germ cells [43]. This important result is in concordance with the previous findings reported by Kossowska-Tomaszczuk et al. [42], who did not observe markers for pluripotent stem cells characteristic of the germ cells, such as NANOG, VASA and STELLA. Therefore, the hypothesis that the stem cells found in granulosa cells cannot be differentiated in germ cells is strongly supported because of the lack of DAZL, NANOG, VASA and STELLA gene expression [42,43].

Multiple factors are involved in the determination of pregnancy outcome in assisted reproduction including age, sperm quality (male factor), fertilization capacity and number of embryos transferred [4,44]. Also, intrinsic deficiencies of the oocyte and/or embryos account for greater than 50% of failed conceptions suggesting that the developmental competence of the oocytes is a major determinant in the establishment of successful pregnancy in assisted reproduction [4,45]. Two factors contributing to oocyte health are chromosomal constitution and gene expression patterns of the oocyte and the follicular micro-environment in which the oocyte grows and matures [4]. In view of concept of the beneficial effect of granulosa cells on oocyte maturation and the need for independent prognostic markers of better outcomes with conventional IVF for couples with non-male factor infertility, studies were focused on target genes in luteinized granulosa cells of the human ovaries. In fact, there are no morphological or physiological features of oocytes that can predict whether IVF fertilization will be successful, or whether is a need for ICSI unrelated to male factor infertility. Moreover, in some countries, not all retrieved oocytes can be fertilized due to legal limitations [46]. In such situations, predicting embryo quality is even more challenging because the time when the predictive evaluation can be performed is limited to the interval between oocyte retrieval and fertilization. This has prompted the search for additional parameters that can support morphological and metabolic evaluations of the oocyte in order to appropriately select those that have the greater chance of fertilization and development. In this respect, the analysis of granulosa cells is a good approach for providing such supplementary information. We investigated for the first time the correlations between the presence or absence and the levels of Oct-4 gene expression in granulosa cells with infertility clinical background and the assisted reproduction outcomes and we think this was a very intriguing approach [43]. The participating patients in the IVF group gave written consent for some of the Cumulus–Mature Oocyte Complexes (CMOCs) to be used only for the study. Therefore the Cumulus–Mature Oocyte Complexes (CMOCs) were randomly selected and manually denuded separately using a fire-polished tip glass pipette. These granulosa cells were analyzed for each patient separately, but the corresponding mature oocytes were not fertilized because for the IVF procedure the presence of cumulus cells is needed. Only some cumulus – mature oocytes complexes were donated and the cumulus cells were taken only from the mature oocytes per patient. In case of ICSI method, the Cumulus-Mature Oocytes Complexes (CMOCs) were manually denuded from granulosa cells using a fire-polished tip glass pipette. Granulosa cells from all the mature oocytes per patient were collected together. ICSI was performed only in oocytes that were morphologically confirmed to be in metaphase II with the first polar body extruded (mature oocytes). In case of the ICSI the cumulus cells were taken only when they surrounded the mature oocytes and these were the ones we analyzed. Therefore, the populations of both groups were homogenous as the cumulus cells in the ICSI group were only from mature oocytes and not from mature, immature and degenerated oocytes. In this way, we examined the expression of Oct-4 mRNA in

granulosa cells from each patient separately in correlation with duration of ovulation induction, number of follicles aspirated, number of oocytes retrieved, number of mature oocytes retrieved, embryo grade and clinical pregnancy and found a clear clinical significance only for the number of oocytes retrieved suggesting that Oct-4 expression positively affects the oocyte development during ART. The possibility for stem cell contamination during egg retrieval or granulosa cells collection and possible Oct-4 expression should be excluded because of the absence of DAZL gene expression, which is typically expressed in gametes. Our population included only patients with male or tubal factor infertility. Studying any clinical significance in ART of the expression of stem cell markers in luteinized granulosa cells is a new field of knowledge and according to our knowledge the present study is the first one. Previous studies on the same field were not done before. More of the studies were performed to correlate the impact of apoptosis or survival factors in granulosa cells with ART parameters and outcome. The clinical significance of the expressions of apoptotic factors in granulosa cells during art is controversial [47,48]. However, the expression of survival factors in granulosa cells gives some promises. Greenseid et al. [49] found that IGF1, IGF2 and their receptors are down regulated in ovarian granulosa cells of women with Diminished Ovarian Reserve (DOR) compared to those with Normal Ovarian Reserve (NOR) undergoing *In vitro* Fertilization (IVF) [49]. Also, Fujino et al. [50] studied the expression of survivin gene in granulosa cells from infertile Japanese patients and found that the gene expression levels of survivin in patients with endometriosis were significantly lower than in patients with male factor infertility. The gene expression levels of survivin in total pregnant patients were higher than those in total non pregnant patients [51]. Moreover, we studied only normal women (male factor infertility) and women with tubal factor infertility who underwent IVI or ICSI and embryo transfer [51]. Women with endometriosis or polycystic ovarian syndrome were not included in our study since endometriosis and androgens promote apoptosis [47,52]. We found a statistically significant increased expression of survivin in granulosa cells of women who had tubal factor infertility compared to normal women (male factor infertility). Therefore, it seems that survivin acts a protective role in the ovarian micro-environment. It is possible that survivin might try to protect ovaries, with possible influenced perfusion due to ipsilateral salpingectomy. In cases with tubal inflammation or hydrosalpinges survivin might try to protect the ovaries from follicular apoptosis in a paracrine environment [51].

In conclusion, a clear clinical significance for the number of oocytes retrieved and the expression levels of the stem cell marker Oct-4 in human luteinized ovarian granulosa cells is possible to exist. More specifically, the expression of Oct-4 mRNA in granulosa cells appears to play an important role in the regulation of follicular growth during ART. It would be interesting if further studies investigated any clinical significance of Oct-4 gene expression in granulosa cells of patients with Diminished Ovarian Reserve (DOR) as such population was not included in our study [43]. Also, it would be interesting if more studies validated the expression of Oct-4 using Western blot analysis and immune-fluorescence on granulosa cells in order to overcome possible limitations of our study and reinforce our findings [43].

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