Research Article

Differential Expression of Ovarian Superficial Epithelium Markers in Diminished Ovarian Reserve and Normal Women

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Abstract

In the last years, diminished ovarian reserve (DOR) is becoming a frequent pathology in the human reproductive system, which also has a major impact on quality of life and fertility of reproductive age women. However, DOR remains as a disease of unknown etiology. The aim of this study was to assess the differential expression levels of multipotent stem cell markers in the ovarian surface epithelium (OSE) of women with DOR and women with adequate ovarian reserve (AOR) that could be associated with the loss of ovarian functionality.

Methods: The present study was performed by using ovary tissue samples from patients previously diagnosed as AOR and DOR according to the clomiphene citrate challenge test. The expression levels of multipotent stem cell and germline markers were analyzed by immunohistochemistry. Results: Our findings demonstrate that a small number of epithelial cells express the Oct4 stem cell marker and the specific germ line markers (Fragilis, Stella, and VASA) in the OSE of both AOR and DOR patients, but the number of immunostained cells were lower in the women with AOR than DOR individuals. On the other hand, the expression of SOX2 and Nanog stem cell markers was only observed in the OSE of DOR women but not in AOR patients. Conclusions: The overall results of this study show that there is a differential expression of stem cell markers in the OSE cells from DOR women as compared with AOR subjects, suggesting a possible compensatory mechanism for the generation of new multipotent cells to participate in the repair and/or renewal of the population of ovary cells in the OSE of DOR women.
Keywords: ovary, multipotent stem cells, diminished ovarian reserve, adequate ovarian reserve, ovarian surface epithelium

Abbreviations: Ovarian Surface Epithelium (OSE), AOR: Adequate Ovarian Reserve; DOR: Diminished Ovarian Reserve; CC: Clomiphene Citrate; H&E: Hematoxylin-Eosin; BSA: Bovine Serum Albumin; DPC: Days Post Coitum; mESCs: Mouse Embryonic Stem Cells; PGCs: Primordial Germ Cells; EGCs: Embryonic Germ Cells; GSCs: Putative Germline Stem Cells

Introduction

Until recently, it was a dogma that mammals are provided with a non-renewable pool of oocyte containing follicles at birth which are exhausted during postnatal life, leading to ovarian failure or premature menopause. In humans has been reported the possibility of the postnatal oogenesis coming to be one of most controversial fields of reproductive and developmental biology currently. However, a growing body of evidence, including the isolation of germline stem cells (GSC) in many mammals, including humans. [1-13]. Recent studies have shown the presence germline stem cells with proliferative potential in adult mammalian ovaries [1,2,12]. The presence of germline stem cells in the adult ovary suggests a renewal mechanism. The ovarian surface epithelium (OSE) has been proposed as a source of ovarian stem cells in humans [6,12,14-16] and mice [17], meaning that this cell layer can be considered as a “germinal epithelium” [16,18-20]. The OSE is a natural source of germ cells in the fetal period of life [21,22]. Scanning and transmission electron microscopy have revealed numerous germ cells with 10 µm in diameter within the OSE of the human fetus from 7 to 24 weeks of intrauterine life [23], supporting that this cell layer may contain a stem cell niche. Recent evidence as the purification and in vitro propagation of premeiotic germ cells from neonatal and young adult mouse OSE cells, showed that are capable of generating a developmentally competent oocyte in the transplanted host female supporting evidence for this idea [24]. These results indicate that both neonatal and adult mouse ovaries carry stem cells capable of producing functional oocytes [24,25]. It has been widely reported that during aging a diminution of ovarian function occurs as a result of the reduction of ovarian follicles. In general, the fertility starts to decline after the age of 30 leading to sterility after the age of 40 [26,27]. Of all the major organ systems in the body, the ovaries are the first to exhibit impaired function with advancing age.

It has been recently reported that an early decline in ovarian function produces diminished ovarian reserve in young women. The reproductive capacity of these women is determined by several tests to evaluate their ovarian reserve [26], which includes the evaluation of the anti-Mullerian hormone (AMH) and follicle stimulating hormone (FSH) levels and, the antral follicle count. In particular, a serum FSH level of ≥ 10 mIU/ml is usually considered as a marker for diminished ovarian reserve [28].

In a previous study of our group, we demonstrated that patients with diminished ovarian reserve did not presented alterations neither in apoptosis nor in proliferation markers as compared with normal patients with adequate ovarian reserve [29], thus indicating that the mechanisms leading to diminished ovarian reserve are complex and an important area of research. Furthermore, there are no current studies evaluating the presence of GSC in women with diminished ovarian reserve, which is of great interest as the loss or the renewal of ovarian follicules could explain the early loss of ovarian function in these patients.

The systematic study of different stem cell markers in the normal ovary and during pathological conditions such as diminished ovarian reserve could help us to have a better understanding of the pathogenesis of the disease and may lead to the discovery of new strategies for the treatment of diminished ovarian reserve. Therefore, the aim of the present study was to assess the presence of multipotent stem cell markers in the OSE cell populations of women with diminished ovary reserve and women with adequate ovarian reserve.
Materials and Methods

Ovary tissue samples

Ovary tissue samples were obtained from 15 patients from 28 to 39 years old who had benign gynecological pathology not involving the ovaries and that were previously reported [29]. This study was approved by the Investigation Review Committee of the Gynecology and Obstetrics Hospital #3 of the Mexican Institute of Social Security in Mexico City. Written informed consent was obtained from all patients.

Clinical classification

The patients used in this study and their clinical characteristics were previously described [29]. In brief: Before surgery, patients were divided into two groups according to basal FSH levels measured on the third day of the menstrual cycle, and FSH concentrations determined on the 10th day of the menstrual cycle after taking 100 mg of clomiphene citrate (CC) orally on days 5-9, according to CC challenge test. Patients who showed basal FSH ≥ 10 mIU/mL or FSH concentrations on the 10th day of the menstrual cycle that exceeded 12 mIU/mL were considered to have a diminished ovarian reserve (DOR). Patients who had basal FSH levels <10 mIU/mL or FSH concentrations on the 10th day of the menstrual cycle <12 mUI/mL under the CC challenge test were considered as the control group [29].

Hormone determinations

Blood samples for basal FSH, LH, and E2 obtained on the third day of the menstrual cycle and were taken from an antecubital vein. After this procedure, 100 mg of CC was orally administered on days 5-9 and venous blood samples were taken again to determine FSH levels. The FSH, LH, and E2 levels were measured using the Immulite kit (Diagnostic Products Corporation, Los Angeles, CA). All determinations were conducted in duplicate. The inter- and intra-assay coefficients of variation were 6.6 % and 5.4 % for FSH, 10 % and 6.5 % for LH, and 5.4 % and 4.4 % for E2, respectively.

Specimens

Ovaries samples were obtained during surgery and processed for immunohistological analysis and were obtained from a previous study [29]. In brief, tissues were processed and paraffin embedded as previously described. Slides containing 5 µm sections hematoxylin-eosin (H & E) stained were used for histomorphology analysis. Additionally, series of sections in parallel were processed for immunohistochemistry assays as previously described [29].

Immunohistochemistry

Immunohistochemistry was performed using our general approach that has been described in the literature with small modifications [29]. Specifically, all specimens were sectioned at 5 µm and mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Each section was cut before immunostaining and attached to a slide by heating in an oven at 60°C for 1h. All slides were deparaffinized in xylene (two changes, 5 min each), hydrated in graded alcohols (absolute, 95 %, 90 %, 80 %, 70 %, H2O, (for 3 min each), and placed in phosphate buffered saline (PBS, pH 7.4). Subsequently, the slides were placed in a plastic coplin jar microwave-compatible filled with 0.01 M citric acid antigen retrieval solution (0.01 M citric acid, pH 6.0) and heated for 5 min in a microwave at high power. After 5 min of heating, the antigen retrieval fluid level was checked, the evaporated buffer was replaced, and each slide was subjected to another heating cycle of 5 min. Then, the slides were cooled to room temperature, and rinsed with distilled water. Later endogenous peroxidase activity of the tissue samples was quenched using an aqueous solution of 3 % hydrogen peroxide for 30 min, followed by one rinse with distilled water for 5 min. Tissues were permeabilized with 1 % Triton X-100 (Sigma-Aldrich Co. Po, St. Louis. MO) in PBS for 10 min, followed by one rinse
of PBS for 5 min. Then nonspecific immunoglobulin binding was blocked by incubating sections in 1% bovine serum albumin (BSA albumin bovine Reagent Pure; Research Organics, Cat # 1331A, Cleveland, OH 44125) for 2h at room temperature to reduce non-specific background staining. After blocking, sections were incubated with the primary polyclonal antibodies overnight at 4°C in a humidified chamber. The following antibodies were used to detect stem cells: Oct4 (1:500 dilution, rabbit ab19857; Abcam, UK) SOX2 and NANOG (1:300 dilution, R & D Systems, Minneapolis; goat AF2018, and goat AF1997, respectively). To detect primordial germ cells, the following antibodies were used: Fragiilis (1:500 dilution rabbit ab15592; Abcam, UK), Stella (1:500 dilution rabbit ab19878; Abcam, UK), DDX4/MVH (VASA) (1:500 dilution, rabbit ab13840; Abcam, UK).

Slides were incubated with a biotin conjugated anti-goat or anti-rabbit secondary antibody at a 1:200 dilution (Vectastain ABC Elite kit, Vector) for 1h at room temperature and rinsed in PBS four times. A peroxidase-labeled avidin-biotin was then added for an additional 1h followed by four washes with PBS. A diaminobenzidine supersensitive substrate kit (Peroxidase substrate kit DAB, Vector Laboratories, Inc., Burlingame, CA) was used to visualize the antigen-antibody complex. Slides were counterstained for 5 min in Mayer’s hematoxylin, rinsed in distilled water, dehydrated in graded alcohols (50%, 70%, 95%, and absolute ethanol for 3 min each) and soaked in xylene before attaching coverslips using Clarion Mounting Medium (Biomeda Corp. Foster City, CA). For the negative controls, tissue sections were incubated with 1% goat serum instead of primary antibodies. For positive controls, mouse fetal gonads of 13 days post coitum (dpc) were used. The slides were analyzed under a Nikon E600 microscope (Nikon, Inc., Melville and N.Y.). Image J program was used to analyze the images. The number of immunopositive cells for each marker was evaluated in the OSE of AOR and DOR ovaries.

**Statistical analysis**

Due to a limitation for tissue sample obtaining and ethical aspects, the results described in this study were semiquantitative. However, all images were analyzed by to expert pathologist independently in a double-blind analysis. Quantification of immunopositive cells was performed using the Image J. Data program, and probability values were subsequently calculated with the Prism 5.0 program (Graph Pad, San Diego, CA). P ≤ 0.05 was considered as significantly.

**Results**

The patients were grouped as AOR or DOR in relation to gonadotropins hormonal serum levels after clomiphene citrate test as previously described by one of us [29], and the patients included as the DOR presented significantly higher FSH levels up to 10 UI/L during the test, as compared with AOR subjects.

The expression of three main stem cell markers, Oct4, Nanog and SOX2, as well as markers of germ line, Fragiilis, Stella, VASA, in the ovaries of both AOR and DOR women were analyzed.

Oct4 protein expression in AOR ovaries was observed in the nucleus of oocytes from primordial follicles (Figure 1A). In the case of the VASA protein, immunolabeling was observed at the cytoplasm of primordial follicles (Figure 1E). Fragiilis marker was observed at the nuclear membrane (perinuclear) of the oocyte (Figure 1I), whereas Stella expression was extended in the nucleus and perinuclear of the oocytes (Figure 1M). The same location of protein markers described above was observed in the primordial follicles of DOR patients (Figures 1B, 1F, 1J, and 1N). There were a lower number of primordial follicles in DOR ovaries compared with AOR ovaries (Figure 1). However, due to scarce biological material, primordial follicles were not quantified. Fetal mouse gonads (13 dpc), in which germ cells (oogonia or spermatogonia) were positive for these markers (Figures 1C, 1G, 1K and 1O) respectively, and were used as positive control. For the negative controls, primary antibody was omitted, or preabsorbed with blocking peptide and these slides were negative to immunolabeling (Figures 1D,1H,1L, and 1P).
Figure 1: Immunodetection of germline and stem cell markers in follicles of AOR and DOR women. Oct4, Vasa, Fragilis and Stella proteins were localized in primordial follicles (A, E, I and M) and DOR (B, F, J, and N) patients. Immunopositive labeling is indicated by arrows. The Oct4 and Stella protein was detected at the nucleus of the oocyte, the VASA protein at the cytoplasm, and the Fragilis proteins at the nuclear membrane of the oocyte. Positive controls (C+) are 13-day post coitum mouse gonads (C, G, K, and O), in which a positive signal for each of the markers can be observed. Negative controls (C-) are ovaries from AOR and DOR patients in which the primary antibody was omitted (D, H, L and P). All experiments were performed in triplicate. Bars= 50 µm.

In the epithelium of the AOR ovaries, scarce Oct4, Fragilis, Stella and VASA immunolabeling was observed, and immunostaining was distributed throughout the OSE (Figure 2). Interestingly, SOX2 and Nanog were not observed in the OSE or in the follicles of the AOR ovaries. In contrast, in the OSE of DOR ovaries the presence of Oct4, Fragilis, Stella, VASA, but also Nanog and SOX2 proteins was observed (Figure 2).
Figure 2: Germline and stem cell markers are present in the OSE of AOR and DOR patients. Immunodetection of protein markers correspond to: Oct4 (A,B), Sox-2 (D,E), Nanog (G,H), Fragilis (J,K) Stella (M,N) and VASA (P,Q) and its respectively negative controls (C,F,I,L,O and R). Immunopositive cells labeling is indicate by arrows. The OSE in the human ovary presented a simple and continuous cubic epithelium. All experiments were performed in triplicate. Bars=100 µm.
One of the most relevant findings of this study was that only in the DOR ovaries the presence of multi-layered epithelial crypts was observed (Figure 3), in which there were Oct4 positive cells (data not show).

**Figure 3:** The human ovarian surface epithelium. The epithelial cells on the ovarian surface (OSE) are supported along the tunica albuginea (TA) through a basement membrane; they are recognized as flat monolayer (A). Also shows the clear presence of primordial follicles, which were observed in both conditions, however those shown correspond to the DOR patients (B). Structural changes in the human ovarian surface epithelium; during repairs of ruptured follicles, produce deep cortical fissures are formed in the cortical stroma what are they covered by surface epithelium cells. Some large and small inclusion cyst with flat and cuboidal epithelium arises from the superficial fissure (C,D). In AOR patients the OSE is pseudostratified cuboidal/flat, the nucleus is round to oval (E), while in DOR patients is pseudostratified columnar/ciliated (F). Hematoxylin & eosin stain, original magnification 20X (A, B), 10X (C, D) and 20X (E, F).
Interestingly, while there was no significant difference in the number of positive cells for the germ line marker (VASA, Fragilis and Stella) in the OSE of AOR or DOR patients (Figure 4). In contrast, in the case of Nanog and SOX2 stem cell markers, it's were present in DOR but any in AOR patients (Figure 4).

Figure 4: Expression of stem cell and germline cell markers in OSE cells from AOR and DOR patients. For the stem cell markers, there was no significant difference in the expression of Oct4 protein between groups (A). However, the SOX2 (B) and Nanog (C) proteins were only present in DOR patients. In the germline cell markers Fragilis (D), Stella (E) and VASA (F), there were no significant differences in the number of positive cells for these proteins between AOR and DOR patients. Data are expressed as mean ± SEM.

Discussion

The results of the present study provide evidence for the presence of multipotent cell markers in the OSE of adult human ovaries of both DOR and AOR subjects. The Oct4, VASA, Stella and Fragilis were detected in both DOR and AOR individuals were lower in AOR than in DOR patients. Interestingly there was a differential expression of Nanog and Sox2 positive cells in the OSE cells from DOR individuals, while they were absent in AOR subjects, suggesting that this might be a possible compensatory mechanism with generation of new multipotent capacity cells for the repair and or renewal of ovary cells in DOR patients.

In regard to the Oct4, Stella, Fragilis, and VASA markers expression, it has been previously reported that Oct4 [30], Stella [1,31], Fragilis [1], and VASA [32,33] were present respectively in germline cells of fetal rodents. It has also been seen in mESCs that Nanog, Oct4, and Sox2 are the core regulators of mouse, and their capacity to induce pluripotency; likewise, in hESCs the same capacity, and it has been assumed that these genes have important and conserved functions [34-36]. According to the prevailing model Nanog, Oct4 and Sox2 cooperatively maintain the regulatory network responsible for self-renewal and pluripotency cell capacity [37].

A recent study in human adult ovaries showed that 75-100 % of the ovarian superficial epithelium cells expressed NANOG, SFRP1, LHX9 stem cells markers [38]; other publications have reported small round cells isolate from OSE scrapings of postmenopausal women and women with primary premature ovary insufficiency, spontaneously differentiate into oocyte-like cells in the presents of follicular fluid under estrogenic stimuli [12,39-42].
Furthermore, the oocyte-like cells identified in these studies demonstrated that they expressed pluripotency (OCT4, SOX2, NANOG, NANOS), and germ cell markers (Ckit, VASA, STELLA SCP1-3).

On the other hand, it was surprising that we detected that Nanog and SOX2 markers only expressed in the OSE of DOR patients. Whether these markers indicate some capacity of these cells in the programing of multipotent capacity in pathophysiological conditions such as DOR, deserve further investigation.

Various studies suggest that OSE is not involved for maintaining the number of ovarian follicles [7,43]. In contrast, Bukovsky et al. claimed that ovarian stem cells exist in the OSE in ovaries from young women, based primarily on morphological observation and immunohistochemical studies [14]. Our results are on line with this proposal, and strongly support that there are cells expressing stem-like markers in the ovarian surface epithelium.

Primordial germ cells (PGCs) in the fetal ovary express most but not all of the markers associated with pluripotent cells [10] and can develop into pluripotent stem cells, such as EGCs and embryonic carcinoma cells (ECCs) [13]. The possible role of ovarian stem cells in oogenesis and de novo folliculogenesis in the adult ovary still remains to be clarified. So far, there are two facts providing the greatest support for the idea of de novo oogenesis and folliculogenesis in the adult human ovary: primarily, the presence of stem cells in the human adult OSE [12,42], besides, the phenomenon of epithelial-mesenchymal transitions. It has already been confirmed that the OSE shows characteristics of both mesenchymal and epithelial cells, and that under mostly unknown conditions epithelial cells can be transformed into mesenchymal cells [11,44].

Gene expression profiling supports the hypothesis that human ovarian surface epithelial cells are multipotent and capable of serving as ovarian cancer-initiating cells [45]. These OSE cells were found positive for multipotent markers, and in terms of morphological characteristics, they had a well-defined round shape and a prominent nucleus. However, some of these cells have a fusiform nucleus, which corresponds to the previously reported cells by Zamboni [46] and Chuaire et al. [47] for germ cells with mitotic activity. In similar manner that it has been observed in this study, Oct4 pattern expression in the OSE of both AOR and DOR ovaries was previously reported in the literature in relation to women with natural menopause onset or premature ovarian failure [5,24,42]. However, there are no previous studies which compare AOR and DOR ovaries. We demonstrated that primordial germ cells were positive for Oct4, in a manner similar to that of cells arriving to the gonadal ridge [3,48]. Additionally, we detected the expression of Oct4 in the nuclei of germ cells, as well as in the putative germline stem cells (GSCs) of the OSE. On the other hand, another group reported the location and size of the GFP-Oct4 positive cells in both neonatal and adult mouse ovary, suggesting the presence of germline stem cells in postnatal ovary. In the same sense, we detected VASA expression, which is a general germ cell marker, and thus is in agreement with the identification of germ line cells in the ovary of fetal mice [49] and of fetal and adult humans [50].

The results obtained in this study provide additional evidence for the multipotential nature of OSE stem cells. Data provided by recent work of Parte and cols. [11] indicate the presence of putative stem cells in the OSE and their ability to differentiate spontaneously in vitro into an oocyte-like structure. These results offer indirect evidence to support postnatal oogenesis in females. However, it is important to remember that the OSE is an important structure of the human ovary and is involved in both reproductive function and ovarian tumor formation. Besides, they can be involved in the formation of ovarian cancer and other pathologies. This might be one of the reasons for the higher incidence of epithelial ovarian cancer in older, postmenopausal woman [13].

In conclusion, the results of the present study suggest that humans have multipotent cells in the OSE of AOR and DOR patients: Ovarian OSE multipotent cells could have profound implications in the treatment of ovarian dysfunctions. While, the presence of stem cells in the OSE promote the formation of epithelial ovarian cancer in
postmenopausal women. Data from this study suggest that the presence of multipotent cells in DOR patients may represent a compensatory mechanism providing a generation of new cell reservoir. This mechanism could be the key to address this gynecological pathology, which is currently increasing due to the lifestyle of women today. Additionally, to evaluating the presence of germline markers, as in the present study, we believe that future clinical and histopathological studies should attempt to clarify the functional roles of the different biomarkers associated with the multipotentiality in the ovarian reserve dynamics.

The overall results of the present study have improved our capacity for the understanding of ovarian physiopathology and the possible design of new strategies in human infertility. This could possibly also contribute to the development of new therapeutic approaches in the treatment of ovarian dysfunctions such as polycystic ovary syndrome, abnormal ovarian reserve, premature ovarian failure and ovarian carcinoma.

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