Repeated ovarian stimulation does not affect the expression level of proteins involved in cell cycle control in mouse ovaries and fallopian tubes

Gianluca Di Luigi · Gianna Rossi · Annalisa Castellucci ·
Pietro Leocata · Gaspare Carta · Rita Canipari ·
Stefania Annarita Nottola · Sandra Cecconi

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Abstract

Purpose To understand if repeated cycles (2–4 rounds) of gonadotropin stimulation could affect intracellular localization/content of proteins controlling cell cycle progression in mouse fallopian tubes (FT) and ovaries.

Methods FT and ovaries of estrous mice (control) and of stimulated mice were analyzed to detect Oct-3/4, Sox-2, p53, β -catenin, pAKT and cyclin D1 localization/content. Spindles and chromosome alignment were analyzed in ovulated oocytes.

Results After round 4, FT and ovaries of control and stimulated groups showed no differences in Oct-3/4, Sox-2 and β -catenin localization nor in Oct-3/4, Sox-2, p53, β -catenin and pAKT contents. Cyclin D1 level increased significantly in FT of treated mice. Oocytes number decreased meanwhile frequency of abnormal meiotic spindles increased with treatments.

Conclusions Repetitive stimulations affected oocyte spindle morphology but did not induce changes in a set of proteins involved in cell cycle progression, usually altered in ovarian cancer. The significant increase of cyclin D1 in the FT requires further investigation.

Keywords Fallopian tubes · Infertility · Ovarian stimulation · Ovary

Introduction

More than 15 % of couples will likely face infertility problems during their reproductive life, and by 2025 the number of women sharing this problem will be about seven million, worldwide [1].

Stimulation regimens are used because they increase oocyte availability by inducing the development of multiple follicles. In mice, repetitive hormonal treatments induced a decrease in oocyte quality [2]. In infertility patients, no data on oocyte quality following repetitive stimulations have been reported in the literature, and the reduction of pregnancy rate after more than three IVF treatments can be mainly due to age-dependent effects [3].

In recent years, public opinion expressed great concern about the long-term effects of fertility drugs on women health, and in particular about the risk of developing ovarian cancer (OC) following infertility treatments [4–7]. Indeed, infertility per se increases the risk for OC [4], and nulliparous women or women who used fertility drugs to achieve pregnancy seem to have a higher risk of developing OC than women with children and/or those that have used oral contraceptives [8]. Large epidemiological studies evidenced a weak but not significant association between infertility drugs and OC risk [5, 9–11], even if an increased risk of borderline ovarian tumors in IVF groups compared with the general population has been reported [10]. As a consequence, it is necessary to evaluate how the need of motherhood could reconcile with the side effects of these therapies.

In fact, OC is the ninth most frequently occurring cancer among women [12]. Although its origin is still debated, many factors are implicated in the etiology of this disease, and many

Capsule In mouse ovary and fallopian tubes, four rounds of gonadotropin stimulation did not modify cell cycle proteins contents, usually altered in ovarian cancer.

G. Di Luigi · G. Rossi · A. Castellucci · P. Leocata · G. Carta ·
S. Cecconi (✉)
Department of Life, Health and Environmental Sciences,
University of L'Aquila, L'Aquila, Italy
e-mail: sandra.cecconi@univaq.it

R. Canipari · S. A. Nottola
Department of Anatomy, Histology, Forensic Medicine and
Orthopedics, School of Pharmacy and Medicine, "Sapienza"
University of Rome, Rome, Italy

hypothesis have been developed in search of early diagnosis and effective therapies [13]. Multiple ovulations, high levels of gonadotrophins as well as of steroid hormones [14], mutations of tumor suppressor genes and environmental factors [15] are considered to play a key role in OC development. Other reports support the inflammatory or the stromal origin of this disease [13]. Recent findings evidenced the possibility that the majority of OC arises from the spread to the ovary of high-grade intraepithelial serous carcinomas originated in the Fallopian tubes (FT) [16–18]. The finding that gene expression profile of high-grade serous carcinoma is more closely related to FT than to ovarian surface epithelium [19] is changing the approach to the etiology and clinical management of this malignancy.

It is of importance to identify proteins playing a role in cancer transformation, and/or correlated with promotion and progression of OC. Several evidences supported that Oct-3/4 (Pou5f1: POU domain class five transcription factor 1, 45 kDa isoform) and Sox-2 (SRY (sex determining region Y)-box 2) expressions progressively increased in malignant transformation from normal ovarian epithelium to invasive carcinoma [20, 21]. Indeed, both transcription factors modulate embryonic stem (ES) cell populations by influencing growth and differentiation [22, 23], and are expressed by stem cancer cells [24, 25]. To date, no definitive conclusion about the association between fertility drugs and OC risk has been drawn. In this study, we evaluated the possibility that repetitive gonadotropin treatments could modify expression levels of proteins implicated in cell cycle control as Oct-3/4, Sox-2, p53, phosphorylated AKT (pAKT), β -catenin and cyclin D1 in the FT and ovaries of naturally-ovulating mice and of mice undergoing several rounds of ovarian stimulation.

Materials and methods

Chemicals

All the chemicals were of the purest analytical grade and were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated. Rabbit polyclonal Sox-2, mouse monoclonal Oct-3/4, mouse monoclonal p53, rabbit polyclonal cyclin D1 and rabbit polyclonal β -catenin primary antibodies were purchased from Santa Cruz Biotechnology (CA, USA), rabbit polyclonal phospho-Akt (T308), from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies were purchased from Molecular Probes (Invitrogen, Carlsbad, CA).

Collection of oocytes and tissues

Adult female mice (2–3 month old, Swiss CD1 Harlan Italy, Udine, Italy; $N=64$) were housed in the animal facility under

controlled temperature (21 ± 1 °C) and light (12 h light/day) conditions, with free access to food and water. Animals in which estrous cycle was evaluated by examination of the vagina and vaginal smears ($N=16$) were used as control (Ctr). Repetitive cycles of ovarian stimulation were performed according to the protocol of Van Blerkom and Davis [2]. Briefly, mice were injected i.p. with 5 IU of PMSG (Folligon, Milano, Italy) and 48 h later with 5 IU of hCG (Corulon, Milano, Italy), and two to four rounds (2R, 3R, 4R) of stimulations were performed with intervals of 1 week between each. For each experiment, control (Ctr; $N=4$) and hyperstimulated ($N=12$; 4/round) mice were sacrificed. The experiment was replicated four times and the total number of mice was 16 for the Ctr and 48 treated.

Naturally ovulated (Ctr) oocytes were collected from FT of animals in estrous, while ovulated oocytes were recovered 14 h post hCG from FT of mice undergoing 2R, 3R and 4R of gonadotropin stimulation. Oocytes were fixed for spindle analysis. From each mouse, one ovary and its neighboring FT were separately stored for molecular analysis, while the contralateral organs stored for immunofluorescence analysis.

Animals were maintained in accordance with the Italian Department of Health Guide for Care and Use of Laboratory Animals. Experimental protocols were approved by the local committees for the animal care and use, according to accepted veterinary medical practice.

Immunofluorescence analysis

FT and ovaries from control and hyperstimulated mice were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek; Qiagen, Valencia, CA, USA), snap frozen in liquid nitrogen/isopentane, and cryosectioned at 12 μ m. Frozen samples were stored at -80 °C until use. To block nonspecific binding, 1 % bovine serum albumin (BSA)/PBS was used. Sections were incubated overnight with mouse monoclonal Oct-3/4 antibody or rabbit polyclonal Sox-2 antibody (1:500 in 1 % BSA) at 4 °C. Then anti-rabbit IgG Alexa Fluor 488 and anti-mouse IgG Alexa Fluor 633 (1:200 in 1 % BSA/PBS) were added for 1 h at 37 °C. Nonspecific binding was blocked by incubating the sections in normal goat or donkey serum, depending on the secondary antibody used. Chromosomes were labelled by using DNA-specific label, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:5000) for 5 min at room temperature. Images were taken by confocal microscope Leica TCS SP5 II (Leica, Germany).

Western blotting

FT and ovaries from control and hyperstimulated mice were resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM

NaCl, 1 mM EDTA and 1 % Igepal) containing protease inhibitors (1 mM phenylmethylsulphonylfluoride, 1 µg/ml leupeptin and 1 µg/ml aprotinin) and phosphatase inhibitors (1 mM sodium fluoride, 10 mM sodium pyrophosphate and 1 mM sodium orthovanadate) for 30 min. Lysates (80 µg /sample) were separated by electrophoresis and transferred to nitrocellulose membranes (Hybond C Extra, Amersham, Little Chalfont, UK). Membranes were incubated overnight at 4 °C with specific primary antibodies: Oct-3/4, Sox-2, p53, β -catenin, cyclin D1(1:200) and pAkt (1:500) and then for 1 h with a peroxidase-conjugated anti-mouse (1:5000) or rabbit secondary antibody (1:10000). After detection using a chemiluminescence reagent (ECL, Pierce, Rockford, USA), densitometric quantification was performed with the public domain software NIH image v.1.62 and standardized using β actin (1:200) as a loading control.

Fig. 1 Expression of Oct-3/4 in the fallopian tubes (FT) obtained from control estrous mice (Ctr) or from mice undergoing 2–4 rounds (2R, 3R, 4R) of gonadotropin stimulation. **a**

Immunofluorescence detection of Oct-3/4 and DAPI is shown in Ctr and 4R. Low intranuclear staining is evident. **b** Representative Western blot showing the expression of Oct-3/4 in FT collected from Ctr or from mice undergoing 2–4 rounds of gonadotropin stimulation. Data represent densitometric quantification of optical density (OD) of Oct-3/4 signal normalised with the OD values of β -actin, used as loading control and are the mean $\pm$ SEM of four independent experiments. Values are expressed as fold change over control

Analysis of meiotic spindles

To detect meiotic spindle and chromosomes, MII oocytes were labelled according to the protocol described by Rossi et al. [26]. The different groups of oocytes were incubated for 1 h at 37 °C with anti α/β tubulin primary antibody (1:100) to detect microtubules, and then with anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (1:800). Chromosomes were labelled with Hoechst 33342 (1 µg/ml). Oocytes were analysed using a fluorescence microscope (40X objective; Axioplan 2; Zeiss) with digital images collected with Leica DFC350 FX camera interfaced with IM500 Leica software.

Statistical analysis

All experiments were performed four times and data were expressed as the mean $\pm$ SEM. Differences between groups

were analysed for statistical significance using ANOVA with Tukey–Kramer multiple comparison test as a post test, or two-tailed t-test when comparing data derived from two groups. Results were considered significantly different if $p < 0.05$.

Results

Protein localization and expression

Intracellular localization and protein level of Oct-3/4 and Sox-2 were determined in the FT collected from estrous mice (Ctr) and from mice undergoing several rounds (2R, 3R, 4R) of ovarian stimulation. As shown in Fig. 1a, Oct-3/4 had a weak staining, and appeared prevalently localized in the nucleus in both control (Ctr) and stimulated mice (4R).

Western blot analysis confirmed that Oct-3/4 content was unaffected by repetitive hormonal treatments (Ctr vs. 2, 3, 4R: $p > 0.05$; Fig. 1b). In the FT of both control (Ctr) and stimulated mice (4R), Sox-2 staining was evident in both cytoplasm and nucleus (Fig. 2a). Repetitive gonadotropin treatments did not significantly affect Sox-2 content (Fig. 2b).

As shown in Fig. 3, the content of β -catenin was comparable in the FT of control (Ctr) mice and of mice undergoing 2–4R of gonadotropin stimulation ($p > 0.05$), while that of cyclin D1 increased significantly but only after 4R (+20 %; $p < 0.05$). The levels of p53 and pAkt were undetectable in all the experimental conditions tested (not shown).

In ovaries, no significant differences in the levels of Sox-2, Oct-3/4, β -catenin and cyclin D1 contents were found among control and treated mice (Fig. 4).

Fig. 2 Expression of Sox-2 in the fallopian tubes (FT) obtained from control estrous mice (Ctr) or from mice undergoing 2–4 rounds (2R, 3R, 4R) of gonadotropin stimulation. **a**

Immunofluorescence detection of Sox-2 and DAPI is shown in Ctr and 4R. Low cytoplasmic and nuclear staining are evident. **b** Representative Western blot showing the expression of Sox-2 in FT collected from Ctr or from mice undergoing 2–4 rounds of gonadotropin stimulation. Data represent densitometric quantification of optical density (OD) of Sox-2 signal normalised with the OD values of β -actin, used as loading control and are the mean $\pm$ SEM of four independent experiments. Values are expressed as fold change over control

Fig. 3 Western blot analysis of β -catenin and cyclin D1 in mouse fallopian tubes (FT). Protein levels are determined in the FT obtained from control estrous mice (Ctr) or undergoing 2–4 rounds (2R, 3R, 4R) of gonadotropin stimulation. Data represent densitometric quantification of optical density (OD) of β -catenin and cyclin D1 signals normalised with the OD values of β -actin, used as loading control and are the mean $\pm$ SEM of four independent experiments. Values are expressed as fold change over Ctr. * $p < 0.05$ vs. Ctr

Spindle analysis in ovulated oocytes

The effects of increasing rounds of ovarian stimulation on the final number of MII oocytes, on spindle morphology and chromosomal alignment were shown in Fig. 5. About 97 % of control oocytes showed focused spindles with aligned chromosomes (Fig. 5a–c), while a progressive increase in the frequency of spindle anomalies occurred with repetitive

rounds of ovarian stimulation. In fact, on round 4, about 80 % of spindles appeared disorganized and asymmetric, and chromosomes not aligned on metaphase plate or detached from microtubules (Fig. 5d–f; $p < 0.05$).

Discussion

Here we reported the effects of repeated cycles of ovarian stimulation on oocyte meiotic spindle morphology and on the expression levels of proteins known to be involved in cell cycle control and cancer development, namely Oct-3/4, Sox-2, β -catenin, pAkt, cyclin D1 and p53 in mouse ovaries and FT. The number of 4 cycles of ovarian stimulation used in the present experiments is that utilized by Van Blerkom and Davis [2] and by Combelles and Albertini [27]. According to both studies, we observed a progressive increase in the percentage of spindle anomalies after repetitive stimulations, with about 80 % of oocytes showing disorganized spindles and chromosomes detached from equatorial plane on round 4. Moreover, Van Blerkom and Davis [2] found that also repeated rounds spaced weeks apart have deleterious effects on oocyte quality, by increasing the frequency of abnormal fertilization. Combelles and Albertini [27] demonstrated that repeated ovarian stimulations impaired the development of both somatic and germinal compartments by reducing ATP production. However, since pre- and post-implantation rates were unchanged, the authors hypothesized the existence of still unknown mechanisms that, at least in mice, can maintain oocyte capacity to achieve successfully post-implantation development.

From our results, it is evident that mouse ovaries and FT did not show statistically different levels of Oct-3/4, Sox-2, β -catenin, pAKT and p53. By contrast, in the FT, cyclin D1

Fig. 4 Western blot analysis of Sox-2, Oct-3/4, β -catenin and cyclin D1 in mouse ovary. Protein levels are determined in the ovaries obtained from control estrous mice (Ctr) or undergoing 2–4 rounds (2R, 3R, 4R) of gonadotropin stimulation. Data represent densitometric quantification of optical density (OD) of Sox-2, Oct-3/4, β -catenin and cyclin D1 signals normalised with the OD values of β -actin, used as loading control and are the mean $\pm$ SEM of four independent experiments. Values are expressed as fold change over Ctr

Fig. 5 Frequency of spindle defects in MII oocytes collected after 2 and 4 rounds (2R, 4R) of gonadotropin treatment. Spindles are labelled with anti α , β tubulin antibody (**a, d**) and chromosomes with Hoechst 33342 (**b, e**; merge: **c, f**). The different percentages of normal (**c**) and abnormal (**f**) configurations have been reported in the table. Arrow indicates PB1. A total number of 80–100 oocytes was analyzed in three different experiments. Different superscripts indicate $p < 0.05$ within each column. Data are expressed as mean $\pm$ SEM. Original magnification: X40

		α, β tubulin	Hoechst	Merge

Conditions tested	No. oocytes/mouse	% Spindle morphology		
		Normal	Abnormal	
Ctr	10 $\pm$ 2 ^a	97 $\pm$ 3 ^a	3 $\pm$ 1 ^a	
2R	13 $\pm$ 2 ^a	60 $\pm$ 2 ^b	40 $\pm$ 3 ^b	
4R	8 $\pm$ 1 ^b	20 $\pm$ 3 ^c	80 $\pm$ 2 ^c	

content significantly increased after round 4 (4R) of stimulation.

Oct3/4 is a transcription factor which plays important roles in ES cells and, together with Sox-2, acts as potent regulators of cell differentiation, especially during peri-implantation development [28] and during ESC differentiation [29]. In addition, the transcription factor Sox-2 regulates the complex transcriptional network that maintains the unique characteristics of embryonic stem cells [30] and the anti-apoptosis property of human cancer stem cells [31]. Literature data show that expression of both these transcription factors is modified in an expanding list of cancers [32, 33]. In humans, the normal ovarian and FT epithelia are characterized by negative or weak Oct4 and Sox-2 expression, that are conversely greatly enhanced during malignant transformation [18, 19]. Although obtained in mice, the findings that both these proteins are expressed at comparable levels in FT and ovaries independently of hormonal stimulation, without changes in morphology and intracellular localization, suggest that repetitive ovarian stimulation does not target FT. This conclusion has been further confirmed by the similar levels of β -catenin and by the absence of pAKT and p53 in FT of control and hyperstimulated groups. In humans, the abnormal activation of the PI3K/AKT pathway [34] and increased nuclear β -catenin levels have been described to occur in OC [35]. Also in mice, high-grade serous OC can

arise from the FT following deletion of PTEN, a negative regulator of PI3K pathway [36]. Moreover, aggressive OC display *TP53* mutations in over 80 % of cases, although the role of p53 signature as a precursor lesion remains to be elucidated thus far. It is noteworthy that, in attempt to explain the genesis of ovarian high-grade serous carcinomas, Kurman and Shih [14] recommended the analysis of p53 expression also in the FT.

As above reported, in our experiments cyclin D1 content increased by 20 % over control in the FT after 4R of ovarian stimulation. By considering that human ovarian carcinoma tissues have an estimated +46 % of cyclin D1 overexpression, that has been correlated with an ascending clinical stage and poor prognosis [37], we can hypothesize that such a more limited increase might highlight a latent precursor phase, that predisposes to potential cancer development. Indeed, epidemiological data evidenced an increased risk for borderline ovarian tumors in infertile women treated with IVF [10], and it is noteworthy that about 2/3 of serous borderline ovarian tumors are characterized by *kras* mutations that determines a significant increase of cyclin D1 expression [38]. On the other hand, we cannot exclude the possibility that, in our experimental model, cyclin D1 increase could be a hypertrophic response of epithelial cells of the FT to supra-physiological gonadotropin stimulation, without any established role in the causation of OC.

In conclusion, our results indicate that in mice several cycles of ovarian stimulation while affecting oocyte quality do not modify the content of several proteins involved in cell cycle control, with the exception of cyclin D1 after 4R of stimulation. Despite these reassuring information, we cannot reject the possibility that in a small percentage of susceptible women also a relatively low increase of cyclin D1, as that recorded in mice, could sensitize epithelial cells towards malignant transformation. As a consequence, even if repetitive hormonal stimulation is not “per se” cause of OC, it remains ethically proper to inform women at risk, as those with a family story of solid cancers, about the potential consequences of infertility treatments.

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S.C. dedicates this paper in the memory of Daniela Lombardi (1956–2012).

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