REPRODUCTION
RESEARCH

Increased rounds of gonadotropin stimulation have side effects on mouse fallopian tubes and oocytes

Valentina Di Nisio[1], Gianna Rossi[1], Maria Grazia Palmerini[1], Guido Macchiarelli[1], Gian Mario Tiboni[2] and Sandra Cecconi[1]

[1]*Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy and* [2]*Department of Medicine and Aging Science, University 'G. D'Annunzio', Chieti-Pescara, Chieti, Italy*

Correspondence should be addressed to S Cecconi; Email: sandra.cecconi@univaq.it

Abstract

In this study, it was evaluated if increased rounds of gonadotropin stimulation could affect in mice: (i) expression levels of proteins regulating cell cycle and DNA repair in fallopian tubes and (ii) meiotic spindle morphology of ovulated oocytes. To this end, adult female mice were subjected or not (Control) to 6 or 8 rounds of gonadotropin stimulation. Ovulated oocytes were incubated with anti A/B tubulin to evaluate spindle morphology. Fallopian tubes were analyzed to detect Cyclin D1, phospho-p53/p53, phospho-AKT/AKT, phospho-GSK3B/GSK3B, SOX2, OCT3/4, phospho-B-catenin/B-catenin, phospho-CHK1 and phospho-H2A.X protein levels. After 6 rounds, Cyclin D1, p53 and phospho-p53 contents were higher than Control. After 8 rounds, the contents of phosphorylated AKT, GSK3B and p53 as well as of total p53, Cyclin D1 and OCT3/4 significantly increased in comparison with Control. Conversely, SOX2 and B-catenin were similarly expressed among all experimental groups. The finding that phospho-CHK1 and phospho-H2A.X protein levels were undetectable supported the absence of extensive DNA damage. Oocytes number and percentage of normal meiotic spindles drastically decreased from 6 rounds onward. Altogether, our results demonstrated that 6 and 8 cycles of gonadotropin stimulation reduce mouse reproductive performances by inducing over-expression and over-activation of proteins controlling cell cycle progression in fallopian tubes and by impairing oocyte spindle.

Reproduction (2018) **155** 245–250

Introduction

Retrieval of human oocytes during assisted reproductive technology requires the use of stimulation protocols (Pacchiarotti *et al.* 2016, Alper & Fauser 2017). Among these, the mild ovarian stimulation (MOS) protocol (Fauser *et al.* 1999) became the preferable option for IVF treatments thanks to the reduction of patient discomfort, risk of developing ovarian hyperstimulation syndrome and costs, as well (Pennings & Ombelet 2007). However, the debate about mild and conventional ovarian stimulation (COS) protocols is nevertheless still controversial. In fact, several studies comparing ongoing pregnancy and live birth rates between MOS and COS protocols revealed that COS was more successful and gave better chance of positive outcome in comparison with MOS (Revelli *et al.* 2011, Crawford *et al.* 2016, Siristatidis *et al.* 2017).

Moreover, women frequently have to undergo repeated cycles of ovarian stimulation. The side effects of these treatments have been highlighted by Homburg and collaborators who found that pregnancy rate was hampered by increasing number of cycle attempts. Indeed, a drastic decline from 25% to 17% after 4 rounds (R) of stimulation, and to 11% after more than 12R was recorded (Homburg *et al.* 2009).

In recent years, there has been a great concern about the risk of developing reproductive cancers, i.e. breast, ovarian and endometrial cancer, following IVF treatments (Brinton *et al.* 2012). For what concerns ovarian cancer (OC), results on the association between repetitive treatments and the risk of developing OC are discrepant. Sanner and collaborators reported that the protocols utilizing gonadotropins were associated with increased risk of OC (Sanner *et al.* 2009). By contrast, other authors reported no elevated risk (Jensen *et al.* 2009, Gadducci *et al.* 2013), while Brinton and collaborators sustained a possible incidence in the development of borderline ovarian tumors (Brinton *et al.* 2012).

The etiological and clinical approach to OC deeply changed following the discovery that OC can also arise from the spread of high-grade intraepithelial serous carcinoma originated in fallopian tubes (FT) epithelium (Kurman & Shih 2010, Cibula *et al.* 2011, Kim *et al.* 2012, Hua *et al.* 2016). Indeed, the genetic expression profile of this kind of cancer is much more related to FT rather than ovarian epithelium (Marquez *et al.* 2005, Eckert *et al.* 2016).

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-17-0687
Online version via www.reproduction-online.org

Discrepancies in clinical data on women undergoing fertility treatments are mainly due to the presence of biases like parity/nulliparity, kind of treatment and, most of all, infertility (Jensen *et al.* 2009, Sanner *et al.* 2009, Gadducci *et al.* 2013). The use of an animal model, such as the Swiss CD1 mouse, avoids the biases mentioned before, highlighting the damages exclusively linked to the treatment.

In our previous study (Di Luigi *et al.* 2014), by using the gonadotropin full-stimulation protocol usually adopted for mice (Hogan *et al.* 1994, Van Blerkom & Davis 2001), we demonstrated that 4 rounds (4R) of gonadotropin stimulation induced a significant increase of Cyclin D1 content in the FT. On the other hand, the expression levels of p53, AKT and of malignant transformation markers such as B-catenin, OCT3/4 and SOX2 remained unchanged (Di Luigi *et al.* 2014). In addition, we observed that 4R of gonadotropin stimulation altered oocytes meiotic spindle (Di Luigi *et al.* 2014). Starting from these results, here, we evaluated if further increasing to 6 and to 8 the number of repeated stimulations could modulate the expression levels of above-mentioned proteins, as well as of GSK3B, CHK1 and H2A.X.

Materials and methods

Chemicals

All the chemicals were of the purest analytical grade and were purchased from Sigma Chemical Company, unless otherwise indicated. Mouse monoclonal p53 and OCT3/4, rabbit polyclonal Cyclin D1, phospho-B-catenin (Thr41/Ser45), B-catenin, phospho-AKT (Ser473), AKT, phospho-GSK3B (Ser9), GSK3B and SOX2 primary antibodies were purchased from Santa Cruz Biotechnology; mouse monoclonal phospho-p53 (Ser15), rabbit monoclonal phospho-CHK1 (Ser345) and phospho-H2A.X (Ser139) were purchased from Cell Signaling Technology. Specific secondary antibodies were purchased from Santa Cruz Biotechnology.

Collection of oocytes and fallopian tubes

Mus musculus Swiss CD1 adult female mice (2–3-month-old; Harlan Italy, Udine, Italy; $N=27$) 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 early luteal phase of estrous cycle was confirmed by examination of the vaginal smears (Caligioni 2009, Byers *et al.* 2012, Cora *et al.* 2015) ($N=9$) were used as Control (Ctr). Repetitive cycles of ovarian stimulation were performed according to the protocol utilized in our previous study (Di Luigi *et al.* 2014). Five IU of PMSG (Folligon, Milano, Italy) were injected in mice. After 48 h, 5 IU of hCG (Corulon, Milano, Italy) were injected in animals. Six and eight rounds (6R, 8R) of stimulation were performed with intervals of 1 week between each. Fallopian tubes (FT) were collected from Control (Ctr; $N=9$) and hyperstimulated ($N=18$; 9 for 6R, 9 for 8R) mice, snap-frozen and stored at −80°C for Western blotting analysis.

Naturally ovulated oocytes were collected from FT of Ctr mice. Oocytes ovulated from hyperstimulated mice were recovered 14 h after the last hCG injection. In total, almost 100 oocytes were fixed for spindle analysis.

All experimental procedures involving animals and their care were performed in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ 358, 1 Dec 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana n. 40, Feb 18, 1992; National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985). The project was approved by the Italian Ministry of Health and the internal Committee of the University of L'Aquila. The method of killing consisted of an inhalant overdose of carbon dioxide (CO_2, 10–30%), followed by cervical dislocation. All efforts were made to minimize suffering.

Western blotting

FT samples 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 inhibitor (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). Membranes were incubated overnight at 4°C with specific primary antibodies: phospho-CHK1 (1:1000), phospho-H2A.X (1:1000), phospho-p53 (1:1000), p53 (1:200), Cyclin D1 (1:200), phospho-B-catenin (1:200), B-catenin (1:200), phospho-AKT (1:200), AKT (1:200), phospho-GSK3B (1:200), GSK3B (1:200), OCT3/4 (1:200), SOX2 (1:200) and then for 1 h with a peroxidase-conjugated anti-mouse (1:5000) or anti-rabbit (1:10000) secondary antibody. After detection using a chemiluminescence reagent (ECL, Pierce), the nitrocellulose membranes were examined using the Alliance LD2-77WL imaging system (Uvitec, Cambridge, UK). Densitometric quantification was performed with the public domain software NIH image v.1.62 and standardized using actin (1:200) as a loading control.

Analysis of meiotic spindles

To detect meiotic spindle morphology, oocytes and chromosomes were labeled as previously described (Rossi *et al.* 2006, Di Luigi *et al.* 2014). Briefly, oocytes were incubated for 1 h at 37°C with anti-A/B tubulin primary antibody (1:100) and then with anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (1:800). Chromosomes were labeled with Hoechst 33342 (1 µg/mL). The analysis was performed using a fluorescence microscope (40× objective; Axioplan 2; Zeiss) with digital images collected with Leica DFC350 FX camera interfaced with IM500 Leica software.

Statistical analysis

The experiments were replicated at least 3 times, and data were expressed as mean ± S.E.M. Differences between groups were analyzed for statistical significance using ANOVA with Tukey–Kramer multiple comparison test as post-test. Results were considered significantly different when $P<0.05$.

Results

Analysis of proteins involved in the control of cell cycle and DNA repair in FT

Comparable levels of total AKT were found in Ctr and repeatedly stimulated mice (Fig. 1A, Ctr vs 6R vs 8R, $P>0.05$). Conversely, phospho-AKT/AKT ratio significantly increased after 8R (+58%) when compared with Ctr and 6R (Fig. 1A; Ctr and 6R vs 8R, $P<0.05$). Moreover, there was a noticeable increase in AKT2 isoform phosphorylation level (Fig. 1A).

Total and phospho-kinase GSK3B contents were determined in Ctr and repeatedly stimulated mice. No variation of total GSK3B expression was found in all experimental groups (Fig. 1B; Ctr vs 6R vs 8R, $P>0.05$). Conversely, phospho-GSK3B/GSK3B ratio increased significantly after 8R of stimulation (+67%; Fig. 1B; Ctr vs 6R, $P>0.05$; Ctr, 6R vs 8R, $P<0.05$).

A very low Cyclin D1 level was found in Ctr (Fig. 1C), while a +27% and a +31% increases were recorded after 6R and 8R, respectively (Fig. 1C; Ctr vs 6R, 8R, $P<0.05$).

In contrast to the low level found in Ctr, a dose-dependent increase of total and phospho-p53 content occurred after 6R (+42%, +46%) and 8R (+67%, +63%) (Fig. 2A, B and C; 6R, 8R vs Ctr, $P<0.05$).

Prolonged treatments did not modulate total and phospho-B-catenin levels, while phospho-CHK1 and phospho-H2A.X remained undetectable (data not shown).

Expression levels of transcription factors SOX2 and OCT3/4 were reported in Fig. 3A and B. SOX2 was unaffected by treatments (Fig. 3A; Ctr vs 6R vs 8R, $P>0.05$). By contrast, a +31% increase of OCT3/4 occurred after 8R (Fig. 3B; Ctr, 6R vs 8R, $P<0.05$).

Spindle analysis in ovulated oocytes

The percentage of Ctr oocytes with normal, focused spindles and chromosome aligned on metaphase II plate was 98% (Fig. 4A, B and C). The percentage of oocytes with disorganized and asymmetric spindles arose concomitantly with rounds of stimulation. After 8R, the number of recovered oocytes drastically dropped (Fig. 4; Ctr vs 6R, $P<0.05$; Ctr vs 8R, $P<0.05$; 6R vs 8R, $P<0.05$).

Discussion

Results presented here demonstrate that in fallopian tubes (FT), the contents of phospho-AKT, phospho-GSK3B and phospho-p53, as well as of Cyclin D1, p53 and OCT3/4 increase together with the number of gonadotropin treatments.

Repetitive gonadotropin stimulation does not modulate total AKT expression, whilst kinase phosphorylation is enhanced after 8 rounds (8R). Interestingly, AKT2 is the isoform that is predominantly phosphorylated. Despite both AKT isoforms 1 and 2 sharing similar substrates (Cecconi *et al.* 2012), phospho-AKT2 seems to be more implicated in cancer cell invasiveness than phospho-AKT1 (Arboleda *et al.* 2003). To date, we do not know the reasons and consequences of AKT2 activation on FT epithelial cells functions. However, the fact that both AKT2 amplification and overexpression occur in OC (Bellacosa *et al.* 1995, Khabele *et al.* 2014) suggests

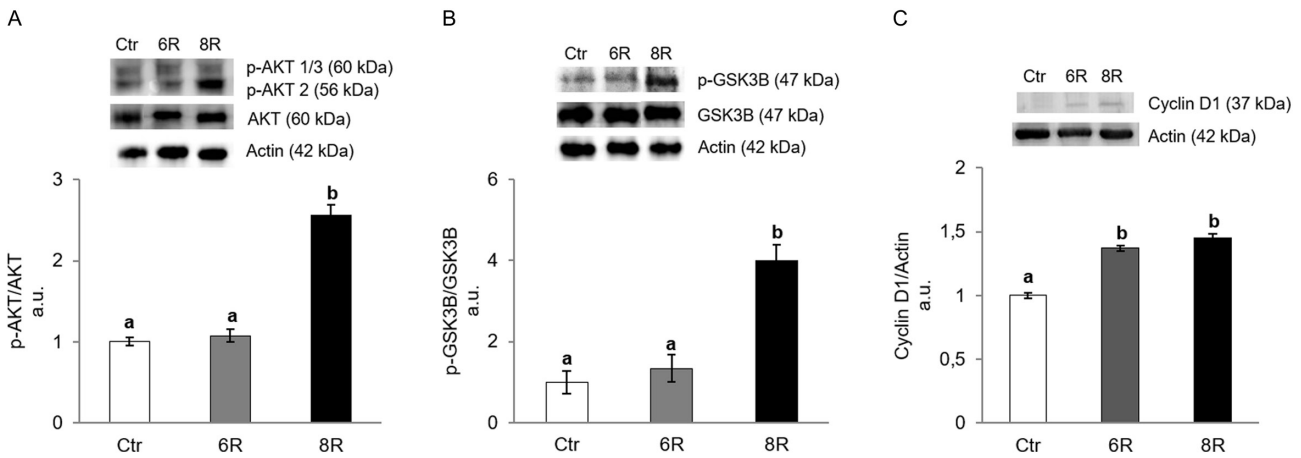


Figure 1 Western blot analysis of proteins involved in cell cycle control in fallopian tubes (FT). Ctr, Control mice; 6R and 8R, mice undergoing 6 rounds and 8 rounds of gonadotropin stimulation. p-AKT/AKT (A); p-GSK3B/GSK3B (B); Cyclin D1 (C). Data are expressed as arbitrary units (a.u.) of phosphorylated/total protein ratio (A and B) and protein/actin (C), used as loading control. The results are presented as mean ± S.E.M. of at least three independent experiments. Different letters: $P<0.05$.

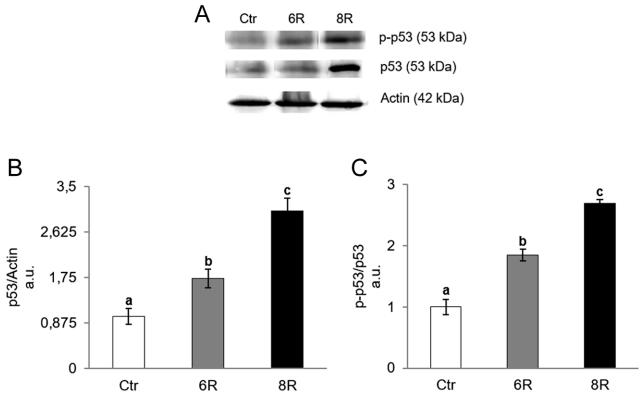


Figure 2 Western blot analysis of p53 and p-p53 in fallopian tubes (FT). Ctr, Control mice; 6R and 8R, mice undergoing 6 rounds and 8 rounds of gonadotropin stimulation (A). Data are expressed as arbitrary units (a.u.) of p53/actin (B), used as loading control and p-p53/p53 (C). The results are presented as mean ± S.E.M. of at least three independent experiments. Different letters: $P<0.05$.

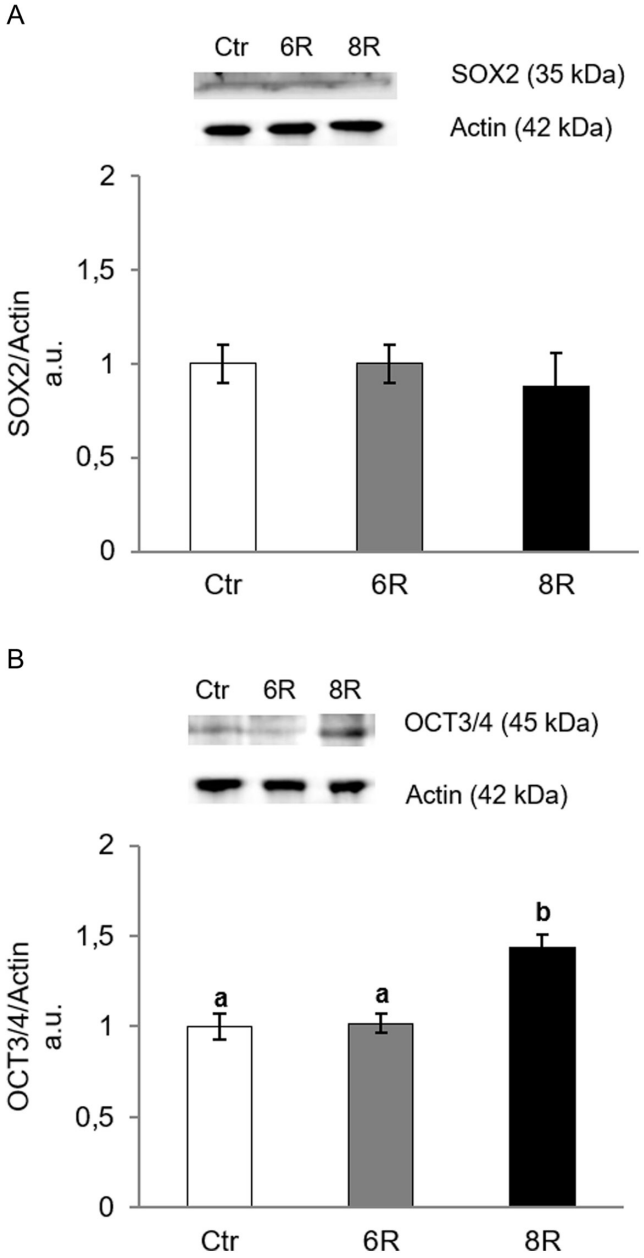
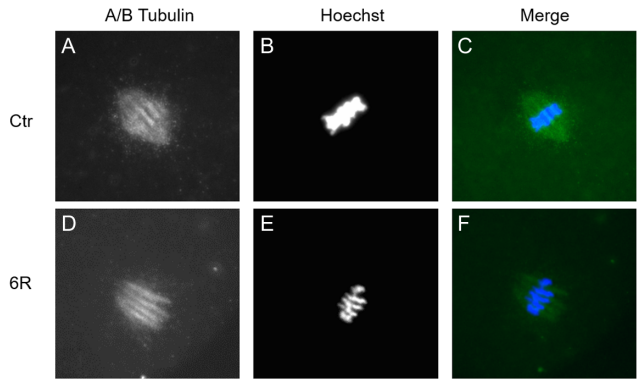


Figure 3 Western blot analysis of transcription factors in fallopian tubes (FT). Ctr, Control mice; 6R and 8R, mice undergoing 6 rounds and 8 rounds of gonadotropin stimulation. SOX2 (A); OCT3/4 (B). Data are expressed as arbitrary units (a.u.) of each protein/actin, used as loading control. The results are presented as mean ± S.E.M. of at least three independent experiments. Different letters: $P<0.05$.

that dysregulation of kinase activity might predispose to/take part in uncontrolled proliferation of tubal epithelial cells. After 8R, phospho-AKT is able to inactivate its substrate GSK3B, which is a regulator of Cyclin D1 phosphorylation and proteolytic turnover (Diehl *et al.* 1998). Since in FT the higher increase of Cyclin D1 content occurs after 8R, it could be possible that the high phospho-GSK3B level inhibits Cyclin D1 degradation (Diehl *et al.* 1998, Chang *et al.* 2003). Likely, the maximum increase of Cyclin D1 content recorded after 8R (+31% over Ctr) is insufficient to induce cancer.

It is of interest to note that elevated levels of gonadotropins are detected during post-menopause, when OC frequency rises (https://www.cancer.org/cancer/ovarian-cancer/causes-risks-prevention/risk-factors.html, Webb & Jordan 2017). Therefore, such physiological age-dependent modifications could contribute to determine conditions that, concomitantly with the presence of mutations/deletions of specific genes, facilitate OC onset (Corney *et al.* 2008, Bast *et al.* 2009, Kim *et al.* 2016).

The demonstration of stem cells in FT (Paik *et al.* 2012, Snegovskikh *et al.* 2014) prompted us to investigate the levels of expression of OCT3/4, B-catenin and SOX2, all key factors linked to stemness and usually overexpressed during malignant transformation in several adult tissues (Palma *et al.* 2008, Wang *et al.* 2014, Zeineddine *et al.* 2014, Hellner *et al.* 2016). In our previous study (Di Luigi *et al.* 2014), we found that 4R of gonadotropin stimulation did not modify the low expression levels of these proteins in FT. Conversely, a +31% increase of OCT3/4 content occurred after 8R, thereby supporting the existence of a relationship between repeated gonadotropin stimulation and overexpression of this transcription factor also in non-tumoral adult somatic cells. Our results support an interaction among OCT3/4, AKT phosphorylation and Cyclin D1. This observation is consistent with results obtained in embryonic stem cells (ESC) (Lin *et al.* 2012, Liu *et al.* 2017). In these cells, phospho-AKT and Cyclin D1 action can be correlated to the inhibition of OCT3/4 proteasomal degradation, thereby leading to the accumulation of this transcription factor (Lin *et al.* 2012, Liu *et al.* 2017). Based on the literature



Conditions tested	No. oocytes/mouse	% Spindle morphology	
		Normal	Abnormal
Ctr	18 ± 2[a]	98 ± 2[a]	2 ± 3[a]
6R	10 ± 2[b]	33 ± 1[b]	67 ± 2[b]
8R	1 ± 1[c]	0[c]	100[c]

Figure 4 Frequency of spindle defects in MII oocytes collected after 6 and 8 rounds (6R, 8R) of gonadotropin treatment. Spindles were labelled with anti A/B Tubulin antibody (A and D) and chromosomes with Hoechst 33342 (B and E; merge C and F). The different percentages of normal (C) and abnormal (F) configurations have been reported in the table. Data are expressed as mean ± S.E.M. of three independent experiments. Original magnification: ×40. Within each column, different superscripts indicate $P<0.05$.

data and on our results, we can hypothesize that the significant increase of phospho-AKT, Cyclin D1 and OCT3/4 contents after 8R could be representative of altered differentiation processes of FT epithelial cells.

The detrimental effects of 6R and 8R on the production of good-quality oocytes are confirmed by reduction of ovulated oocytes and increased meiotic spindle abnormalities. Hence, excessive gonadotropin administrations are able to perturb follicle development and acquisition of oocyte developmental competence (Van Blerkom & Davis 2001, Di Luigi *et al.* 2014).

In conclusion, our study indicates that, in mouse, a number of cycles from 6 to 8 can induce a different modulation of AKT, GSK3B, Cyclin D1, p53 and OCT3/4, as well as an impairment of ovulation and oocytes spindle organization. It should be noted that 6R of gonadotropin stimulation seems to be the threshold number of cycles without irreversible consequences on oocytes quality and on the expression levels of the proteins here analyzed.

These observations could be of interest if translated from a mouse model to young healthy donors approaching uncontrolled and repetitive stimulations for oocyte donation. Indeed, the possibility that such protocols could increase the risk of developing gynecological cancers in adulthood cannot be ruled out and should be carefully considered.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by Ministero dell'Università e della Ricerca (RIA 2015-16) to S C and G M T.

Acknowledgements

Authors would like to thank Gianfranco Ciccone and Anna Rita Nardecchia of the animal staff of University of L'Aquila for providing animal care.

References

Alper MM & Fauser BC 2017 Ovarian stimulation protocols for IVF: is more better than less? *Reproductive BioMedicine Online* **34** 345–353. (https://doi.org/10.1016/j.rbmo.2017.01.010)

Arboleda MJ, Lyons JF, Kabbinavar FF, Bray MR, Snow BE, Ayala R, Danino M, Karlan BY & Slamon DJ 2003 Overexpression of AKT2/protein kinase B beta leads to up-regulation of B1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Research* **63** 196–206.

Bast RC Jr, Hennessy B & Mills GB 2009 The biology of ovarian cancer: new opportunities for translation. *Nature Reviews Cancer* **9** 415. (https://doi.org/10.1038/nrc2644)

Bellacosa A, De Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V *et al*. 1995 Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *International Journal of Cancer* **64** 280–285. (https://doi.org/10.1002/ijc.2910640412)

Brinton LA, Sahasrabuddhe VV & Scoccia B 2012 Fertility drugs and the risk of breast and gynecologic cancers. *Seminars in Reproductive Medicine* **30** 131–145. (https://doi.org/10.1055/s-0032-1307421)

Byers SL, Wiles MV, Dunn SL & Taft RA 2012 Mouse estrous cycle identification tool and images. *PLoS ONE* **7** 1–5. (https://doi.org/10.1371/journal.pone.0035538)

Caligioni C 2009 Assessing reproductive status/stages in mice. *Current Protocols in Neuroscience* **48** A.4I.1–A.4I.8. (https://doi.org/10.1002/0471142301.nsa04is48)

Cecconi S, Mauro A, Cellini V & Patacchiola F 2012 The role of AKT signalling in the mammalian ovary. *International Journal of Developmental Biology* **56** 809–817. (https://doi.org/10.1387/ijdb.120146sc)

Chang F, Lee JT, Navolanic PM, Steelman LS, Shelton JG, Blalock WL, Franklin RA & McCubrey JA 2003 Involvement of PI3K/AKT pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia* **17** 590–603. (https://doi.org/10.1038/sj.leu.2402824)

Cibula D, Widschwendter M, Májek O & Dusek L 2011 Tubal ligation and the risk of ovarian cancer: review and meta-analysis. *Human Reproduction Update* **17** 55–67. (https://doi.org/10.1093/humupd/dmq030)

Cora MC, Kooistra L & Travlos G 2015 Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicologic Pathology* **43** 776–793. (https://doi.org/10.1177/0192623315570339)

Corney DC, Flesken-Nikitin A, Choi J & Nikitin AY 2008 Role pf p53 and Rb in ovarian cancer. In: *Ovarian Cancer. Advances in Experimental Medicine and Biology*, edn 1, vol **622**, pp. 99–117. Eds G Coukos, A Berchuck and R Ozols. New York: Springer. (https://doi.org/10.1007/978-0-387-68969-2_9)

Crawford NM, Sahay KM & Mersereau JE 2016 Mild stimulation versus conventional IVF: a cost-effectiveness evaluation. *Open Journal of*

Obstetrics and Gynecology **6** 180–188. (https://doi.org/10.4236/ojog.2016.63023)

Di Luigi G, Rossi G, Castellucci A, Leocata P, Carta G, Canipari R, Nottola SA & Cecconi S 2014 Repeated ovarian stimulation does not affect the expression level of proteins involved in cell cycle control in mouse ovaries and fallopian tubes. *Journal of Assisted Reproduction and Genetics* **31** 717–724. (https://doi.org/10.1007/s10815-014-0198-z)

Diehl JA, Cheng M, Roussel MF & Sherr CJ 1998 Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes and Development* **12** 3499–3511. (https://doi.org/10.1101/gad.12.22.3499)

Eckert MA, Pan S, Hernandez KM, Loth RM, Andrade J, Volchenboum SL, Faber P, Montag A, Lastra R, Peter ME *et al*. 2016 Genomics of ovarian cancer progression reveals diverse metastatic trajectories including intraepithelial metastasis to the fallopian tube. *Cancer Discovery* **6** 1342–1351. (https://doi.org/10.1158/2159-8290.CD-16-0607)

Fauser BC, Devroey P, Yen SS, Gosden R, Crowley WF Jr, Baird DT & Bouchard P 1999 Minimal ovarian stimulation for IVF: appraisal of potential benefits and drawbacks. *Human Reproduction* **14** 2681–2686. (https://doi.org/10.1093/humrep/14.11.2681)

Gadducci A, Guerrieri ME & Genazzani AR 2013 Fertility drug use and risk of ovarian tumors: a debated clinical challenge. *Gynecological Endocrinology* **29** 30–35. (https://doi.org/10.3109/09513590.2012.705382)

Hellner K, Miranda F, Fotso Chedom D, Herrero-Gonzales S, Hayden DM, Tearle R, Artibani M, KaramiNejadRanjbar M, Williams R, Gaitskell K *et al*. 2016 Premalignant SOX2 overexpression in the fallopian tubes of ovarian cancer patients: discovery and validation studies. *EBioMedicine* **10** 137–149. (https://doi.org/10.1016/j.ebiom.2016.06.048)

Hogan B, Beddington R, Costantini F & Lacy E 1994 *Manipulating the Mouse Embryo. A Laboratory Manual*. Edn 2. New York: Cold Spring Harbor Laboratory Press.

Homburg R, Meltcer S, Rabinson J, Scharf S, Anteby EY & Orvieto R 2009 Is there a limit for the number of in vitro fertilization cycles for an individual patient? *Fertility and Sterility* **91** 1329–1331. (https://doi.org/10.1016/j.fertnstert.2008.03.010)

Hua Y, Choi PW, Trachtenberg AJ, Ng AC, Kuo WP, Ng SK, Dinulescu DM, Matzuk MM, Berkowitz RS & Ng SW 2016 Epithelialization of mouse ovarian tumor cells originating in the fallopian tube stroma. *Oncotarget* **7** 66077–66086. (https://doi.org/10.18632/oncotarget.12296)

Jensen A, Sharif H, Frederiksen K & Kjær SK 2009 Use of fertility drugs and risk of ovarian cancer: Danish population based cohort study. *BMJ* **338** b249. (https://doi.org/10.1136/bmj.b249)

Khabele D, Kabir SM, Dong Y, Lee E, Rice VM & Son DS 2014 Preferential effect of AKT2-dependent signaling on the cellular viability of ovarian cancer cells in response to EGF. *Journal of Cancer* **5** 670–678. (https://doi.org/10.7150/jca.9688)

Kim J, Coffey DM, Creighton CJ, Yu Z, Hawkins SM & Matzuk MM 2012 High-grade serous ovarian cancer arises from fallopian tube in a mouse model. *PNAS* **109** 3921–3926. (https://doi.org/10.1073/pnas.1117135109)

Kim M, Lu F & Zhang Y 2016 Loss of HDAC-mediated repression and gain of NF-κB activation underlie cytokine induction in ARID1A- and PIK3CA-mutation.driven ovarian cancer. *Cell Reports* **17** 275–288. (https://doi.org/10.1016/j.celrep.2016.09.003)

Kurman RJ & Shih I-M 2010 The origin and pathogenesis of epithelial ovarian cancer – a proposed unifying theory. *American Journal of Surgical Pathology* **34** 433–443. (https://doi.org/10.1097/PAS.0b013e3181cf3d79)

Lin Y, Yang Y, Li W, Chen Q, Li J, Pan X, Zhou L, Liu C, Chen C, He J *et al*. 2012 Reciprocal regulation of AKT and Oct4 promotes the self-renewal and survival of embryonal carcinoma cells. *Molecular Cell* **48** 627–640. (https://doi.org/10.1016/j.molcel.2012.08.030)

Liu L, Michowski W, Inuzuka H, Shimizu K, Nihira T, Chick JM, Li N, Geng Y, Meng AY, Ordureau A *et al*. 2017 G1 cyclins link proliferation, pluripotency and differentiation of embryonic stem cells. *Nature Cell Biology* **19** 177–188. (https://doi.org/10.1038/ncb3474)

Marquez RT, Baggerly KA, Patterson AP, Liu J, Broaddus RR, Frumovitz M, Atkinson EN, Smith DI, Hartmann L, Fishman D *et al*. 2005 Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. *Clinical Cancer Research* **11** 6116–6126. (https://doi.org/10.1158/1078-0432.CCR-04-2509)

Pacchiarotti A, Selman H, Valeri C, Napoletano S, Sbracia M, Antonini G, Biagiotti G & Pacchiarotti A 2016 Ovarian stimulation protocol in IVF: an up-to-date review of the literature. *Current Pharmaceutical Biotechnology* **17** 303–315. (https://doi.org/10.2174/1389201017666160118103147)

Paik DY, Janzen DM, Schafenacker AM, Velasco VS, Shung MS, Cheng D, Huang J, Witte ON & Memarzadeh S 2012 Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serous cancer initiation. *Stem Cells* **30** 2487–2497. (https://doi.org/10.1002/stem.1207)

Palma I, Peña RY, Contreras A, Ceballos-Reyes G, Coyote N, Eraña L, Kofman-Alfaro S & Queipo G 2008 Participation of OCT3/4 and beta-catenin during dysgenetic gonadal malignant transformation. *Cancer Letters* **263** 204–211. (https://doi.org/10.1016/j.canlet.2008.01.019)

Pennings G & Ombelet W 2007 Coming soon to your clinic: patient friendly ART. *Human Reproduction* **22** 2075–2079. (https://doi.org/10.1093/humrep/dem158)

Revelli A, Casano S, Salvagno F & Delle Piane L 2011 Milder is better? Advantages and disadvantages of "mild" ovarian stimulation for human in vitro fertilization. *Reproductive Biology and Endocrinology* **9** 25. (https://doi.org/10.1186/1477-7827-9-25)

Rossi G, Macchiarelli G, Palmerini MG, Canipari R & Cecconi S 2006 Meiotic spindle configuration is differentially influenced by FSH and epidermal growth factor during in vitro maturation of mouse oocytes. *Human Reproduction* **21** 1765–1770. (https://doi.org/10.1093/humrep/del074)

Sanner K, Conner P, Bergfeldt K, Dickman P, Sundfeldt K, Bergh T, Hagenfeldt K, Janson PO, Nilsson S & Persson I 2009 Ovarian epithelial neoplasia after hormonal infertility treatment: long-term follow-up of a historical cohort in Sweden. *Fertility and Sterility* **91** 1152–1158. (https://doi.org/10.1016/j.fertnstert.2008.01.073)

Siristatidis C, Salamalekis G, Dafopoulos K, Basios G, Vogiatzi P & Papantoniou N 2017 Mild versus conventional ovarian stimulation for poor responders undergoing IVF/ICSI. *In Vivo* **31** 231–237. (https://doi.org/10.21873/invivo.11050)

Snegovskikh V, Mutlu L, Massasa E & Taylor HS 2014 Identification of putative fallopian tube stem cells. *Reproductive Sciences* **21** 1460–1464. (https://doi.org/10.1177/1933719114553448)

Van Blerkom J & Davis P 2001 Differential effects of repeated ovarian stimulation on cytoplasmic and spindle organization in metaphase II mouse oocytes matured in vivo and in vitro. *Human Reproduction* **16** 757–764. (https://doi.org/10.1093/humrep/16.4.757)

Wang H, Wang H, Makki MS, Wen J, Dai Y, Shi Q, Liu Q, Zhou X & Wang J 2014 Overexpression of β-catenin and cyclinD1 predicts a poor prognosis in ovarian serous carcinomas. *International Journal of Clinical and Experimental Pathology* **7** 264–271.

Webb PM & Jordan SJ 2017 Epidemiology of epithelial ovarian cancer. *Best Practice and Research: Clinical Obstetrics and Gynaecology* **41** 3–14. (https://doi.org/10.1016/j.bpobgyn.2016.08.006)

Zeineddine D, Hammoud AA, Mortada M & Boeuf H 2014 The Oct4 protein: more than a magic stemness marker. *American Journal of Stem Cells* **3** 74–82.

American Cancer Society 2014 What Are the Risk Factors for Ovarian Cancer? Available at: https://www.cancer.org/cancer/ovarian-cancer/causes-risks-prevention/risk-factors.html. Accessed on 10 September 2017.

Received 9 November 2017
First decision 8 December 2017
Revised manuscript received 15 December 2017
Accepted 4 January 2018