Effect of physiological levels of phytoestrogens on mouse oocyte maturation in vitro

Naoko Yoshida · Katsushige Mizuno

Received: 29 November 2010 / Accepted: 14 June 2011 / Published online: 26 June 2011
© Springer Science+Business Media B.V. 2011

Abstract Phytoestrogens are a group of naturally occurring compounds that have weak estrogenic activity. Genistein and daidzein are major phytoestrogens produced by soybeans. It has been reported previously that at high concentration, some phytoestrogens inhibit cell cycle progression of mouse germinal vesicle (GV) oocytes, but the environmentally relevant level is much lower. Here we show the effects of low concentrations of the isoflavones genistein, daidzein and the daidzein metabolite, equol, on mouse oocyte maturation. GV oocytes denuded of cumulus cells were cultured in TaM medium containing low levels (5 μ M) of genistein, daidzein, or equol. In all cases, the oocytes underwent normal GV break down, first polar body extrusion and became arrested at metaphase II (mII). As judged by fluorescence microscopy, the treated mII oocytes exhibited normal distributions of actin microfilaments, cortical granules and metaphase spindle formation with condensed metaphase chromatin. Moreover, mRNA expression levels of the cytostatic factors Emi2 and Mos were similar to those of their respective controls. These data suggest that exposure of maturing GV oocytes to environmental levels of genistein, daidzein or equol in vitro do not cause

negative effects on maturation to produce mII oocytes.

Keywords Genistein · Daidzein · Equol · Mouse · Oocyte maturation · Meiosis · Metaphase arrest

Abbreviations

GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
IVM	In vitro maturation
mI	First metaphase
mII	Second metaphase
CSF	Cytostatic factor

Introduction

In mammals, oogonia enter meiosis during embryonic development or around birth, but become arrested at the diplotene stage of the first prophase after birth. These meiotically quiescent oocytes carry a large nucleus, called the germinal vesicle (GV). Resumption of meiosis in GV oocytes within fully grown Graafian follicles is triggered by a preovulatory surge of luteinizing hormone (LH) from the pituitary gland (Eppig et al. 2004; Sun et al. 2009). Meiotic resumption includes chromatin condensation, disintegration of nuclear membrane (germinal vesicle breakdown, GVBD), chromosome segregation, extrusion of the first polar body (the first meiotic division) and a wealth of cytoplasmic changes, many of which

N. Yoshida (✉) · K. Mizuno
Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan
e-mail: nyoshida@sk.ritsume.ac.jp

are poorly characterized. After the completion of the first meiotic cell cycle, matured fertilizable oocytes arrested at the second metaphase (mII) are released from the ovary (ovulation). Matured oocytes are prevented from continuing the cell cycle and arrested at mII by cytostatic factor (CSF), thereby preventing parthenogenesis. This mII arrest correlates with the kinase activity of maturation promoting factor (MPF), a heterodimer of Cyclin B and the cyclin dependent kinase Cdc2 (Perry and Verlhac 2008). CSF stabilizes the activity of MPF and results in a cell cycle arrest that is unique to mII oocytes. Mos and endogenous meiotic inhibitor 2 (Emi2) are principal CSFs present in mouse oocytes and depleting either factor impairs the establishment of mII arrest (Colledge et al. 1994; Hahimoto et al. 1994; Choi et al. 1996; Shoji et al. 2006; Suzuki et al. 2010).

Ovarian function in adults is controlled by hormones circulating in the body. The primary hormone responsible for cyclicity in mammals is estrogen, which mostly produced in the ovary. Soybeans contain isoflavones often termed phytoestrogens according to their estrogenic activity. Phytoestrogens effectively bind to the estrogen receptors, although weakly compared to the physiological estrogens, and initiate estrogen-dependent transcription (Kuiper et al. 1998). The predominant phytoestrogens found in soy are genistein and daidzein. Neonatal mice treated with genistein cause alteration in ovarian development (Jefferson et al. 2005, 2006). Genistein or daidzein at high concentration inhibit meiotic resumption in vitro (Van Cauwenberge et al. 2000). Apart from these negative instances, effects on oocyte maturation at environmentally relevant dose have not yet been investigated precisely. Regarding the amount of phytoestrogens taken from diet, serum genistein has been measured in the range of 92.9 nM in Asian women, slightly lower than this in vegetarian women, and under 7 nM in non-vegetarian women (Jefferson and Williams 2011). Circulating levels of genistein in human infants exposed orally at the dose of 6–9 mg/kg-d is 1–5 μ M (Jefferson and Williams 2011).

Here, an in vitro assay was performed to determine what, if any, was the direct effect of low dose phytoestrogens on mouse oocyte maturation. Mouse GV oocytes undergo spontaneous meiotic resumption after isolation from Graafian follicles and can be cultured in a suitable medium in vitro. To eliminate

the influence from oocyte-surrounding cumulus cells, GV oocytes were denuded and cultured in control medium TaM which contains only BSA as a protein source (Miki et al. 2006). The phytoestrogens, genistein, daidzein or the daidzein metabolite equol were continuously present during the culture of test (but not control) oocytes throughout. Matured mII oocytes were evaluated carefully by analyzing morphology and mRNA levels. In vitro culture of denuded, meiotically competent oocytes is a useful model that may allow for the assessment of direct chemical effects, and methods adopted in this study could be used as simple indicators to determine maturation level of mII oocytes.

Materials and methods

Collection and culture of oocytes

Fully grown germinal vesicle stage (GV) oocyte-cumulus cells complexes were collected from ovaries of 8- to 10-week-old B6D2F₁ female mice (SHIMIZU Laboratory Supplier, Japan) intraperitoneally injected with pregnant mare serum gonadotrophin (PMSG) 44–48 h previously. Antral follicles were punctured using a 27 G needle in M2 medium (Sigma, USA). After 1 h culture in TaM medium (mixture of TYH and α MEM) (Miki et al. 2006) supplemented with 3 mg/mL bovine serum albumin (BSA) (Sigma), 10% (v/v) fetal bovine serum (FBS) (GIBCO Invitrogen, USA) and 150 μ M isobutylmethylxanthine (IBMX) (Sigma), cumulus cells were displaced from associated GV oocytes by repeated pipetting. GV oocytes were washed carefully to remove IBMX and subsequently cultured in 3 mg/mL BSA containing TaM control medium under mineral oil (Sigma) and incubated for 16 h in humidified CO₂ [5% (v/v) in air] at 37 °C (Suzuki et al. 2010). Stock solutions of 100 mM genistein (Wako, Japan), daidzein (Fujicco, Japan) or equol (Extrasynthese, France), each in DMSO, were diluted in control medium for each experiment respectively to 5, 25 or 100 μ M final concentration. Oviductal in vivo matured metaphase II (mII) oocytes were collected after 16 h human chorionic gonadotrophin (hCG) injection and cumulus cells were removed by hyaluronidase (Sigma) treatment. Superovulation was induced by standard serial injections of PMSG followed 48 h later by hCG.

Fluorescence staining of mII oocytes

Oocytes were fixed using 4% (w/v) paraformaldehyde for 15 min at room temperature. After washing with 0.1% BSA-PBS, samples were subsequently permeabilized and blocked in 0.1% Triton X-100/3% normal goat serum (Gibco Invitrogen)/0.1% BSA-PBS solution for 90 min. Staining for actin was performed using rhodamine-conjugated phalloidin (Molecular Probes, USA), cortical granules using FITC-conjugated *Lens culinaris* agglutinin (LCA) (Sigma) and tubulin using anti-mouse monoclonal α -tubulin antibody (Sigma) followed by anti-mouse IgG-Alexa 488 conjugate secondary antibody (Molecular Probes). All samples were double-stained with Hoechst No. 33258 (Sigma). Fluorescence was visualized using Zeiss Axiovert 200 microscope equipped with a BioRad Radiance 2100 laser scanning confocal system. Images were acquired using LaserSharp 2000 software and projection data exported as 8-bit TIFF files and processed using Adobe Photoshop CS4.

Real time PCR (qPCR)

3–4 mII oocytes were collected in 1 μ L 0.2% (w/v) sarkosyl and subjected to cDNA synthesis using Superscript III (Invitrogen) as described previously (Shoji et al. 2006). Prepared cDNA was used for ratiometric quantification of mRNAs by ABI Prism 7500 Sequence Detection System using Power SYBR Green PCR Master Mix (ABI, USA) and following primer pairs (5'–3'): H2afz, gcgtatcacccctcgtcacttg and tctctgtgtcctttctcccg; Mos, cagtgttgctacaatctgcg and agccttgaggtcccttgagg; Emi2, agtggtgagcagggtccaactctg and tgtttactccgtaggtgggtgagg. More than three independent samples per group were analyzed in duplicate per primer pair and data normalized with respect to H2afz.

Results

In order to assess any effects of phytoestrogen concentration on oocyte maturation, cumulus-free GV oocytes were cultured in vitro in TaM control medium containing either genistein, daidzein or equol from 1 to 100 μ M. After 16 h, oocytes were observed for any phytoestrogen effect on oocyte maturation. Gross maturation rates in the presence of

phytoestrogens at 1 μ M were the same as those at 5 μ M (data not shown). This titration indicated 5 μ M to correspond to the lower limit for this study. Appearances of representative IVM oocytes are shown in Fig. 1 and maturation rates for three different phytoestrogens at 5, 25 and 100 μ M are shown in Fig. 2. Oocytes were categorized into four groups according to the developmental stages, (1) GV: with clear germinal vesicle nucleus, (2) mI: GVBD oocytes without polar body, (3) mII: with polar body, (4) other: abnormal shape oocytes. Most (respectively 74.7 and 76.7%) of the oocytes cultured in 5 μ M genistein or daidzein underwent normal first polar body extrusion and matured to mII oocytes, although some stopped at mI (Fig. 1a, c). In contrast, none of the oocytes cultured in 100 μ M genistein matured to mII stage and all stopped either at mI or GV (Figs. 1b, 2a). The inhibitory effect of 100 μ M daidzein was smaller compared to 100 μ M genistein: 34.7% of the oocytes became mII, whilst the remainder arrested development (Figs. 1d, 2a). Analogous analyses with equol, a metabolite of daidzein, produced similar results as those of daidzein (Fig. 2b), and the daidzein effect may therefore indirectly have reflected equol cytotoxicity. Taken together, genistein, daidzein or equol clearly inhibited cell cycle progression of GV oocytes during IVM at high concentration (100 μ M), although <5 μ M had no significant effect; GV oocytes underwent, GVBD and first meiosis, with first polar body extrusion and maturation to mII oocytes at similar rates as controls.

The quality of mII oocytes matured in 5 μ M genistein, daidzein or equol was evaluated by checking both nuclear and cytoplasmic features in comparison with both untreated IVM and in vivo matured control oocytes. To visualize characteristic structures in mII oocytes, fluorescence staining was performed using specific dyes and antibodies. In mammalian oocytes, condensed metaphase chromosomes migrate to a cortical region just beneath the surface of plasma membrane. Characteristic actin microfilaments were observed at the plasma membrane proximal to condensed mII chromosomes (Fig. 3a). Also characteristically, LCA-labeled cortical granules were absent from the chromosome region (Fig. 3b). Mature metaphase spindle formations with chromosomes in the center were detected by α -tubulin immunostaining (Fig. 3c). There were no obvious differences in morphological structures (in particular,

Fig. 1 Effect of phytoestrogens on oocyte in vitro maturation (IVM). Images of Hoffman modulation contrast microscopy after 16 h culture in TaM control media containing different concentration of phytoestrogens. **a** 5 μ M genistein; **b** 100 μ M

genistein; **c** 5 μ M daidzein; **d** 100 μ M daidzein. Representative oocytes at different developmental stage are indicated as followed. White arrowhead: mII, black arrow: mI, black arrowhead: GV. Scale bar: 50 μ m

Fig. 2 Dose dependent inhibitory effect of phytoestrogens on oocyte development. After 16 h culture, oocytes were categorized into 4 groups according to the developmental stages. Oocytes showing abnormal appearances were grouped as the

other (striped area). Total numbers of scored oocytes are indicated at the top of each bar diagram. **a** genistein and daidzein treated oocytes; **b** equol treated oocytes. Control: TaM control medium, FBS: Control + 10% FBS

microtubules and chromosomes) between oocytes matured in vivo and 5 μ M genistein, daidzein (Fig. 3) or equol (data not shown).

We further evaluated cytoplasmic maturation in treated mII oocytes by determining mRNA levels for

the key meiotic regulators, Mos and Emi2 by real time PCR (qPCR). Mos and Emi2 are CSFs that establish and maintain mII in mammalian oocytes. IVM mII oocytes possessed slightly higher *Mos* and lower *Emi2* levels than respective levels of control

Fig. 3 Morphological characteristics of mII oocytes after IVM. Fluorescence images of confocal microscopy. In vitro matured mII oocytes were collected after 16 h culture. The concentration used for genistein or daidzein treatment was 5 μ M. **a** actin stained with phalloidin (red); **b** cortical granule stained with *Lens culinaris* agglutinin (LCA) (green); **c** tubulin

stained with anti- α -tubulin antibody (green); **a-c** mII chromosomes double-stained with Hoechst No. 33258 (blue). Distribution of LCA, configuration of actin microfilaments and tubulin spindle formation in IVM mII oocytes were similar to those of in vivo matured oocytes

IVM oocytes (Fig. 4). This could reflect small differences in the length of maturation time in vitro and in vivo. Under test conditions here, mII oocytes were evaluated after 16 h culture in the presence of 5 μ M genistein, daidzein or equol. For IVM samples, mII oocytes were also collected after 16 h following hCG injection to match the time from GV to mII. However, cell growth and development generally becomes slower in vitro and thus IVM mII oocytes could be at slightly immature stage of mII compared to in vivo. The expression levels of *Mos* are higher in IVM samples than that of in vivo samples because *Mos* decline accompanies to mII oocyte aging (Suzuki et al. 2010). Nevertheless, the addition of 5 μ M genistein, daidzein or equol did not show significant difference in either *Mos* or *Emi2* mRNA levels when compared to control IVM mII oocytes.

Discussion

The present work is the first to test the effects on oocyte maturation of the environmental phytoestrogens daidzein and its metabolite equol in comparison with genistein at a relevant level (5 μ M). Genistein is

an inhibitor of protein tyrosine kinases (PTKs) and affects diverse cell functions (Dixon and Ferreira 2002). The inhibitory affect of genistein on the maturation of mammalian oocytes has been reported previously (Jung et al. 1993; Van Cauwenberge and Alexandre 2000). However, the reported effects were not necessarily due to PTK inhibition, because daidzein, which is a structurally related isoflavone, which does not inhibit PTK but did interfere with IVM. In both genistein and daidzein, meiotic inhibition was recorded at 100–200 μ M (Van Cauwenberge and Alexandre 2000). These results coincide with ours, in which addition of genistein or daidzein each at 100 μ M inhibited cell cycle progression and IVM (Figs. 1b, d, 2a). Genistein induces G2/M arrest in mammalian cells by a different mechanism than PTK inhibition that is due in part to the inhibition of Cdc2 and Cdk2 kinase activities (Matsukawa et al. 1993; Choi et al. 1998; Balabhadrapathruni et al. 2000).

We also evaluate the, daidzein metabolite, equol (Fig. 2b). Yeast trans-activation, E-screen and ER binding assays, suggest that genistein and equol possess more profound estrogenic activity than daidzein (Choi et al. 2008). Also, genistein and equol are ranked higher than daidzein for estrogenic

Fig. 4 Real time PCR (qPCR) analysis showing cytotaxic factor (CSF) mRNA levels in mII oocytes after IVM. In vitro matured mII oocytes were collected after 16 h culture. The concentration used for genistein, daidzein or equol treatment was 5 μ M. **a** genistein and daidzein treated oocytes; **b** equol treated oocytes. Each value was normalized against H2afz and presented relative to the value for in vivo matured mII oocytes. Control: TaM control medium, FBS: Control + 10% FBS. Data represent means $\pm$ SEM ($n = 3$)

potency against human breast cancer cells (Pugazhendhi et al. 2008). Accordingly, addition of 100 μ M genistein effectively blocked oocyte maturation to mII. Compared to genistein, the effect of 100 μ M daidzein was less efficient and GV oocytes matured to mII in some ($\sim 34.7\%$) cases. In spite of the stronger estrogenic activity of equol, the IVM rate in its presence was not very different from that of daidzein (Fig. 2). It has often been reported that genistein induces apoptosis in cancer cells cultured in vitro, nevertheless little, if any, cell death or apoptosis was observed in oocyte culture here (Balabhadrapathruni et al. 2000; Khan et al. 2010).

Studies have been performed investigating the effect at low concentration of genistein (Chan 2009) on mouse, and daidzein (Galeati et al. 2010) on pig IVM, fertilization and development, but using cumulus-oocyte complexes (COCs), although in the

present study, cumulus-oophorous complexes were completely removed to eliminate the possibility that cumulus cells might be responsible for the buffering of cytotoxicity. In these earlier studies, Genistein produced a slight decrease in IVM in a dose dependent manner between 1 and 10 μ M (Chan 2009). As there was no significant difference between control and 5 μ M genistein in our system (Fig. 2a), this discrepancy may indeed have occurred due to the buffering effect of cumulus cells, rather than due to a direct effect on oocytes. Neither 1 nor 10 μ M daidzein induced significant variations in IVM, although P4 steroid production by cumulus cells was significantly inhibited (Galeati et al. 2010).

Within ovarian follicles, oocytes form gap junctions with granulosa cells (cumulus cell precursors) and meiotic resumption is prevented by mechanisms that include the maintenance of relatively high levels of cyclic adenosine 3', 5'-monophosphate (cAMP) (Sun et al. 2009). The preovulatory surge of LH that triggers mammalian ovulation and meiotic resumption, also stimulates an increase of follicular steroid production (Tsafiri and Motoka 2007). In rat and mouse follicles, LH or hCG stimulation induces a transient increase in progesterone and estrogen at a crucial time before meiosis (Su et al. 2006). Yet studies with steroid receptor- or with steroid synthetic enzyme-deficient mice revealed that, despite impaired ovarian development and function, oocyte maturation was unaffected (Fisher et al. 1998; Conneely et al. 2001; Barnett et al. 2006). Collectively these studies and ours suggest that steroids play more important roles in ovarian follicular development than oocyte maturation itself.

We corroborated this by evaluation of sub-cellular structures and CSF mRNA levels in mII oocytes that had matured under the conditions of the experiments (Figs. 3, 4). Nuclear maturation of mII oocytes was monitored via GVBD, first polar body extrusion and metaphase spindle formation with condensed chromosomes (Fig. 3). Whilst *Mos* and *Emi2* transcript levels are a valuable initial indicator of functional and healthy oocytes, they are insufficient parameters for a full evaluation. To confirm full oocyte maturation to a functional state, it is desirable to perform in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Yoshida and Perry 2007) with IVM oocytes, and observe responses to the sperm-borne activation signal and monitor subsequent embryogenesis.

Acknowledgments We appreciate Dr. Tony Perry for reading the manuscript and giving us critical comments.

References

- Balabhadrapathruni S, Thomas TJ, Yurkow EJ, Amenta PS, Thomas T (2000) Effects of genistein and structurally related phytoestrogens on cell cycle kinetics and apoptosis in MDA-MB-468 human breast cancer cells. *Oncol Rep* 7:3–12
- Barnett KR, Schilling C, Greenfield CR, Tomic D, Flaws JA (2006) Ovarian follicle development and transgenic mouse models. *Hum Reprod Update* 12:537–555
- Chan WH (2009) Impact of genistein on maturation of mouse oocytes, fertilization, and fetal development. *Reprod Toxicol* 28:52–58
- Choi T, Fukasawa K, Zhou R, Tessarollo L, Borror K, Resau J, Vande Woude GF (1996) The Mos/mitogen-activated protein kinase (MAPK) pathway regulate the size and degradation of the first polar body in maturing mouse oocytes. *Proc Natl Acad Sci USA* 93:7032–7035
- Choi YH, Zhang L, Lee WH, Park KY (1998) Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. *Int J Oncol* 13:391–396
- Choi SY, Ha TY, Ahn JY, Kim SR, Kang KS, Hwang IK, Kim S (2008) Estrogenic activities of isoflavones and flavones and their structure-activity relationships. *Planta Med* 74:25–32
- Colledge WH, Carlton MB, Udy GB, Evans MJ (1994) Disruption of *c-mos* causes parthenogenetic development of unfertilized mouse eggs. *Nature* 370:65–68
- Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ (2001) Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. *Mol Cell Endocrinol* 179:97–103
- Dixon RA, Ferreira D (2002) Genistein. *Phytochemistry* 60:205–211
- Eppig JJ, Vivieros MM, Marin-Bivens C, De La Fuente R (2004) Regulation of mammalian oocyte maturation. In: Leung PCK, Adashi EY (eds) *The Ovary*, 2nd edn. Elsevier Academic Press, Amsterdam, pp 113–129
- Fisher CR, Graves KH, Parlow AF, Simpson ER (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. *Proc Natl Acad Sci U S A* 95:6965–6970
- Galeati G, Vallorani C, Bucci D, Bernardini C, Tamanini C, Parmeggiani A, Spinaci M (2010) Daidzein does affect progesterone secretion by pig cumulus cells but it does not impair oocytes IVM. *Theriogenology* 74:451–457
- Hahimoto N, Watanabe N, Furuta Y, Tamemoto H, Sagata N, Yokoyama M, Okazaki K, Nagayoshi M, Takeda N, Ikawa Y, Aizawa S (1994) Parthenogenetic activation of oocytes in *c-mos*-deficient mice. *Nature* 370:68–71
- Jefferson WN, Williams CJ (2011) Circulating levels of genistein in the neonate, apart from dose and route, predict future adverse female reproductive outcomes. *Reprod Toxicol* 31:272–279
- Jefferson WN, Padilla-Banks E, Newbold RR (2005) Adverse effects on female development and reproduction in CD-1 mice following neonatal exposure to the phytoestrogen genistein at environmentally relevant doses. *Biol Reprod* 73:798–806
- Jefferson WN, Newbold R, Padilla-Banks E, Pepling M (2006) Neonatal genistein treatment alters ovarian differentiation in the mouse: inhibition of oocyte nest breakdown and increased oocyte survival. *Biol Reprod* 74:161–168
- Jung T, Fulka JJ, Lee C, Moor RM (1993) Effect of the protein phosphorylation inhibitor genistein on maturation of pig oocytes in vitro. *J Reprod Fertil* 98:529–535
- Khan N, Adhami VM, Muhtar H (2010) Apoptosis by dietary agents for prevention and treatment of prostate cancer. *Endocr Relat Cancer* 17:R39–R52
- Kuiper GG, Lemmen JG, Carlsson BO, Corton JC, Safe SH, van der Saag PT, van der Burg V, Gustafsson JA (1998) Interaction estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* 139:4252–4263
- Matsukawa Y, Marui N, Sakai T, Satomi Y, Yoshida M, Matsumoto K, Nishino H, Aoki A (1993) Genistein arrests cell cycle progression at G2-M. *Cancer Res* 53:1328–1331
- Miki H, Ogonuki N, Inoue K, Baba T, Ogura A (2006) Improvement of cumulus-free oocyte maturation in vitro and its application to microinsemination with primary spermatocytes in mice. *J Reprod Dev* 52:239–248
- Perry ACF, Verlhac M-H (2008) Second meiotic arrest and exit in frogs and mice. *EMBO Rep* 9:246–251
- Pugazhendhi D, Watson KA, Mills S, Botting N, Pope GS, Darbe PD (2008) Effect of suphation on the estrogen agonist activity of the phytoestrogens genistein and daidzein in MCF-7 human breast cancer cells. *J Endocrinol* 197:503–515
- Shoji S, Yoshida N, Amanai M, Ohgishi M, Fukui T, Fujimoto S, Nakano Y, Kajikawa E, Perry ACF (2006) Mammalian Emi2 mediates cytostatic arrest and transduces the signal for meiotic exit via Cdc20. *EMBO J* 25:834–845
- Su YQ, Nyegaard M, Overgaard MT, Qiao J, Giudice LC (2006) Participation of mitogen-activated protein kinase in luteinizing hormone-induced differential regulation of steroidogenesis and steroidogenic gene expression in mural and cumulus granulosa cells of mouse preovulatory follicles. *Biol Reprod* 75:859–867
- Sun QY, Miao YL, Schatten H (2009) Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. *Cell Cycle* 8:2741–2747
- Suzuki T, Suzuki E, Yoshida N, Kubo A, Li H, Okuda E, Amanai M, Perry ACF (2010) Mouse Emi2 as a distinctive regulatory hub in second meiotic metaphase. *Development* 137:3281–3291
- Tsafiriri A, Motoka S (2007) Are steroids dispensable for meiotic resumption in mammals? *T Endocrinol Metabol* 18:321–327
- Van Cauwenberge A, Alexandre H (2000) Effect of genistein alone and in combination with okadaic acid on the cell cycle resumption of mouse oocytes. *Int J Dev Biol* 44:409–420
- Yoshida N, Perry ACF (2007) Piezo-actuated mouse intracytoplasmic sperm injection (ICSI). *Nat Protoc* 2:296–304