Reprod Med Biol (2014) 13:153–159
DOI 10.1007/s12522-014-0177-1

ORIGINAL ARTICLE

# Effect of single-oocyte culture system on in vitro maturation and developmental competence in mice

**Manami Nishio · Yumi Hoshino · Kentaro Tanemura · Eimei Sato**

Received: 25 June 2013 / Accepted: 27 January 2014 / Published online: 14 February 2014


**Abstract**
*Purpose* To investigate whether single-culture systems influence the quality of in vitro-matured oocytes, we examined the maturation and developmental competence of oocytes obtained by grouped in vitro maturation (IVM) or single IVM.
*Methods* In vitro-matured oocytes were obtained using the culture drop (CD) method for the grouped IVM experiments, and the CD and hanging drop (HD) method for the single IVM experiments. To evaluate oocyte developmental competence, we performed in vitro fertilization and culture, and counted the number of blastocysts. To evaluate the oocyte cytoplasmic maturation, we measured the maturation promoting factor (MPF) expression levels.
*Results* Oocytes cultured singly had lower maturity and developmental competence than the grouped IVM oocytes. However, enhanced oocyte fertility and blastocyst quality was achieved by the HD single IVM method. Additionally, the MPF activity level increased in all culture methods, compared to the control; however, it lagged behind nuclear maturation.
*Conclusions* These results suggest that the HD method is efficient for single IVM.

**Keywords** Hanging drop · In vitro maturation · Mouse oocyte · Oocyte quality · Single culture

M. Nishio · Y. Hoshino (✉) · K. Tanemura · E. Sato
Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan
e-mail: hoshimi@bios.tohoku.ac.jp

## Introduction

In vitro maturation (IVM) technologies using mammalian oocytes such as those from cattle and mice [1–3] have been widely developed and modified to allow the effective use of immature oocytes in follicles. The IVM technology is important to many fields, including the animal industry, preservation of wild animals, and assisted human reproduction technology. It has been intensely studied to assess the efficiency and quality of in vivo embryo development following in vitro fertilization and in vitro culture (IVF–IVC). However, at present, the fertility and developmental competence of in vitro-matured oocytes are lower than that of in vivo-matured oocytes [4]. Therefore, it is important to improve IVM conditions.

The number of cumulus-oocyte complexes (COCs) in IVM affects the oocyte maturation rate because the cumulus cells and oocytes maintain the supply of paracrine and autocrine factors, which are essential for oocyte development [5]. The results of a previous study suggest that high paracrine and autocrine activities are achieved in cultures with more than 20 COCs [5]. However, grouped oocyte culture has several drawbacks for application to the animal industry and experimental animal research studies. This is because grouped IVM oocytes are indistinguishable from each other, and therefore, it is very difficult to differentiate them. In animal research studies, grouped IVM produces unsatisfactory results, as it requires the use of many animals and is, therefore, against the 3R principles: replacement, reduction, and refinement. Moreover, it is inefficient to use many oocytes for one experiment.

Single IVM can overcome many problems of grouped IVM. In laboratory research, single IVM requires smaller numbers of oocytes, and therefore, a smaller number of animals for each individual experiment, thereby making it

applicable for high-throughput testing. Additionally, it is easy to modify and control the microenvironment. Therefore, it is of great importance that an efficient single-oocyte culture system be developed.

Previously, we have investigated the influence of droplet size and number of COCs in the IVM experiment on mouse oocyte quality [6]. Our results demonstrated no significant difference between the groups with 20 COCs/100 μL and five COCs/200 μL, and we suggest that ≤5 COCs in the IVM culture produces greater blastocyst numbers.

In our previous study, we used the IVM culture drop (CD) method [6]. In this method, a microdroplet is placed on the bottom of a dish under oil. Although the CD method is widely used, it possesses some shortcomings. Therefore, all cumulus cells are able to expand, and their maturation and developmental competence is high. The lower cumulus cells are degenerated, leading to reduced oocyte maturation and developmental competence.

In this study, we utilized the hanging drop (HD) method in addition to the CD method. Generally, the suspension culture methods can achieve mass embryonic body production [7–9]. The HD method is an effective technique for differentiating embryonic stem cells into a number of cell types [10–12], and it has been extensively used to culture embryonic bodies.

Therefore, the aim of the present study was to establish a culture system that could improve the quality of oocytes matured by single IVM. Initially, we examined the maturation rates of oocytes cultured in a droplet in the presence or absence (control) of degenerated oocytes. Then, we compared the results obtained from grouped IVM and single IVM by using the CD and HD methods. To this end, we investigated the maturation and developmental competence of oocytes matured by the CD and HD methods.

## Materials and method

### Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO, USA).

### Animals

ICR mice were purchased from Japan SLC, Inc. (Shizuoka), and bred in our laboratory. Immature 20- to 22-day-old mice were used for all experiments. The experimental procedures described in this report were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by Tohoku University. The Tohoku University's approval number of these animal experiments are '2011-1' and '2012-1'.

### Oocyte collection

Oocyte collection was performed according to previously described methods [6]. The immature COCs were collected from small antral follicles, at 48 h following intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (Teikoku Hormone MFG, Tokyo). The ovaries were dissected and collected in Leibovitz's L-15 medium (Invitrogen, Grand Island, NY), supplemented with 0.1 % polyvinyl alcohol (PVA). To prevent spontaneous resumption of meiosis, 4 mM hypoxanthine was added to this medium. The COCs were mechanically isolated by puncturing antral follicles with a 26-gauge needle. Only COCs that consisted of an oocyte surrounded by a compact cumulus cell mass were selected for the experiments.

### In vitro maturation

In vitro maturation of oocytes was performed according to previously described methods [6]. The culture medium consisted of Waymouth's MB752/1 medium (Invitrogen) supplemented with 5 % fetal calf serum (Gemini Bio, CA), 0.23 mM pyruvic acid, 75 mg/L penicillin G (Meiji Seika, Tokyo), 50 mg/L streptomycin sulfate (Meiji Seika), 4 mM hypoxanthine, 100 mIU/mL follicle-stimulating hormone (FSH), and 0.1 % PVA. The COCs were cultured in droplets of the culture medium overlaid with paraffin liquid (Nacalai Tesque, Kyoto) in a humidified atmosphere of 5 % CO2 in air at 37 °C. For the IVM and IVM–IVF–IVC experiments, the COCs were cultured for 18 h, whereas for the experiments evaluating nuclear and cytoplasmic maturation, the COCs were cultured for 12, 15, 18, and 21 h. At the end of the incubation period, the oocytes were removed from the cumulus cells by treatment with 0.1 % hyaluronidase at room temperature.

### Culture methods

We used the CD and HD methods for the IVM experiments. In the CD method, 100-μL droplets of the culture medium were placed on the bottom of the 35-mm dish [6, 13]. In the HD method, 10 μL of the culture medium was placed in each well of the plate. The maximum volume put in one-well is 10 μL. In this study, we used three experimental groups: control (20 COCs cultured by the CD method), CD-1 (one COC was cultured by the CD method), and HD-1 (one COC was cultured by the HD method).

### In vitro fertilization and culture

In vitro fertilization and embryo cultures were performed according to previously described methods with slight

modifications [6]. In the IVF experiments, we examined oocytes incubated for 18 h. Spermatozoa were collected from the cauda epididymis and preincubated for 1–2 h in 400 μL of human tubal fluid (HTF) medium to allow capacitation before insemination. After capacitation, the spermatozoa were introduced into 200-μL droplets of the HTF medium at a final concentration of 700 spermatozoa/μL. At 4 h after insemination, the penetration of the sperms into the oocytes was confirmed by microscopic examination; subsequently, the oocytes were thoroughly washed five times and cultured in the potassium simplex optimized medium (KSOM). All embryos were incubated in 100-μL droplets of KSOM in a humidified atmosphere of 5 % CO2 in air at 37 °C. In the control group, all metaphase II (MII) oocytes collected after IVM were examined for each IVF experiment. In the CD-1 and HD-1 experiments, 16–18 of the MII oocytes were examined in each IVF experiment.

### Blastocyst cell counting

Blastocysts were quantified according to previously described methods [6]. Blastocysts were fixed with 2 % paraformaldehyde in Dulbecco's phosphate-buffered saline containing 0.1 % PVA and 0.2 % Triton X-100 at room temperature. Next, the embryos were stained with 5 μg/mL Hoechst 33342. The stained blastocysts were transferred to glass slides, and a glass cover was placed atop. The slides were examined under an ultraviolet fluorescence microscope.

### MPF activity measurements

At 12, 15, 18, and 21 h after incubation, 20 cumulus-free MII oocytes from each of the three groups were transferred into microtubes containing 5 μL of cell lysis buffer supplemented with 1 mM phenyl-methyl-sulfonyl fluoride. The samples were frozen at −80 °C until use for further analysis. Just before the assay, 30 μL of Milli-Q water was added to the microtubes, and the oocytes were completely destroyed by ultrasonographic treatment (3 × 30 s). The MPF activity in the oocytes was measured using a MESACUP cdc2 kinase enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems).

### Statistical analysis

Each experiment was performed at least four times for every group. The data were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's test. All data are expressed as the mean ± standard deviation (SD). A $P$ value of <0.05 was considered statistically significant.

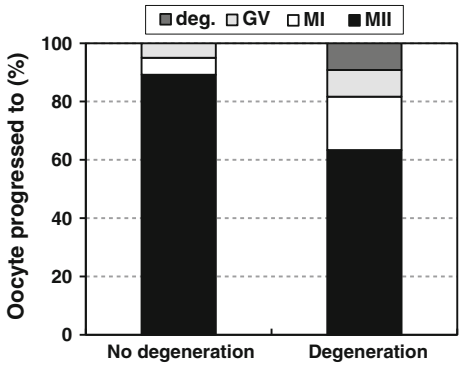


**Fig. 1** Degenerated oocytes in a droplet suppressed the maturation of other oocytes. Twenty oocytes were cultured by the culture drop (CD) method for 18 h. Two degenerated oocytes were added into the culture medium of the degenerated group. Values represent the mean of six replicates (total 120 oocytes). *Deg*. degenerated oocytes, *GV* germinal vesicle, *MI* metaphase I, *MII* metaphase II

## Results

### Effect of degenerated oocytes on the maturation of other oocytes

To determine whether the degenerated oocytes affect the maturation of other oocytes, we examined the maturation rates of oocytes cultured in a droplet in the presence or absence (control) of degenerated oocytes. The maturity of oocytes was evaluated by observing the emission of the first polar body. The maturation rates of the oocytes are shown in Fig. 1. The maturation rate in the absence of the degenerated oocytes (*No degeneration*) was 89.2 %, whereas that in the presence of the degenerated oocytes (*Degeneration*) was lower by 16 % (63.3 %). Indeed, the ratio of metaphase I(MI) oocytes in the *Degeneration* group was 3-fold greater than that in the control.

These results suggest that the presence of degenerated oocytes delays or arrests the meiotic maturation of the other oocytes. This finding demonstrates a weakness of using grouped IVM.

### Evaluation of oocyte maturation competence

Next, we assessed the influence of the culture methods on oocyte maturation and developmental competence. The maturity of the oocytes was evaluated by observing the first polar body emission. Specifically, we regarded oocytes with absent nuclear membranes and first polar bodies as having undergone germinal vesicle breakdown (GVBD). The ratios of oocytes from each treatment group are shown in Table 1. In the CD-1 experiments, both the GVBD and maturation rates were significantly less than those observed in the control ($P < 0.05$). Comparatively, in the HD-1

**Table 1** Effects of different IVM methods on oocyte maturation

| Experimental group | Total number of oocytes | GVBD (% ± SD) | MII (% ± SD) |
|---|---|---|---|
| Control | 120 | 95.9 ± 2.0 | 89.2 ± 3.8 |
| CD-1 | 123 | 87.8 ± 2.7* | 77.3 ± 3.4* |
| HD-1 | 121 | 95.0 ± 4.4 | 77.8 ± 3.4* |

Values represent the mean of six replicates ± SD

*CD* culture drop, *HD* hanging drop, *GVBD* oocytes having undergone germinal vesicle break down, *MII* oocytes progressed to metaphase II

* Values that are significantly different from control ($P < 0.05$; ANOVA)

experiments, the maturation rate was significantly less than that of the control ($P < 0.05$), but the GVBD rate showed no significant difference. These results indicate that single IVM produces reduced the number of mature oocytes; however, the resumption of meiotic maturation was normal in the HD method.

### Evaluation of early embryonic developmental competence

To determine whether the IVM methods affect early embryonic development, we performed IVF–IVC using MII oocytes that were matured via the HD and CD methods. As illustrated in Table 2, data obtained in CD-1 were significantly lower than those in the control ($P < 0.05$). The pronuclei formation rates and the rate of development to the two-cell embryo stage of the HD-1 cells did not differ from that of the control; however, the rate of development to blastocysts was significantly less than that of the control.

Next, we quantified the blastocysts in each group to evaluate their quality (Fig. 2). The total cell number of blastocysts in the HD-1 group was not significantly different from that of the control. These results suggest that although the numbers of blastocysts obtained by single IVM were decreased, the quality in the HD-1 group did not differ from that of the control.

### Effects of different IVM methods on the nuclear and cytoplasmic maturation of oocytes

To investigate the reason for the deterioration of oocyte fertility and early embryonic development competence in the single IVM methods, we evaluated the maturation kinetics of oocytes.

First, we observed the maturation kinetics to test whether different culture methods affect nuclear maturation rates. These experiments were repeated 10 times (total 200 oocytes), and Fig. 3 displays the oocyte maturation rates at 12, 15, 18, and 21 h. The rate of oocyte nuclear maturation at 12 h was higher in the single IVM method than in the control group, and the HD-1 group showed the highest rate among all the IVM methods. However, at 18 h after incubation, the maturation rate in the control group exceeded the single IVM groups.

Next, we measured the MPF activity of the in vitro-matured oocytes to test whether different culture methods affect cytoplasmic maturation rates. High MPF activity is required for MII arrest until its fertilization [14]. These experiments were repeated 10 times (total 200 MII oocytes). We set the basal values (the control group at 18 h after incubation) as 100 % and calculated the percentage of each group at 12, 15, 18, and 21 h. As illustrated in Fig. 4, the MPF level in the CD-1 group peaked at 15 h, and was at its highest in the control and HD-1 groups at 18 h. Interestingly, the MPF level in the HD-1 group exceeded the amounts in the control group at 18 h after incubation.

## Discussion

In the present study, we developed a new culture method, namely, the HD method. The HD method was utilized to culture embryonic bodies [7–9] and follicles [15]. The HD

**Table 2** Effects of the culture methods on oocyte fertilization and embryonic development

| Experimental group | MII oocytes | Twi-pronuclei (% ± SD)[a] | Multiple-pronuclei (% ± SD)[a] | Two-cell embryos (% ± SD)[b] | Blastocysts (% ± SD)[b] |
|---|---|---|---|---|---|
| Control | 97 | 93.8 ± 5.6 | 4.1 ± 3.2 | 95.5 ± 5.5 | 82.3 ± 9.4 |
| CD-1 | 102 | 82.6 ± 5.1* | 10.1 ± 4.3* | 83.5 ± 9.9* | 53.6 ± 5.4* |
| HD-1 | 98 | 89.7 ± 3.5 | 5.2 ± 2.5 | 92.2 ± 5.1 | 47.7 ± 4.1* |

Values are expressed as the mean of six replicates ± SD

*CD* culture drop, *HD* hanging drop

* Values that are significantly different from control ($P < 0.05$; ANOVA)

[a] Values per MII oocytes

[b] Values per two-pronuclei

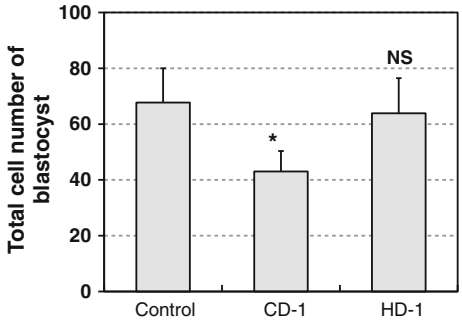


**Fig. 2** Effects of different IVM conditions on the number of blastocysts after IVF and IVC. Values represent the mean of 20 blastocysts ±SD. *TCN* total cell number, *CD* culture drop, *HD* hanging drop, *NS* no significant difference

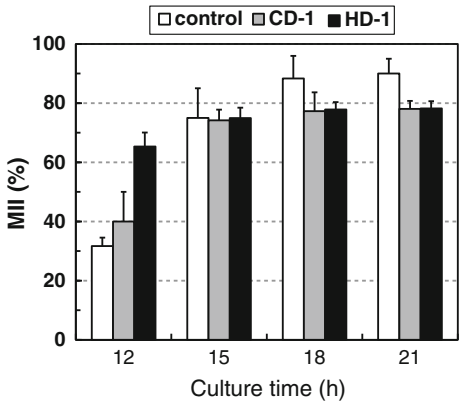


**Fig. 3** Effects of the different IVM conditions on the maturation rate of the in vitro matured oocytes. Values represent the mean ± SD of three replicates (60 oocytes). *CD* culture drop, *HD* hanging drop, *MII* metaphase II

culture was recently used to culture ovarian follicles [16]. According to this report, HD methods were able to maintain the follicular architecture much better than the two-dimensional culture method. Additionally, the maturation rate was also described to have been high as compared to the two-dimensional culture. These results suggest that COCs obtained from HD might be better able to maintain their structures after in vitro maturation.

First, we evaluated the maturity of oocytes cultured in grouped or single IVM, and the fertility and developmental competence of the mature oocytes. Compared with the control group, the oocyte maturation rate of the single IVM group was significantly decreased. These results were consistent with those of our previous study [6]. However, in single IVM, no paracrine system exists between oocytes, and therefore, oocytes in our study with low maturity did not mature, and the oocyte maturation rate was decreased.

The mechanisms of oocyte meiotic resumption with cumulus expansion have been investigated in previous studies. It is important for cytoplasmic maturation that a

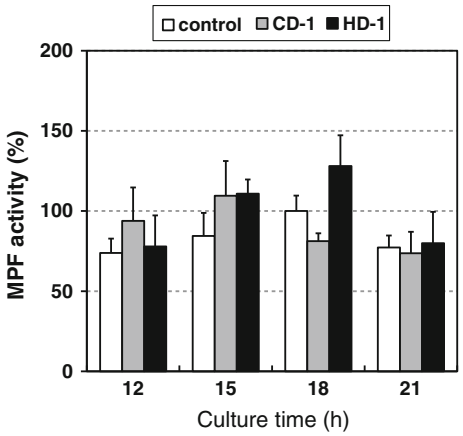


**Fig. 4** Effects of the different IVM conditions on the MPF activity of the in vitro-matured oocytes. Values represent the mean ± SD of 10 replicates (200 oocytes). The ratio of the MPF activity was calculated using the CD-20 value at 18 h as 100 %. *MPF* maturation promoting factor, *CD* culture drop, *HD* hanging drop, *MII* metaphase II

cumulus cell fully carries out expansion [17, 18]. So, if the cumulus expansion was limited, oocyte maturation would be prevented, and the fertility and developmental competence of the oocyte would be decreased [5, 19].

In the present study, there was a significant difference in the rate of two-pronuclei formation and cleavage of the CD-1 and HD-1 groups. Choi et al. [16] reported that preantral follicles cultured in HD became bigger than in the CD method. We believe that this was attributable to the different three-dimensional structures of cumulus expansion. Our study also demonstrated that the total cell number of blastocysts in the HD-1 group was not significantly different from that in the control group, and that the total cell number of blastocysts increased during the period of blastocyst expansion and hatching [20]. It is generally accepted that a high quality of blastocysts is attained with many cells. Therefore, we suggest that the quality of the blastocysts in the HD-1 group was similar to that in the control group.

Although the developmental competence of the embryo did not appear to be significantly different in the control and HD-1 group until the two-cell stage, as shown in Table 2, the rate of blastocyst formation in the HD-1 group was significantly reduced. These results showed that there was some stimulus effective in embryonic development from the surrounding oocytes or cumulus cells in the control group during in vitro maturation.

MPF is composed of the catalytic subunit $p34^{cdc2}$ that possesses histone H1 kinase activity and a cyclin as the regulatory subunit [21]. Generally, the level of MPF activity plateaus at the end of the first meiotic M-phase [21, 22]. A transient decline in MPF activity occurs during the transition between meiosis I and meiosis II. MPF is rapidly reactivated to promote the beginning of meiosis II and is

maintained at a high level during the metaphase II arrest [14]. However, if the oocytes are not fertilized at the appropriate time, the MPF activity of the oocyte is decreased with oocyte aging [23]. In other words, MPF activity is at its highest during the proper timing of oocyte fertilization.

In the HD-1 group, the MPF activity of the mature oocytes was the highest at 18 h after incubation, and it was higher than the control group at 18 h. Vanhoutte et al. [23] reported that human embryos on day 3 after three-dimensional prematuration culture (PMC) were of a better quality and had fewer nuclear abnormalities than the two-dimensional PMC group; they attributed this finding to heightened cytoplasmic maturation of the oocytes. Although the oocytes cultured in this report were denuded and at the germinal vesicle-stage, these findings support our results that demonstrated that the MPF activity of the oocytes cultured by the HD method was higher than those cultured by the CD method because the three-dimensional structure of COCs was maintained in the HD-1 group.

However, the developmental competence in the HD-1 group was lower than that in the control group. We concluded that this was because of the gap in nuclear and cytoplasmic maturation of the oocytes. In the HD-1 group, the MPF activity was the highest at 18 h after incubation, but the nuclear maturation rate had already begun to plateau at 15 h after incubation; this shows that oocytes may be over-matured at 18 h. One approach to optimizing the developmental competence post-IVM is by temporarily inhibiting spontaneous meiotic maturation in vitro [24, 25]. To this end, oocytes are exposed to a meiosis inhibitor to allow time for promoting cytoplasmic maturation and synchronization of the cytoplasmic and nuclear maturation processes within each individual oocyte [26]. Therefore, to use the HD IVM method, it is important to synchronize the lag between the cytoplasmic and nuclear maturation processes.

Our results suggest that the maturity, fertility, and developmental competence of single-cultured oocytes were lower than group-cultured oocytes. Therefore, we need to analyze a greater number of markers of oocyte cytoplasmic maturation and determine more appropriate culture conditions.

**Acknowledgments** This work was supported by a grant from the Japan Society for the Promotion of Science to E. Sato (Grant No. 21248032). This work was also supported in part by a Grant-in-Aid for Young Scientists (B) to Y. Hoshino (No. 21780250) from the Ministry of Education, Science and Culture, Japan, and by research funding from the Kanzawa Medical Research Foundation to Y. Hoshino.

**Conflict of interest** The authors declare that they have conflicts of interest.

## References

1. van de Sandt JJ, Schroeder AC, Eppig JJ. Culture media for mouse oocyte maturation affect subsequent embryonic development. Mol Reprod Dev. 1990;25(2):164–71.
2. A'Arabi SY, Roussel JD, Chandler JE. Chromosomal analysis of mammalian oocytes matured in vitro with various culture systems. Theriogenology. 1997;48(7):1173–83.
3. Hughes PM, Morbeck DE, Hudson SB, Fredrickson JR, Walker DL, Coddington CC. Peroxides in mineral oil used for in vitro fertilization: defining limits of standard quality control assays. J Assist Reprod Genet. 2010;27(2–3):87–92.
4. Eppig JJ, Schroeder AC. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. Biol Reprod. 1989;41(2):268–76.
5. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction. 2001;122(6):829–38.
6. Nishio M, Hoshino Y, Sato E. Effect of droplet size and number of oocytes examined on mouse oocyte quality in in vitro maturation. J Mamm Ova Res. 2011;28(1):53–60.
7. Jungling K, Nagler K, Pfrieger FW, Gottmann K. Purification of embryonic stem cell-derived neurons by immunoisolation. FASEB J Off Publ Fed Am Soc Exp Biol. 2003;17(14):2100–2.
8. Sakai Y, Yoshiura Y, Nakazawa K. Embryoid body culture of mouse embryonic stem cells using microwell and micropatterned chips. J Biosci Bioeng. 2011;111(1):85–91.
9. Chen M, Lin YQ, Xie SL, Wu HF, Wang JF. Enrichment of cardiac differentiation of mouse embryonic stem cells by optimizing the hanging drop method. Biotechnol Lett. 2011;33(4):853–8.
10. Maltsev VA, Rohwedel J, Hescheler J, Wobus AM. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech Dev. 1993;44(1):41–50.
11. Maltsev VA, Wobus AM, Rohwedel J, Bader M, Hescheler J. Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. Circ Res. 1994;75(2):233–44.
12. Abranches E, Bekman E, Henrique D, Cabral JM. Expansion and neural differentiation of embryonic stem cells in adherent and suspension cultures. Biotechnol Lett. 2003;25(9):725–30.
13. Hoshino Y, Sato E. Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. Dev Biol. 2008;314(1):215–23.
14. Brunet S, Maro B. Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. Reproduction. 2005;130(6):801–11.
15. Merkwitz C, Ricken AM, Losche A, Sakurai M, Spanel-Borowski K. Progenitor cells harvested from bovine follicles become endothelial cells. Differ Res Biol Divers. 2010;79(4–5):203–10.
16. Choi JK, Agarwal P, He X: In vitro culture of early secondary preantral follicles in hanging drop of ovarian cell-conditioned medium to obtain MII oocytes from outbred deer mice. Tissue engineering Part A 2013.
17. Larsen WJ, Wert SE, Brunner GD. A dramatic loss of cumulus cell gap junctions is correlated with germinal vesicle breakdown in rat oocytes. Dev Biol. 1986;113(2):517–21.
18. Isobe N, Maeda T, Terada T. Involvement of meiotic resumption in the disruption of gap junctions between cumulus cells attached to pig oocytes. J Reprod Fertil. 1998;113(2):167–72.
19. Gomez MN, Kang JT, Koo OJ, Kim SJ, Kwon DK, Park SJ, Atikuzzaman M, Hong SG, Jang G, Lee BC. Effect of oocyte-secreted factors on porcine in vitro maturation, cumulus

expansion and developmental competence of parthenotes. Zygote. 2012;20(2):135–45.
20. Sherbahn R, Frasor J, Radwanska E, Binor Z, Wood-Molo M, Hibner M, Mack S, Rawlins RG. Comparison of mouse embryo development in open and microdrop co-culture systems. Hum Reprod. 1996;11(10):2223–9.
21. Verlhac MH, Kubiak JZ, Clarke HJ, Maro B. Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development. 1994;120(4):1017–25.
22. Choi T, Aoki F, Mori M, Yamashita M, Nagahama Y, Kohmoto K. Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. Development. 1991;113(3):789–95.
23. Vanhoutte L, Nogueira D, De Sutter P. Prematuration of human denuded oocytes in a three-dimensional co-culture system: effects on meiosis progression and developmental competence. Hum Reprod. 2009;24(3):658–69.
24. Lonergan P, Fair T, Khatir H, Cesaroni G, Mermillod P. Effect of protein synthesis inhibition before or during in vitro maturation on subsequent development of bovine oocytes. Theriogenology. 1998;50(3):417–31.
25. Anderiesz C, Fong CY, Bongso A, Trounson AO. Regulation of human and mouse oocyte maturation in vitro with 6-dimethylaminopurine. Hum Reprod. 2000;15(2):379–88.
26. Vanhoutte L, Nogueira D, Dumortier F, De Sutter P. Assessment of a new in vitro maturation system for mouse and human cumulus-enclosed oocytes: three-dimensional prematuration culture in the presence of a phosphodiesterase 3-inhibitor. Hum Reprod. 2009;24(8):1946–59.