—Full Paper—

Long-Term Effects of *In Vitro* Growth of Mouse Oocytes on Their Maturation and Development

Yayoi OBATA^{1,2)}, Yuhei MAEDA³⁾, Izuhito HATADA⁴⁾ and Tomohiro KONO^{1,2)}

¹⁾Department of BioScience, Tokyo University of Agriculture, Tokyo 156-8502, ²⁾Bio-oriented Technology Research Advancement Institution (BRAIN), Tokyo 105-0001, ³⁾Department of Animal Science, Tokyo University of Agriculture, Kanagawa 243-0034 and ⁴⁾Biological Genome Resource Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma 371-8512, Japan

Abstract. Since very few oocytes grow completely *in vivo*, *in vitro* growth (IVG) of ovarian oocytes may provide a new source of functional oocytes. The long-term effects of *in vitro* maturation (IVM) of oocytes and *in vitro* culture of fertilized eggs have been reported; however, the effects of IVG of oocytes are unknown. Here in, we report the long-term effects of IVG of oocytes. Ovaries from 1-day-old mice containing non-growing oocytes were cultured for 10 days; the isolated follicles were then cultured for 11 days. Secondary follicles from 10-day-old mice were also cultured for 11 days. The nuclei of oocytes collected from the IVG and Graafian follicles of adult mice were transferred to enucleated oocytes grown *in vivo*, respectively. Developmental competence was examined following IVM of the reconstituted oocytes. Chronologically, oocytes of 1-day-old, 10-day-old and adult mice were cultured for 22, 12 and 1 day(s). The result showed that the reconstituted eggs developed into pups at high rates after nuclear transfer and *in vitro* fertilization (IVF) in all the experimental groups (29–45%). However, the pups from reconstituted eggs containing the nuclei of 22-day cultured oocytes were heavier than the control pups ($P < 0.05$). We concluded that long-term culture of oocytes did not affect their nuclear ability to develop to term; however, fetal growth was affected by the culture duration or culture conditions during the initial phase of follicular growth.

Key words: Full-term development, *In vitro* culture, Long-term effect, Mouse, Nuclear transfer, Oocyte growth

(J. Reprod. Dev. 53: 1183–1190, 2007)

Oocytes are the only cells that can generate embryos and produce individuals. Even in terminally differentiated cells, nuclear transfer into the ooplasm can induce reprogramming, thereby allowing the cloned embryos to acquire competence to develop to term [1]. In recent years, oocytes have been utilized for purposes such as research in the field of human reproductive medicine and studies on regenerative medicine using

embryonic stem (ES) cells derived from cloned blastocysts [2, 3]. However, only a limited number of oocytes grow completely and acquire competency because a species-specific number of follicles is selected from a large pool of primordial follicles for entry into the growth phase *in vivo* [4–8]. Therefore, there is an imminent critical shortage of oocytes.

Several studies endeavoring to produce competent oocytes *in vitro* have been reported [9–11]. Hubner *et al.* demonstrated that mouse ES cells, which have infinite proliferative potential, can mor-

phologically develop into oocytes *in vitro* [12]. However, oocyte-like cells derived from ES cells in culture failed to arrest at metaphase in second meiosis (MII) and underwent parthenogenetic cleavage. Thus far, *in vitro* growth (IVG) and *in vitro* maturation (IVM) of primordial follicles are considered to be the most realistic models for producing competent oocytes and increasing the number of female gamete sources. Eppig *et al.* [13, 14], devised a 2-step culture method for production of produce oocytes from the primordial follicles of 1-day-old mice. First, the ovaries of 1-day-old mice are cultured intact for 8 days, and the obtained follicles are subsequently cultured for 14 days. These oocytes are sufficiently competent to undergo meiotic maturation, fertilization and development into live pups. We have also demonstrated successful development of pups from oocytes that were differentiated from the pre-meiotic female germ cells of mouse foetuses at 12.5 days post coitum (dpc) using a nuclear transfer technique combined with Eppig's 2-step culture method [15]. In this strategy, to overcome the cytoplasmic deficiency of IVG oocytes, the nuclei of oocytes that were differentiated after ovarian culture for 17 days and follicular culture for 11 days were transferred into ooplasm grown and matured *in vivo*. The key factor in this method is the provision of a large number of maternal genomes.

On the other hand, it has been suggested that *in vitro* culture of ruminant embryos causes a high frequency of multiple abnormalities, which are collectively referred to the large offspring syndrome [16, 17]. Several studies have reported that assisted reproductive technology (ART) is associated with an increased risk of low-birth-weight infants and birth defects in humans [18–20]. The issue regarding whether the abnormalities are associated with the duration of culture and the degree of complications encountered while manipulating the gametes is of particular interest.

In the present study, in order to investigate whether the period of *in vitro* culture during oocyte growth affects post-implantation and post-natal development, the following series of experiments were performed: (1) oocytes at various stages derived from 1-day-old, 10-day-old and adult mice were cultured for 22, 12 and 1 days, respectively; (2) the nuclei of oocytes cultured *in vitro* were transferred into ooplasm grown and matured *in vivo*; (3) the reconstituted M II oocytes were subjected to *in*

vitro fertilization (IVF); and (4) the blastocysts obtained after 4-day *in vitro* culture were transferred to pseudopregnant female mice.

Materials and Methods

Animals

All procedures described in this study were reviewed and approved by the Tokyo University of Agriculture Institutional Animal Care and Use Committee and were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments as promulgated by the Science Council of Japan. BDF1 (C57BL/6N × DBA/2; Clea Japan, Tokyo, Japan) hybrid mice were used for all the experiments.

IVG of non-growing oocytes from newborn mice (Experiment 1)

The viability, growth, and maturation of oocytes after *in vitro* culture were assessed using alpha minimum essential medium (alpha-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 50 mg/l streptomycin sulphate and 75 mg/l penicillin G. Ovaries obtained from 1-day-old mice were cultured in medium containing 10% fetal bovine serum (FBS) on Coaster Transwell polycarbonate membranes (3.0 μ m pore size, 24 mm diameter; Corning, Lowell, MA, USA) in 5% CO₂ and 95% air at 37 C [13]. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.7–0.8 ml of the medium was added to the surface of the membrane. A total of 6 ovaries were placed on each membrane. On day 10, secondary follicles were mechanically isolated from the ovaries and cultured for 11 days in medium containing 5% FBS, 0.1 IU/ml follicle-stimulating hormone (FSH; Sigma-Aldrich, St. Louis, MO, USA), 5 μ g/ml insulin, 5 μ g/ml transferrin and 5 ng/ml selenium (ITS; Roche, Basel, Switzerland) on Coaster Transwell-COL membranes (3.0 μ m pore size, 12 mm diameter; Corning) in 5% CO₂ and 95% air at 37 C. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.5 ml of the medium was added to the surface of the membrane. Half of the culture media was replaced every 3 days. An interference microscope (200 × magnification; Nikon, Tokyo, Japan) and a eyepiece micrometer (10 × A; Nikon) were used to measure the diameters of the oocytes grown *in vitro* (Fig.1).

Fig. 1. Schematic diagram illustrating the experimental design and production of functional oocytes from immature oocytes. *In vivo* and *in vitro* derivatives are pink and yellow, respectively. With regard to the donor oocytes, primordial (experiment 1), secondary (experiment 2) and Graafian's follicles (control 1) were cultured for 21, 11 and 0 days, respectively, in order to attain the same chronological age. Serial nuclear transfer to cytoplasm grown and matured *in vivo* was conducted at the GV and MII stages, respectively. Following IVF, the resultant blastocysts were transferred to pseudopregnant females, and the long-term effects were assessed.

IVG of growing oocytes from 10-day-old mice (Experiment 2)

Secondary follicles isolated from the ovaries of 10-day-old mice were cultured as mentioned above. In short, secondary follicles were mechanically isolated and cultured for 11 days in medium

containing 5% FBS, 0.1 IU/ml FSH and ITS on Coaster Transwell-COL membranes. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.5 ml of the medium was added to the surface of the membrane (Fig.1).

Table 1. Development of oocytes after IVG

	Culture duration	No. of oocytes				No. of embryos developed to	
		cultured	undergone GVBD	matured into MII	normally fertilized	2-cell	blastocyst
Primordial follicles of 1-day-old mice	22 days	91	52 ^a (57%)	19 ^a (21%)	1 ^a (5%)	0 (0%)	0 (0%)
Secondary follicles of 10-day-old mice	12 days	140	105 ^b (75%)	51 ^c (36%)	19 ^b (37%)	8 (42%)	0 (0%)

a *vs.* b: $P < 0.01$. a *vs.* c: $P < 0.05$.

Nuclear transfer and IVM

Adult female mice were injected with 5 IU equine chorionic gonadotropin (eCG) (Serotropin®; Teikoku Zoki, Tokyo, Japan), and 5 IU human chorionic gonadotropin (hCG) (Puberogen®; San-kyo Zoki, Tokyo, Japan) was injected 48 h after the eCG injection. Freshly ovulated MII oocytes were collected from the oviducts 14–16 h after the hCG injection. Fully grown oocytes were collected at the germinal vesicle (GV) stage from the ovaries 44–48 h after the eCG injection.

Serial nuclear transfers were performed as described previously [15, 21]. In order to prevent GV breakdown, the GV-stage oocytes were manipulated in M2 medium containing 240 μ M dibutyryl cAMP (Sigma). Karyoplasts obtained from the oocytes, which were cultured for 21 days (Experiment 1) and for 11 days (Experiment 2), were fused with enucleated fully grown GV-stage oocytes using the inactivated Sendai virus (2700 hemagglutinating activity units/ml). As a control, karyoplasts obtained from fully grown GV-stage oocytes were fused with enucleated fully grown GV-stage oocytes using the inactivated Sendai virus (control 1). The reconstituted oocytes were cultured in alpha-MEM containing 5% FBS in 5% CO₂, 5% O₂ and 90% N₂ at 37 C. After 14 h, the MII chromosomes were transferred from the reconstituted oocytes to the enucleated and ovulated MII-stage oocytes in order to yield cytoplasmic competency (Fig. 1).

IVF, *in vitro* culture and embryo transfer

Sperm were collected from fertile mice, and the reconstituted oocytes were incubated with capacitated sperm for 3 h in T6 medium. After IVF, reconstituted embryos containing single male and female pronuclei were regarded as normally fertil-

ized eggs and were cultured at 37 C for 4 days in M16 medium in 5% CO₂, 5% O₂ and 90% N₂. The resulting blastocysts were transferred to the uterine horns of pseudopregnant female mice at 2.5 dpc. As a control, freshly ovulated oocytes (control 2) were subjected to the same sequence of IVF, *in vitro* culture and embryo transfer as that performed for the reconstituted oocytes. The ability of these blastocysts to develop into pups was analysed at 19.5 dpc by performing a caesarean section (Fig. 1). All the experiments described above were repeated at least three times.

Statistical analysis

Data on the growth of the oocytes and weight of the pups were examined using the Student's *t*-test. The rate of follicular viability, oocyte maturation and embryonic development were analyzed using the chi-square test.

Results

Growth of oocytes cultured in alpha-MEM medium

When the ovaries of 1-day-old mice were cultured for 10 days, the size (mean $\pm$ SD) of the oocytes enclosed within the secondary follicles was $51.7 \pm 3.86 \mu\text{m}$ ($n=179$). Subsequently, the secondary follicles were cultured for 11 days. On day 21, 69% of the oocytes were enclosed within the follicles; they were now $68.6 \pm 3.87 \mu\text{m}$ ($n=124$) in size. These oocytes underwent the first meiotic division (19/91) but were not successful with regard to IVF (1/19). The mean diameter of the oocytes enclosed within intact secondary follicles isolated from the 10-day-old mice was $53.8 \pm 3.91 \mu\text{m}$ ($n=250$). Some of the oocytes ovulated by 11 days after follicular culture and 56% of the oocytes were enclosed

Table 2. Development of oocytes reconstituted with nuclei from oocytes after IVG and IVM

	Culture duration	No. of oocytes					No. of embryos		No. of living pups
		NT	fused	matured into MII	NT	fused	normally fertilized	developed to blastocyst	
Primordial follicles of 1-day-old mice (Experiment 1)	22 days	133	94 (71%)	89 (95%)	79	66 (84%)	49 (74%)	47 (96%)	14 (29%)
Secondary follicles of 10-day-old mice (Experiment 2)	12 days	187	168 (90%)	138 (82%)	121	106 (88%)	84 (79%)	77 (92%)	25 (30%)
Graafian's follicles of adult mice (Control 1)	1 day	114	95 (83%)	90 (95%)	86	73 (85%)	55 (75%)	52 (95%)	25 (45%)
M II oocytes of adult mice (Control 2)	0 day	—	—	—	—	—	40 (89%)	39 (98%)	31 (79%)

NT: nuclear transferred.

within follicles. They had reached $69.2 \pm 3.57 \mu\text{m}$ ($n=140$) in size, and had undergone the first meiotic division (51/140). After IVF, 19 eggs extruded a second polar body and formed two pronuclei. No significant differences were observed in the sizes of oocytes of the same chronological age that were cultured for 21 and 11 days. However, the maturation and fertilization rates of the oocytes cultured for 11 days were significantly higher than for those cultured for 21 days ($P<0.05$). In both cases, the resultant eggs after IVF did not develop beyond the 4-cell stage (Table 1).

Post-implantation development of reconstituted eggs containing the nuclei of oocytes produced in vitro

We initially we observed that physically denuded oocytes at the GV stage had high potential to mature to the MII stage (487/528; 92%) similar to cumulus enclosed oocytes (291/307; 95%); however, denuded oocytes had poor ability to achieve fertilization (1/152; 0.6%) and pre-implantation development (0/1). When zonae pellucidae of denuded oocytes were cut, the fertilization rate improved (71/135; 52.6%) but the ability to develop to the blastocyst stage was quite low (3/70; 4%). These results and Table 1 indicate that IVG and IVM of oocytes and denudation of cumulus cells caused zona hardening and critical cytoplasmic deficiency. Therefore, we conducted serial nuclear transfer experiments to analyze the nuclear competence of oocytes grown and matured *in vitro* and to investigate whether the duration of culture affects post-implantation development (Fig. 1).

In order to attain the same chronological age for the donor oocytes, primordial (experiment 1), secondary (experiment 2) and Graafian follicles (control 1) were cultured for 21, 11 and 0 days, respectively (Fig. 1). After nuclear transfer, oocytes reconstituted with the nuclei of the oocytes cultured for 21, 11 and 0 days were highly competent to mature into MII-stage oocytes (95, 82 and 95%, respectively) and develop into blastocysts (96, 92 and 95%, respectively; Table 2). In the post-implantation stage, the competency of the reconstituted oocytes was unaffected by the duration of culture. When the maternal nuclei were donated from oocytes cultured for 22 or 12 days, approximately 30% of the reconstituted eggs developed into pups (14/49 and 25/84, respectively). This observation was made with regard to the developmental rate of the reconstituted eggs containing nuclei obtained from oocytes cultured for 1 day (control 1, 45%; Table 2).

Offspring from oocytes produced in vitro

No obvious abnormalities were observed in any of the pups or placentae. The pups ($1.48 \pm 0.17 \text{ g}$, mean $\pm$ SD) and placentae ($135 \pm 35 \text{ mg}$) from the reconstituted eggs, which contained the nuclei of oocytes cultured for 22 days after their isolation from 1-day-old mice, were heavier than those of control 2 (pups, $1.25 \pm 0.14 \text{ g}$; placentae, $89 \pm 23 \text{ mg}$). In the other experimental group, no significant difference was observed between the weight of the pups and placentae and those of the pups and placentae of control 2 (Fig. 2). In the present

Fig. 2. Weight of the pups (a) and placentae (b) derived from reconstituted oocytes at birth. The bars indicate the mean weights and SDs of the pups (A) and placentae (B) obtained from the reconstituted eggs, which contained the nuclei of oocytes cultured for 22 (experiment 1), 12 (experiment 2), 1 (control 1) and 0 (control 2) day(s) after isolation from 1-day-old, 10-day-old, adult and superovulated adult mice, respectively. A significant difference was only observed in the weights of the pups and placentae that contained the nuclei of oocytes cultured for 22 days compared with those of the pups and placentae in control 2. a vs. b: $P < 0.05$.

experimental series, only 1 pup died at birth (1/64, 1.6%), and all the tested pups were fertile. The lifespan of the obtained pups was 818 days (experi-

ment 1; $n=4$), 765 days (experiment 2; $n=3$), 720 days (control 1; $n=3$) and 810 days (control 2; $n=4$); this appeared to be normal despite the long culture period of the donated oocytes.

Discussion

In the present study, we analyzed whether the duration of culture affects pre- and post-implantation and post-natal development. No significant difference was observed between the size of the oocytes obtained from 1-day-old mice and cultured for a total of 21 days and those obtained from 10-day-old mice and cultured for 11 days. However, the maturation rate of the oocytes cultured for 11 days was significantly higher than that of oocytes cultured for 21 days ($P < 0.05$). The same tendency was observed in the fertilization rate. There are several possibilities to explain the incompetency of the oocytes cultured for 21 days. Formation and maintenance of gap junctional communication, which is mediated by factors such as Connexin 37 [22] and kit ligand [23], between granulosa cells and the oocytes during the initial phase of follicular growth may be insufficient under these culture conditions. Furthermore, zona hardening might occur more severely or the zona structure might be defective in the oocytes cultured for 21 days. Since replacement of the cytoplasm and zona pellucida by nuclear transfer in both cases led to a high rate of successful maturation and fertilization, it is plausible that long-term IVG or *in vitro* culture during the initial phase of follicular growth induced these deficiencies. No significant differences were observed with regard to the rate at which the eggs reconstituted with the nuclei of the oocytes cultured for 22, 12 and 1 days developed into blastocysts and pups (Table 2). In contrast to use of complicated manipulation, application of the nuclear transfer technique may prevent critical long-term effects. However, a significant increase in weight was observed in oocytes from the primordial follicles of 1-day-old mice that were cultured *in vitro* for 22 days (Fig. 2). The overgrowth phenotype at birth exhibits many common features in ruminants and is caused by IVM and IVF [16, 17]. The expression of *Igf2r*, which is an imprinted gene that negatively regulates fetal growth, is decreased in the large offspring of sheep due to abnormal demethylation of *Igf2r* [24]. The *Igf2r* gene is meth-

ylated in the early phase of oogenesis [25]. However, we previously reported the absence of abnormal methylation of the *Igf2r* gene in oocytes cultured for 28 days and the pups from these oocytes [15]. Although the precise mechanisms of overgrowth are not understood, the culture duration of oocytes or culture conditions during the initial phase of follicular growth might induce epimutations in some genes.

In this study, we used a nuclear transfer technique to successfully produce a high rate of live offspring (29%) from embryos that contained the nuclei of oocytes cultured for 22 days after isolation from 1-day-old mice. Many features are essential for formation of a functional oocyte; these include meiotic maturation, cytoplasmic maturation and the establishment of genomic imprinting [21, 26, 27]. In mice, oocyte-specific imprinting is naturally established during oocyte growth up to 60–65 μm in diameter [25, 28]. Therefore, even when the donor oocytes did not grow to their maximum size, as long as they attained a diameter of $\geq 60 \mu\text{m}$ after IVG, our strategy produced functional oocytes. This is potentially applicable for preservation and increase of the number of wild animals, valuable livestock and transgenic/knockout animals. On the other hand, in the UK, USA, France and Australia, it has been reported that the frequency at which ART children are born with Beckwith-Wiedemann

syndrome (BWS) is 4- to 15-fold higher than the population risk or that of non-ART children. In ART children with Angelman syndrome, the loss of methylation in the *Snrpn* gene is observed at a 6-fold higher frequency [29]. Analyses of a large number of IVG oocytes and pups from IVG oocytes in mice can help to elucidate the cause of epimutation and help to establish safe ART.

In conclusion, we demonstrated that (1) oocytes from the primordial follicles of 1-day-old mice and secondary follicles of 10-day-old mice acquired competence to develop into pups at a high rate (29% and 30%, respectively) after IVG, nuclear transfer, IVF and embryo transfer and that (2) the long-term culture of oocytes did not affect their nuclear ability to develop to term; however, fetal growth was affected by the duration of culture or culture conditions during the initial phase of follicular growth.

Acknowledgment

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan to Y.O. and the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan, to T.K.

References

1. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 1996; 380: 64–66.
2. Munsie MJ, Michalska AE, O'Brien CM, Trounson AO, Pera MF, Mountford PS. Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. *Curr Biol* 2000; 10: 989–992.
3. Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P. Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* 2001; 292: 740–743.
4. Baker T. In: Balin H, Glasser S (eds.), *Reproductive Biology*. Amsterdam: Excerpta Medica; 1972: 398–437.
5. Byskov AG. In: Austin CR, Short RV (eds.), *Germ Cells and Fertilization*. Cambridge: Cambridge University Press; 1982: 1–16.
6. Krarup T, Pedersen T, Faber M. Regulation of oocyte growth in the mouse ovary. *Nature* 1969; 224: 187–188.
7. Peters H. The development of the mouse ovary from birth to maturity. *Acta Endocrinol (Copenh)* 1969; 62: 98–116.
8. Peters H, Levy E. Cell dynamics of the ovarian cycle. *J Reprod Fertil* 1966; 11: 227–236.
9. Lenie S, Cortvrindt R, Adriaenssens T, Smitz J. A reproducible two-step culture system for isolated primary mouse ovarian follicles as single functional units. *Biol Reprod* 2004; 71: 1730–1738.
10. Yamamoto K, Otoi T, Koyama N, Horikita N, Tachikawa S, Miyano T. Development to live young from bovine small oocytes after growth, maturation and fertilization *in vitro*. *Theriogenology* 1999; 52: 81–89.
11. Dyce PW, Wen L, Li J. *In vitro* germline potential of stem cells derived from fetal porcine skin. *Nat Cell Biol* 2006; 8: 384–390.
12. Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF,

- 3rd, Boiani M, Scholer HR. Derivation of oocytes from mouse embryonic stem cells. *Science* 2003; 300: 1251–1256.
13. Eppig JJ, O'Brien MJ. Development *in vitro* of mouse oocytes from primordial follicles. *Biol Reprod* 1996; 54: 197–207.
14. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* 2003; 68: 1682–1686.
15. Obata Y, Kono T, Hatada I. Gene silencing: maturation of mouse fetal germ cells *in vitro*. *Nature* 2002; 418: 497.
16. Walker SK, Hartwich KM, Seamark RF. The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenology* 1996; 45: 111–120.
17. Young LE, Butterwith SC, Wilmut I. Increased ovine fetal weight following transient asynchronous embryo transfer is not associated with increased placental weight at day 21 of gestation. *Theriogenology* 1996; 45: 231.
18. Doyle P, Beral V, Maconochie N. Preterm delivery, low birthweight and small-for-gestational-age in liveborn singleton babies resulting from *in vitro* fertilization. *Hum Reprod* 1992; 7: 425–428.
19. Maher ER. Imprinting and assisted reproductive technology. *Hum Mol Genet* 2005; 14 Spec No 1: R133–138.
20. McDonald SD, Murphy K, Beyene J, Ohlsson A. Perinatal outcomes of singleton pregnancies achieved by *in vitro* fertilization: a systematic review and meta-analysis. *J Obstet Gynaecol Can* 2005; 27: 449–459.
21. Bao S, Obata Y, Carroll J, Domeki I, Kono T. Epigenetic modifications necessary for normal development are established during oocyte growth in mice. *Biol Reprod* 2000; 62: 616–621.
22. Simon AM, Goodenough DA, Li E, Paul DL. Female infertility in mice lacking connexin 37. *Nature* 1997; 385: 525–529.
23. Driancourt MA, Reynaud K, Cortvrindt R, Smitz J. Roles of KIT and KIT LIGAND in ovarian function. *Rev Reprod* 2000; 5: 143–152.
24. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001; 27: 153–154.
25. Hiura H, Obata Y, Komiyama J, Shirai M, Kono T. Oocyte growth-dependent progression of maternal imprinting in mice. *Genes Cells* 2006; 11: 353–361.
26. Bao S, Obata Y, Ono Y, Futatsumata N, Niimura S, Kono T. Nuclear competence for maturation and pronuclear formation in mouse oocytes. *Hum Reprod* 2002; 17: 1311–1316.
27. Sorensen RA, Wassarman PM. Relationship between growth and meiotic maturation of the mouse oocyte. *Dev Biol* 1976; 50: 531–536.
28. Obata Y, Kono T. Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J Biol Chem* 2002; 277: 5285–5289.
29. Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet* 2002; 71: 162–164.