Follicle growth and oocyte development after ovary transplantation into back muscle of immune-intact adult castrated male mice

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Abstract

Ovary grafting is not only a method of investigating follicle and oocyte development, but also a useful model to explore the possibility of the re-establishment of the reproductive axis in male-to-female sexual reversal. This study investigated ovary survival and follicle development after mouse ovaries were transplanted into immune-intact castrated male mice. Ten-day-old mouse ovaries were transplanted into the back muscle of adult outbred castrated male mice treated with immunosuppressants. Twenty-two days later, the ovary structure and the number of follicles present was examined by hematoxylin and eosin staining. The oocytes were harvested, and then used for in vitro maturation (IVM) and IVF. The results showed that primordial and antral follicles were mainly found in the grafts, and there were obvious differences compared with 32-day-old fresh ovaries (P < 0.05). Embryos were derived from collected oocytes after IVM and IVF with a 72.4% cleavage rate and 7.9% blastocyst rate; 12 live pups were generated by embryo transfer. The hormone assay showed that plasma concentrations of both estrogen and progesterone increased after ovarian transplantation (P < 0.01). In conclusion, immune-intact adult castrated male mice can support ovary survival and further development of follicles with endocrine function after ovarian transplantation.


Introduction

The ovaries are important reproductive and endocrine organs, which play an important role in mammalian reproduction and sexual activity. Recently, research on ovarian transplantation has advanced. Many researchers have found that grafted ovaries could recover reproductive and endocrine function (Baird et al. 1999, Candy et al. 2000, Shaw et al. 2000, Salle et al. 2002, Lee et al. 2004). In practice, ovary implantation techniques have been used in human medical clinics to help patients who fail to have a normal pregnancy (Radford et al. 2001, Donnez et al. 2004, Meirow et al. 2005, 2007).

It was also found that ovaries or ovarian tissues could grow and develop after being xenotransplanted to another species or even to males (Gosden et al. 1994, Oktay et al. 1998, Shaw et al. 2000, Kaneko et al. 2003, Senbon et al. 2003, Moniruzzaman et al. 2009). For instance, human female ovarian tissue could develop in female mice (Maltaris et al. 2006) or in male mice (Weissman et al. 1999, Hernandez-Fonseca et al. 2004), and mouse ovaries can develop in male rats (Snow et al. 2002). Primordial follicles from cryopreserved porcine ovarian tissues developed to the secondary stage in male mice (Moniruzzaman et al. 2009). For the male recipients, offspring had been obtained after mouse ovarian tissue was grafted into same strain-intact males (Waterhouse et al. 2004). However, Hernandez-Fonseca et al. (2004) found that non-castrated male mice tended to preserve grafted ovarian tissue better than castrated recipients, and that mean estradiol concentrations in serum were not significantly increased in mice with ovarian grafts compared with those in mice without a graft. Thus, questions remain over the ability of castrated male mice to support further development of follicles and endocrine function of the grafted ovaries. We recently tried to investigate the developmental potential of follicles, and examine the endocrine function of grafted ovaries by observing steroid fluctuation. We found that testes, the main source of steroids in...
In recent years, healing granulation tissue has been pressure, and mechanical contact (Donnez et al. 2006). A muscle site might be promising as a choice for ovarian transplantation (Maltaris et al. 2006, Soleimani et al. 2008, Eimani et al. 2009). Such a site was selected in this study.

It is well known that gonadotropins promote follicular growth and oocyte development. Previous reports demonstrated that stimulation with exogenous gonadotropins after ovary transfer enhanced follicular growth, oocyte maturation, and embryo development in female and male mice (Yang et al. 2006, Yuan et al. 2008, 2009). Thus, in our present study, exogenous gonadotropin was given to facilitate the further development of follicles and oocytes.

In this study, using immune-intact adult castrated male mice for ovarian transplantation, our objectives were as follows: to investigate the possibility of oocyte development and follicle growth in the castrated male mice; and to examine the endocrine function of the grafted ovaries after the male host’s gonads were removed.

Results

Physiological status change

After castration of the male recipient mice, the body temperature increased slightly and then returned to normal, and none of them died. Seven days later, no abnormal diet or body temperature change was observed. After ovarian transplantation, the recipients' weights increased from approximately 20 to 28 g at the 22nd day, no obvious change in the main organs was observed on dissection, including the size of the spleen.

Graft development

From the 3rd day after ovarian transplantation, blood vessels gradually generated around the implants. Twenty-two days later, the grafts protruded from the muscle, and different sizes of developed follicles were observable on the surface of the grafts (Fig. 1A); the blood vessels around the grafts were clearly visible (Fig. 1B); the corpus luteum was found on the grafts (Fig. 1C); and some others displayed inflammatory infiltration around the grafts, such as degenerated grafts with a red color and the presence of pus around the grafts (Fig. 1D).

Graft size and recovery rate were examined, the results showed that the diameter of grafts increased to 1861 μm at 22 days after ovary grafting from 816 μm at the very beginning of grafting, i.e. for 10-day-old mouse ovaries; this increase was statistically significant (P<0.01). Also, the number and diameter of the retrieved grafts was obviously higher than that of the grafts from the recipients without immunosuppressant treatment (Table 1).
Histological analysis

Hematoxylin and eosin (H&E) staining showed that before ovarian transplantation, in the 10-day old mice ovaries, most follicles were at the primordial and primary stages (Fig. 2A). In contrast, 22 days after ovary grafting, follicles at different stages were observed at the edge of the grafts (Fig. 2B and C), including morphologically normal primordial follicles (Fig. 2D) and Graafian follicles (Fig. 2E), compared with age-matched ovaries, which contained many follicles (Fig. 2F) at different stages, from primordial follicles to antral follicles (Fig. 2G). However, the grafts disappeared completely in the recipients without immunosuppressants, and granulosa cells and oocytes degenerated in the follicles (Fig. 2H).

The number of follicles (Table 2) and follicular apoptosis (Fig. 3) in the grafts are also shown. In the 10-day-old fresh ovaries, most of the follicles were primordial, few were antral (Table 2). TUNEL staining showed that some primordial and primary follicles were tagged with fluorescence (Fig. 3A1–A2). In contrast, a clear shift to advanced stages was noticed in the grafts 22 days after grafting (Table 2), TUNEL staining showed that many primordial and primary follicles were apoptotic (Fig. 3B1–B2). However, in the age-matched ovaries, TUNEL staining showed that only some advanced follicles were fluorescently tagged (Fig. 3C1–C2), and few primordial and primary follicles were positive.

Oocyte development

In the first set of experiments, oocytes were harvested from 19 grafts (Table 3). The results showed that the 163 oocytes at the germinal vesicle (GV) stage which were derived from 22-day-old grafts had few granulosa cells attached around them (Fig. 4A). After 16 h of in vitro maturation (IVM), 146 oocytes extruded the first polar body (PB1; Fig. 4B). After IVF, 108 embryos were cultured with a 72.4% cleavage rate; in total, 13 blastocysts were obtained with a 7.9% blastocyst rate (Fig. 4C). Subsequent fluorescence staining showed that there were on average 47 cells in the blastocysts. In the control group, from 109 oocytes at the GV stage, 101 oocytes were IVM and IVF, and 89 embryos were in vitro cultured (cleavage rate, 89.1%), and 36 blastocysts were produced (blastocyst rate, 35.8%). In the second set of experiments, a total of 127 embryos were used for embryo transfer; finally, 3 of 14 recipients carried pregnancy to term, and 12 live pups were produced (Fig. 4D). These pups are now healthy adults and have given birth to subsequent generations.

Hormonal analysis

Ten non-grafted castrated and four non-castrated male mice were age-matched to be used as controls. In grafted castrated mice, estrogen (E2) and progesterone (P4)
concentrations were significantly higher than those of the control mice ($P<0.01$; Table 4). Interestingly, the level of testosterone (T4) in recipients was a little higher than in non-grafted castrated mice ($P<0.05$). The level of T4 in both recipients and non-grafted castrated mice was lower than that of the non-castrated male mice ($P<0.01$).

**Discussion**

Ovarian transplantation in mammals provides a broad platform of opportunities for research and new applications in reproductive medicine and conservation biology. Serving as an important technique in experimental endocrinology and pathology, it has great potential. So far, encouraging results have been achieved. Normal oocytes and even offspring have been obtained from the grafts (Gosden et al. 1994, Liu et al. 2000, 2001, Snow et al. 2002, Waterhouse et al. 2004, Hasegawa et al. 2006, Grazul-Bilska et al. 2008, Hosoe et al. 2008).

Previously, ovaries were often transferred into the renal capsule (Liu et al. 2000, Waterhouse et al. 2004, Hosoe et al. 2008), where the graft could easily re-vascularize. Soleimani et al. (2008) found that the back muscle was a promising site for ovary grafts, providing some advantages over the renal site. For example, the surgery...
is more convenient (Eimani et al. 2009), and the host experiences less stress. However, grafts transplanted to muscle are more difficult to fix there. In our study, we found that the back muscle is a feasible site for ovary grafts. We treated the recipients with vitamin E after surgery as this may reduce oxidative stress and facilitate blood vessel regeneration in the grafts (Nugent et al. 1998).

Immunorejection is one of the serious problems in organ transplantation, as the grafts are often rejected by the recipients. Rejection is characterized by leukocyte infiltration around the grafts (Gosden 2007). The problem still occurs when grafting between genetically non-identical sisters (Donnez et al. 2007). In order to avoid immunorejection, inbred strains, immunodeficient or homozygous animals from the same strain were often

Table 2 The number of follicles and follicular densities in the ovarian grafts recovered after transplantation into the back muscle of castrated male mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of primordial follicles (%)</th>
<th>Number of primary follicles (%)</th>
<th>Number of preantral follicles (%)</th>
<th>Number of antral follicles (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grafts</td>
<td>120 ± 13% (71.4)</td>
<td>8 ± 3% (4.8)</td>
<td>6 ± 3% (3.6)</td>
<td>34 ± 2% (20.2)</td>
<td>168 ± 20%</td>
</tr>
<tr>
<td>O₁₀</td>
<td>213 ± 28% (68.5)</td>
<td>57 ± 9% (18.3)</td>
<td>39 ± 3% (12.5)</td>
<td>2 ± 1% (0.64)</td>
<td>311 ± 32%</td>
</tr>
<tr>
<td>O₁₂</td>
<td>151 ± 17% (62.7)</td>
<td>31 ± 4% (12.9)</td>
<td>9 ± 7% (3.7)</td>
<td>50 ± 5% (20.8)</td>
<td>241 ± 26%</td>
</tr>
</tbody>
</table>

The data in the same columns were statistically analyzed by ANOVA post-hoc test. Significant difference (P < 0.05) among groups was indicated by different superscripts (a, b, c). O₁₀, 10-day-old ovary; O₁₂, 32-day-old ovary.

Figure 3 Cell apoptosis determination in grafts after mouse ovarian transplantation. (A₁–A₂) Ten-day-old fresh ovary before grafting. In A₂, fluorescent signals (arrows) showed that a few cells became apoptotic, especially the basal membrane cells around the primordial and primary follicles. (B₁–B₂) Recovered grafts after ovaries were transferred for 22 days. In B₂, apoptotic cells were scattered in the grafts. (C₁–C₂) 32-day-old fresh ovary. In C₂, positive signals were mainly limited to a few late stage follicles (arrows). (D₁) Ovarian tissue without terminal deoxynucleotidyl transferase digestion as a negative control from bright field. (D₂) DNase-treated ovarian tissue as a positive control. A₁, B₁, and C₁, scale bar = 500 μm; A₂, B₂, C₂, D₁, and D₂, scale bar = 200 μm.


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used for ovarian transplantation (Candy et al. 2000, Hosoe et al. 2008, Schubert et al. 2008, Soleimani et al. 2008, Abir et al. 2009, Motohashi et al. 2009). For the immune-intact animals as recipients, immunosuppressant treatment is the first and most effective choice. It was found that cyclosporine A plus steroids was also needed for ovarian allografts to survive (Cornier et al. 1985, Scott et al. 1987). Based on previous reports (Kocik et al. 2004), three suppressants were used in our study, we found that they worked well without serious side effects or infection; the main physiological indexes were normal, including diet, temperature, spleen size, and blood vessel re-establishment around the grafts after transfer. Although there was also an inflammatory infiltration around some grafts with immunosuppressant treatment, our study strongly showed that the recovery rate (23.5%) and structure of the grafts without immunosuppressants were much worse than the control (recovery rate, 67%), and that the immunosuppressive therapy was effective in ovarian transplantation in mice. These results are similar to those of Cornier et al. (1985), who showed that cyclosporine A as an immunosuppressive drug was not teratogenic in animals and was not cytostatic in an appropriate immunosuppressive regimen; it could inhibit immune responses at the cellular level as well as the production of lymphocyte T-dependent antibodies and allow the allografts to be tolerated in 60% of recipients. Considered together, immunosuppressants might ease the rejection after ovarian transplantation to some extent. However, the viability of immunosuppressants in human ovarian transplantation still needs to be substantiated by further studies; immunosuppressants may cause some negative effects in the hosts, such as increasing infection risk or even cancer induction. Although these phenomena were not observed in our study, the problem of infection, safety, and the effect of immunosuppressive therapy on fertility in human clinics warrants further investigation using animal experiments. Fortunately, in the future, it may be possible to find other immunosuppressant compounds with reduced side effects. Thus, the ethical problems about the use of mice xenografted with human ovarian tissues might also be addressed by using humans as recipients with immunosuppressive therapy.

It appears that combined suppression with three suppressants performs well, but follicular development was not as good as in normal age-matched mice, and the follicle density in grafts was noticeably decreased. Moreover, a number of follicles changed their morphology and structure, and the oocytes in the follicles degenerated (data not shown), as observed in a previous study (Liu et al. 2002). Presumably, azathioprine, which interferes with the synthesis of the purines that are required for DNA synthesis, may contribute to the reduction in follicle development. However, there is also a report showing that azathioprine had no effect on the number of oocytes or follicles in mice (Mattison et al. 1981). Therefore, more

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**Table 3** The developmental ability of oocytes recovered from 22-day-old grafts.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of embryos cultured</th>
<th>Cleavage % (mean ± s.d.)</th>
<th>Blastocysts % (mean ± s.d.)</th>
<th>Number of total cells (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grafts</td>
<td>146</td>
<td>72.4 ± 3.8^a</td>
<td>7.9 ± 1.4^b</td>
<td>47.0 ± 4.0^a</td>
</tr>
<tr>
<td>Controls</td>
<td>101</td>
<td>89.1 ± 7.2^a</td>
<td>35.8 ± 3.2^b</td>
<td>67.0 ± 2.0^a</td>
</tr>
</tbody>
</table>

The data in same columns were statistically analyzed by t-test. Values within the same column with different superscripts (a, b) are significantly different (P<0.05).

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**Figure 4** Embryos and live pups derived from the grafts after ovarian transplantation. (A) recovered oocytes with germinal vesicles from the 22-day-old grafts; (B) oocytes with first body (white arrows) after in vitro maturation; (C) blastocysts (arrows) produced by IVF; (D) live pups (white) and their surrogate (black) after embryo transfer. A–C, scale bar = 200 μm; D, scale bar = 8 mm.
antral follicles in mouse ovaries, which was
grafts, primordial follicles could develop to preantral
follicles. Our study also showed that in the 22-day-old
from 10-day-old mouse ovaries, which had few antral
our study strongly indicated that the follicles developed
be induced, an assumption which needs direct evidence
is well known that mammalian primordial follicles are
accessory hypophysis and their main source is from the follicles of gonads,
Aubard et al (2002, 2007). The follicles at late stages were
This is considered to be the main obstacle in ovary
considered to be the main obstacle in ovary
Diss 1999). is well known that mammalian primordial follicles are
gonadotropin treatment helped follicular development. Moreover, gonadotropins might also endow the oocytes in grafts with a developmental potential, which was demonstrated in our study by embryo development and pup birth. These results are consistent with the idea that exogenous gonadotropin stimulation enhanced the competence of follicular growth, oocyte maturation, and embryo development (Yang et al. 2006, Yuan et al. 2009). In our study, we demonstrated the development of early embryos derived from the grafted oocytes with a cleavage rate of 72.4% and a blastocyst rate of 7.9%, which indicated that castrated male recipients support the further development of follicles and oocytes.
interestingly, based on changes in follicular distribution we also found that primordial and antral follicles were numerous in the grafts 22 days after ovary grafting while few primary and preantral follicles were present. It is well known that mammalian primordial follicles are triggered to grow to primary follicles in a gonadotropin-independent manner (McNatty et al. 1999). It was reported that T4 and dihydrotestosterone had a stimulatory effect on primordial follicle recruitment to primary follicle in the primate ovary (Vendola et al. 1999). Human ovarian tissue xenografted to male mice possessed more preantral and antral follicles after stimulation with human menopausal gonadotropin, but there was no difference found in primary follicle number (Weissman et al. 1999). Yuan et al. (2008, 2009) found that primordial follicles were recruited in the intact male mice accepting ovary grafts. Collectively, we assumed that the recruitment of primordial follicles from the follicular reservoir might not work well enough, despite the preantral and antral follicles developing well with the help of exogenous gonadotropins, and that T4 might also stimulate primordial follicle recruitment in mice. Further studies are needed to provide direct evidence, and we suggest that growth factor(s) might be involved and applied in further trials.
As is well known, endocrinology underlies fertility in mammals, which have two important endocrine systems related to reproductive hormone modulation, including the directly associated hypothalamic–pituitary–gonadal axis. E2, P4, and T4 are the most representative steroids, and their main source is from the follicles of gonads, while adrenal tissue is a minor source by several stress-induced synthesis pathways (Rivier & Rivest 1991, Sapolosky et al. 2000, Tilbrook et al. 2000, Goldstein 2004, Gore et al. 2006, Kirby et al. 2009). The gonads could affect the hypothalamic–pituitary–adrenal (HPA) axis at all levels (Viau et al. 2001, Viau 2002, Garcia et al. 2003, Seale et al. 2004, Euvarkerhe et al. 2009). The steady state of T4, E2, and P4 forms the basis for sex and individual differences in mammals (Dallman et al. 1995). In this study, we investigated the endocrine function of grafts, and found that castrated mice without ovarian transplantation had about 420 pmol/l of E2, which was mostly from adrenal tissue, similar to previously reported

Table 4 Steroid hormones changes in castrated male mice after ovary grafting.

<table>
<thead>
<tr>
<th>Hormone tested</th>
<th>Estrogen (pmol/l) (mean ± s.d.)</th>
<th>Progesterone (nmol/l) (mean ± s.d.)</th>
<th>Testosterone (nmol/l) (mean ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grafted</td>
<td>799.45 ± 319.95 *</td>
<td>180.39 ± 13.32 a</td>
<td>3.31 ± 0.60 b</td>
</tr>
<tr>
<td>Non-grafted</td>
<td>427.53 ± 103.95</td>
<td>2.31 ± 1.23</td>
<td>1.26 ± 0.37</td>
</tr>
<tr>
<td>Non-castrated</td>
<td>352.78 ± 49.8 b</td>
<td>0.95 ± 0.35</td>
<td>37.90 ± 7.60 a</td>
</tr>
</tbody>
</table>

The data in same columns were statistically analyzed by ANOVA post-hoc test. Most significant differences (P<0.01) among groups were indicated by different superscripts (a, b, c), but 'b' and 'c' showed significant differences (P<0.05).
data (Son et al. 2008). In contrast, E\textsubscript{2} concentration in grafted recipients was up to 800 pmol/l, which suggests that the follicles in the grafts exert an endocrine function, and the same phenomenon was evident in the significant difference in P\textsubscript{4} level between the recipients and castrated mice (180 vs 2 nmol/l). The endocrinology of ovary-grafted animals could also help us to understand follicular and oocyte development. Previously, there has been little knowledge on any steroid changes in male mice after ovarian transplantation. In this study, the increased level of E\textsubscript{2} and P\textsubscript{4} implied that the follicles were functioning for steroid secretion after ovarian transplantation in castrated male mice.

This study shows that mouse ovarian tissues develop further after they are grafted into immune-intact adult castrated male mice, the follicles exert endocrine function, and that the oocytes derived from the grafts have developmental potential.

**Materials and Methods**

**Animals**

Animals used in the experiments were treated according to the Local Care and Use of animals. Kunming mice, an outbred strain, were used. Ten-day-old female mice were used as ovary donors. Graft recipients were 8–10 weeks old, and weighed ~20 g. The recipients were randomly divided into three groups. Mice in the first group were castrated (n=214), and 7 days later, they were allografted with the intact 10-day-old fresh ovaries into the back muscle. The second group was the non-grafted castrated group (n=21), which was used for hormone assay after castration and surgical operation without ovary grafting. The third group (n=16) comprised of non-castrated male mice with back muscle sham-operated incisions and were age matched for hormone assay. The mice were housed with free access to food and water under a 12 h light:12 h darkness cycle at 18–25 °C in the animal house.

**Recipient and donor treatment**

All the mice used as graft recipients were castrated before ovary grafting. Before castration, the mice were anesthetized with i.p. injection of Sumianxinzhushueye at 0.02 ml/g of body weight (Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, Harbin, People’s Republic of China). The neck skin of the anesthetized mice was lifted, and the mice were shaken slightly in order to ensure that the testes stayed in the scrotum. A 5 mm long incision was made on the mouse scrotum by using an aseptic surgical knife; the two testes were pressed out of the scrotum slightly and then separated bluntly. In order to get the ovaries, the donor mice were killed, and the bilateral ovaries were recovered into culture dishes containing pre-warmed PBS, and kept on a 37 °C heating pad. The whole fresh ovaries were used as the implants. Some other fresh ovaries (n=12) were fixed for histological staining.

**Ovary allografting**

The fresh ovaries from the 10-day-old mice were immediately transplanted to the back muscle of castrated male mice as noted above. In the morning of the grafting day, the recipients were fasted of food and water. The mice were anesthetized as noted above. After shearing and sterilizing two sides of the backbone skin, an incision of about 1 cm long was made on the skin using aseptic scissors, and the muscle beneath the skin was then exposed. A 3–5 mm deep incision was made on the bilateral muscle by using a fine watchmakers’ forceps according to a previously reported method (Soleimani et al. 2008). Two ovaries collected from the same donor were transplanted into two sides of the muscle. The implants were about 10 mm away from the edge of the incision, and superficially attached to the muscle beneath, facing the skin (Israely et al. 2003, 2006). The grafting sites were well smoothed, and the skin was carefully stitched up. The entire grafting procedure was accomplished within 30 min under sterile conditions. The grafts on the allografting day were designated as 0-day-old. Some other castrated and non-castrated mice were treated in the same manner without ovary grafting. After being grafted, the recipient mice were smeared on the back muscle with an oily fluid of vitamin E (BASF AG, Ludwigshafen, Germany) every 3 or 4 days, which was hoped would reduce oxidative stress and facilitate blood vessel regeneration (Nugent et al. 1998).

**Immunosuppression treatment**

Based on the study of Kocik et al. (2004), a combination of cyclosporine, azathioprine, and prednisone was used as immunosuppressants in this study. Before immunosuppressant treatment, the cyclosporine (BBI Co., Kitchener, Ontario, Canada) was fist dissolved in absolute ethanol, and then redissolved in olive oil to a final concentration of 15 mg/ml (4% ethanol; Zhang et al. 2003). Four to eight hours before ovary grafting, the recipient mice were treated with cyclosporine, azathioprine, and prednisone. Specifically, they were injected s.c. with 0.1 ml cyclosporine of 5 mg/ml, followed by the same dose daily afterwards for 7 days, and then with 0.1 ml cyclosporine of 2.5 mg/ml every 3–4 days. Prednisone (Zhejiang Xianju Pharmaceutical Co., Taizhou, China) was dissolved in 0.9% sodium chloride and injected i.m. by 0.1 ml of 0.01 mg/ml every 3 days. Due to the low solubility of azathioprine (GlaxoSmithKline Co.), it was ground into powder and then dissolved in 0.9% sodium chloride to obtain a 1 mg/ml solution; the recipients were drenched with 0.1 ml azathioprine every 3–4 days. The control mice for hormone assay were treated in the same way. Other recipients were not treated with immunosuppressants as a control to observe the recovery rate and size of the grafts.

**Gonadotropin treatment**

Follicular stimulation was carried out according to a previously reported method with minor modifications (Soleimani et al. 2008). The recipients were treated with exogenous gonadotropins to facilitate follicle development, as the preliminary
study produced better results with stimulation by FSH and eCG than with FSH alone (data not shown). Forty-eight hours before ovary grafting, 10 IU FSH (Institute of Zoology, Chinese Academy of Sciences, Beijing, People's Republic of China) were given by i.p. injection. On the 3rd and 7th day after ovary grafting, 10 IU FSH treatment was repeated. On the 12th day after ovary grafting, 10 IU eCG (Calbiochem, San Diego, CA, USA; an Affiliate of Merck KgaA) was given by i.p. injection. Finally, on the 20th day, 10 IU eCG was repeated. The mice in the control group were treated with the same dosage and method.

**Histological staining**

On the 22nd day of ovary grafting, the recipients were autopsied. In total, 152 developmental grafts were recovered from the back muscle sites. Subsequently, 28 grafts were fixed for 48 h in 4% paraformaldehyde at 4 °C, and rinsed for 12 h in running water at room temperature, clarified in xylene, and embedded manually in paraffin. Serial sections of 5 μm thickness were made from 17 of the 28 fixed grafts and stained by H&E staining. The follicles were examined and counted under a microscope. Follicles were classified as primordial, primary, preantral, or antral follicles based on Jones & Krohn (1961) and our previous study (Cheng et al. 2009). Primordial follicles were confirmed when an oocyte was seen inside with one layer of flattened pre-granulosa cells around it; primary follicles were confirmed when an oocyte was seen inside with one layer of cuboidal granulosa cells around it; preantral follicles were confirmed when an oocyte was seen inside with two or more layers of granulosa cells around it without antrum; antral follicles were confirmed when an oocyte was seen inside with an obvious antral cavity, due to the certain size of one follicle, which might be sectioned with the serial sections. In order to prevent over-counting follicles, every five serial sections were considered to be a group, and then, under the microscope, the same follicles within this group were carefully distinguished and counted by two independent individuals. Finally, as with the above method, every section from the whole graft was observed and counted. The mean was obtained from these two independent counts, and an analysis was made among grafts.

**TUNEL staining**

Some other tissue sections from the remaining 11 grafts were subsequently deparaffinized by heating at 37 °C for 20 min and washing twice in xylene for a total of 20 min. The sections were then rehydrated through a graded series of alcohols, and rinsed twice with PBS. In situ TUNEL analysis was carried out according to the instructions of a commercial assay kit (In Situ Cell Death Detection Kit-Fluorescein; Roche) with some modifications. Briefly, tissue sections were incubated in a humidified chamber with 0.1% Triton X-100 (0.1% sodium citrate, freshly prepared; Solarbio, Beijing, People's Republic of China) at room temperature for 20–30 min, and then the slides were rinsed twice with PBS. The TUNEL reaction mixture was added, and the slides were incubated for 1 h at 37 °C with 50 μl TUNEL reaction mixture in a humidified dark chamber. A second set of tissue sections was incubated with 50 μl reaction buffer without terminal deoxynucleotidyl transferase as a negative control. As a positive control, a third set of sections was treated with 200 U/ml RQ1 RNase-free DNase (Promega) for 15–20 min at room temperature to induce non-specific breaks in DNA. The reaction was stopped by washing the sections in PBS three times, and then the tissue sections were examined with a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

**Oocyte collection and IVM**

19 grafts were washed in α-MEM (Sigma), and slightly pierced by a 1 ml syringe connected to a needle of 27½ gage to release the oocytes from the follicles. The recovered oocytes were rinsed, and then matured *in vitro* in α-MEM medium supplemented with 10 IU/ml eCG, 10 IU/ml hCG, 10 ng/ml epidermal growth factor, and 5% FBS (Motohashi et al. 2009) at 37 °C in 5% CO2 in air. Sixteen hours later, the PB1 extrusion was examined. On the 20th day of transplantation, 10 IU eCG was also given to the age-matched female mice, and using the same method, the oocytes were released from the follicles for use as a control for IVM and IVF.

**IVF and embryo culture**

After maturation, the oocytes with PB1 were taken for IVF. Briefly, the oocytes were washed and shifted to equilibrated human tubal fluid (HTF; Quinn et al. 1985). The epididymis was isolated from an adult male mouse, and cut into pieces in HTF medium for IVF. Four to six hours later, the putative fertilized oocytes were washed in HTF and mKSOM respectively (Lawitts & Biggers 1993, Erbach et al. 1994), and cultured in mKSOM in 5% CO2 in air at 37 °C.

In the first set of experiments, after 120 h, embryo development was examined. The blastocystcs were stained with Hoechst 33258 (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China), and photographed under a fluorescence microscope (IX71, Olympus).

**Embryo transfer**

In the second set of experiments, the 104 grafts were used to recover oocytes, and the embryos derived from IVF were transferred to pseudopregnant recipient mice (C57/BL6), 8- to 12-week-old virgins, which were induced by vasectomized males (C57/BL6). Those with vaginal plugs were used as pseudopregnant recipients. The 3.5-day-old embryos were transferred to pseudopregnant recipients. Approximately, eight to ten embryos were transferred to the unilateral uterus with a fine glass pipette. For pup production, the recipients were observed 17–18 days later.

**Hormonal assay**

At the 22nd day after ovarian transplantation, blood samples were collected. The concentration of E2, P4, and T4 was measured by a chemiluminescence immunoassay analyzer (CENTAUR, Bayer HealthCare).
Statistical analysis
The diameter and the number of the retrieved grafts, the number of the follicles in grafts, and the level of hormones were expressed as mean ± s.d., and analyzed with the ANOVA post-hoc tests. The developmental ability of oocytes was measured and expressed in the same way, and the t-test was used to analyze for statistical differences. \( P < 0.05 \) denoted a statistically significant difference, while \( P < 0.01 \) denoted a highly significant difference.

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.

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