Mouse spermatid nuclei can support full term development after premature chromosome condensation within mature oocytes

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The nucleus of round spermatids, the earliest haploid male germ cells, can participate in the formation of normal zygotes when incorporated into activated oocytes. In this study, we injected mouse round spermatids into homologous mature oocytes that were kept arrested at metaphase II to induce premature chromosome condensation (PCC) of the spermatid nuclei. After full condensation of the spermatid chromosomes, the oocytes were activated by Sr2+-containing medium, into which cytochalasin B was added to prevent extrusion of the segregated female and male chromosomes as polar bodies. Out of 142 oocytes examined, 104 (73%) formed two male (pseudo)pronuclei and two female pronuclei. To restore the diploid state of these zygotes, one of the female pronuclei was removed. When cultured in vitro for 72 hours, all (n = 37) of the constructed embryos developed to the morula/blastocyst stage. When 2-cell embryos and morulae/blastocysts were transferred into pseudopregnant females, 14 (13/96) and 24% (9/37), respectively, developed into term offspring. This study indicates that the spermatid chromosomes, which had undergone PCC, moved safely to opposite poles after oocyte activation. Since round spermatids contain no (in the mouse) or little (in patients with spermatogenic failure) oocyte-activating factor, this method may be used to rescue oocytes that fail to be activated at the time of spermatid injection.

Key words: fertilization/mouse/premature chromosome condensation/pronucleus/round spermatids

Introduction

Recent advances in micromanipulation techniques have made it possible to use immature sperm cells (spermatogenic cells) as male gametes. Normal zygotes have been obtained by either intracytoplasmic injection or electrofusion using spermatids (see review in Tesarik et al., 1998a), secondary spermatocytes (Kimura and Yanagimachi, 1995a), or primary spermatocytes (Kimura et al., 1998; Ogura et al., 1998). The spermatogenic cells most frequently used for microfertilization are the round spermatids, which have a set of haploid chromosomes just like mature spermatozoa. Unlike mature spermatozoa, however, round spermatids have a decondensed nucleus, which is more susceptible to metaphase-promoting factor (MPF) in the oocytes. According to Ogura and Yanagimachi (1993) and Kimura and Yanagimachi (1995b), the best protocol for fertilizing hamster and mouse oocytes with round spermatids is to inject spermatid nuclei into oocytes that have been activated previously, thus avoiding MPF-induced premature chromosome condensation (PCC) of the spermatid nucleus. However, if spermatids are injected as little as 2 h after oocyte activation, they fail to undergo nuclear membrane breakdown and their nuclei stay small. In mice, the spermatid nuclei should be incorporated into oocytes at telophase II, which occurs about 45–80 min after activation. This time-limited protocol may, at least in part, be responsible for the lower rate of conception with spermatids than with mature spermatozoa.

Recently, it has been demonstrated that G1-phase chromosomes that undergo premature condensation in mature metaphase II (MII) oocytes moved to either of the poles after oocyte activation (Wakayama et al., 1998). These two groups of the donor chromosomes intermingled at the first mitosis. If it is also true for spermatid G1 chromosomes, they may participate in embryo development following premature condensation and oocyte activation. In this study, mouse spermatids were injected into mature oocytes, which were kept arrested at MII to induce premature condensation of the spermatid chromosomes. The oocytes were activated while extrusion of the polar bodies was prevented, and one of the female pronuclei was removed in order to restore the diploid state. The developmental ability of embryos thus obtained was examined in vivo and in vitro.

Materials and methods

Collection of oocytes

Mature oocytes were collected from the oviducts of B6D2F1 females (SLC Co., Shizuoka, Japan) induced to superovulate with 7.5 IU equine chorionic gonadotrophin (eCG) followed 48 h later with 7.5 IU human chorionic gonadotrophin (HCG). They were placed in CZB medium (Chatot et al., 1989) and treated with hyaluronidase until the cumulus cells dispersed. The oocytes were placed in drops of CZB, covered with silicone oil (Aldrich Chemical Co., Wisconsin, USA) and kept in plastic dishes (Falcon no. 1008; Becton Dickinson, NJ, USA). They were kept under 5% CO2 in air at 37°C unless otherwise stated.

Collection of spermatogenic cells

Spermatogenic cells were mechanically isolated from the seminiferous tubules of C57BL/6J males (SLC Co., Shizuoka, Japan) as described previously (Ogura and Yanagimachi, 1993). The cell suspension was washed by centrifugation twice and stored in Dulbecco’s phosphate-
buffered saline (DPBS) containing 0.5% bovine serum albumin (BSA, fraction V, Calbiochem, CA, USA) at 4°C for up to 4 h.

Microinsemination with round spermatids

The cover of a plastic dish (Falcon no. 1006; Becton Dickinson) was used as a microinjection chamber. A row of four small drops (1–2 µl each), of which two were HEPES-buffered CZB containing 0.1 g/ml polyvinyl alcohol (for oocytes) and two were 12% polyvinylpyrrolidone in HEPES-buffered CZB, was placed on the bottom of the dish and covered with silicone oil. One of the PVP drops contained a spermatogenic cell suspension. The dish was placed on the stage of an inverted microscope (Nikon TE300) equipped with Nomarski differential interference optics. Mouse round spermatids can be easily identified by their small size (about 12 µm in diameter) and round nucleus with a centrally located nucleolar structure. The pairs of spermatid-derived pronuclei (pseudopronuclei) were easily identified by their smaller diameters, fewer nucleoli, and their more distal position from the plasma membrane of the oocyte, as compared to other pairs. In addition, presumptive spermatid-derived nucleoli of a single male pronucleus.

Results

Between 70–90% of the oocytes survived the injection procedure, depending on the experiment. Most oocytes (>95%) remained in the MII state after spermatid injection until they were artificially activated. The spermatid chromosomes underwent premature condensation shortly (<1 h) after injection and were randomly scattered in the spindle that was newly formed in the ooplasm (Figure 2A). Treatment with SrCl2 in the presence of cytochalasin B caused the oocytes to resume meiosis and MII chromosomes started to segregate to form two haploid chromosome masses. At the same time, the spermatid chromosomes each started to move towards the proximal pole (Figure 2B). Within 4 h of activation, one to four pronuclei appeared in each activated oocyte (Table I). The data were analysed with Fisher’s exact probability test.

Figure 1. Diagrams illustrating two methods of microfertilization with round spermatids in the mouse. In A, the nucleus of round spermatids undergoes premature chromosome condensation and forms two (pseudo)pronuclei. Then one of the two female pronuclei is removed to restore the diploid state. In B, the method previously reported, the spermatid nucleus is injected into pre-activated oocytes to obtain a single male pronucleus.
Figure 2. (A) An oocyte 2 h after spermatid injection, showing the female MII chromosomes (F) and the spermatid chromosomes that had been prematurely condensed (M). The spermatid chromosomes are randomly arranged within the spindle. Bar = 20 µm. (B) An oocyte 30 min after activation. The female chromosomes segregate, showing the anaphase configuration (F). The spermatid chromosomes are all moving towards the proximal pole (M). Bar = 20 µm. (C) An oocyte 8 h after activation, showing two spermatid-derived (pseudo)pronuclei (arrowheads) and two female pronuclei (small arrows), one of which is being removed to restore the diploid state. The large arrow indicates a restored diploid zygote with three pronuclei. Bar = 100 µm.

Table I. Pronuclear formation in oocytes injected with spermatids and then activated 2 h later in the presence of cytochalasin B

<table>
<thead>
<tr>
<th>No. of oocytes examined</th>
<th>No. (%) of oocytes with</th>
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<tr>
<td></td>
<td>4 PN</td>
</tr>
<tr>
<td>142</td>
<td>104 (73)</td>
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</table>

PN = pronucleus.

pronuclei often (about 30%) differed in size from each other. One of the female pronuclei was removed from the oocytes 4 or 8 h after activation (Figure 2C). In our preliminary study, we found that separation of one female pronucleus from the other with a glass pipette sooner than 4 h post-activation was impossible due to the rigidity of the microtubules connecting the two pronuclei. They could be separated more easily as time passed after activation, and at 8 h, the separation seemed to cause no mechanical damage to the oocytes. Therefore, we examined whether the time of female pronucleus removal affects the subsequent in-vivo and in-vitro development of restored embryos. As shown in Table II, all the constructed embryos developed to the morula/blastocyst stage after 72 h in culture and some developed to term after transfer into recipient females. The time of enucleation had no effect on their development either in vivo or in vitro. In the second experiment, embryos were transferred into the oviducts at the 2-cell stage, 2 days earlier than in the first experiment. Once again, normal term pups were obtained and the enucleation time had no effect on the in-vivo development (Table III). The rate of term development was lower than, but not significantly different from, that in the first experiment ($P > 0.05$). The implantation rate was also low by unknown causes; in our routine experiments, 50–80% of normally fertilized embryos [in-vitro fertilization (IVF) or in-vivo produced embryos] are implanted after oviductal transfer. All the pups were phenotypically normal and had pigmented eyes, as expected.

Of 18 oocytes that were fixed and stained 20–35 min after activation, four had individually identifiable spermatid chromosomes that were moving toward either of the poles. Of these, two oocytes showed spermatid chromosomes dividing into 15 and 5, one into 14 and 6, and one into 11 and 9. In the remaining 14 oocytes, the spermatid chromosomes were still at the equatorial area or segregated completely to form the telophase configuration.

Discussion
In several mammalian species, including humans, the nucleus of spermatids has the ability to participate in normal fertilization and embryo development (for review see Tesarik et al., 1998a). According to previous studies using round spermatids and
mature oocytes, normal fertilization can only occur when the oocytes are activated shortly before or at the time of spermatid injection. In these protocols, PCC of the spermatid by the ooplasmic MPF was prevented, while breakdown of the spermatid’s nuclear membrane was induced so that a well-developed male pronucleus could form. The present study demonstrates that even after PCC and chromosome segregation into two pronuclei, the paternal genome of the spermatids maintains its ability to support full-term development. The spermatid chromosomes that underwent premature condensation seemed to segregate randomly to either pole. When activated oocytes were investigated at anaphase, a varying number of chromosomes moved toward each pole. This finding is compatible with the fact that in some oocytes (about 30%), the two spermatid-derived pronuclei appeared differently in size.

According to Kimura and Yanagimachi (1995b), the best protocol for spermatid conception in the mouse is to activate oocytes first and inject them with a spermatid nucleus 1 h later. The majority of oocytes (77%) were normally fertilized (two swollen pronuclei and one second polar body), while the remaining oocytes had a small spermatid-derived pronucleus. The abnormal pronuclear formation in the latter was probably due to the persistence of the spermatid nuclear membrane. This was observed more frequently when spermatid nuclei were injected into oocytes at more advanced stages in which the cytoplasmic MPF level had decreased. These small male pronuclei supported embryo development less efficiently than the regular sized male pronuclei (Kimura and Yanagimachi, 1995b). In our new protocol, one of two female pronuclei is removed to restore the diploid state. As far as we could tell, the time of enucleation did not have any effect on the subsequent embryo development. The rates of normal birth after embryo transfer (24 and 14%) were within the range of our previous experiments using pre-activated oocytes (about 10–35%, Ogura et al., 1996a; Tanemura et al., 1997).

In some oocytes, two or three pronuclei were found, instead of four, and we could not perform enucleation. After spermatid injection, the oocytes were kept arrested at MII for about 2 h to induce full condensation and spindle formation. The spermatid chromosomes might have mingled with female chromosomes during this period because of the attractive forces of newly polymerized spindle microtubules, or during early pronuclear development as observed in human zygotes after spermatid injection (Tesarik and Mendoza, 1996; Barak et al., 1998). Our findings have implications for human clinical practice. Sperm-borne oocyte activating factor is present in human round spermatids, but it is absent or deficient in round spermatids from patients with spermatogenic failure (Tesarik et al., 1998a), and, in fact, the fertilization rate after round spermatid injection is significantly lower than that after elongated spermatid injection (25.6 versus 71%) (Kahraman et al., 1998). Therefore, it is very probable that spermatids from these patients would undergo PCC after incorporation into oocytes, which have high MPF activity. These oocytes are classified as ‘unfertilized’ and are presently discarded. The results of this study indicate that these oocytes may be rescued by applying external activation stimulus, followed by removal of a female pronucleus. These rescued oocytes may develop to term if spermatids have no DNA damage, which is often associated with spermiogenesis failure (Tesarik et al., 1998b).

### Table II. Development of oocytes fertilized with round spermatids (uterine transfer after 72 h in culture)\(^a\)

<table>
<thead>
<tr>
<th>Time of female PN removal(^b)</th>
<th>No. of oocytes cultured</th>
<th>No. (%) of oocytes that developed to morulae/blast(^c)</th>
<th>No. (%) of oocytes implanted(^d)</th>
<th>No. (%) of oocytes developed to term(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>20</td>
<td>20 (100)</td>
<td>15 (75)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>8 h</td>
<td>17</td>
<td>17 (100)</td>
<td>12 (71)</td>
<td>4 (24)</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>37 (100)</td>
<td>27 (73)</td>
<td>9 (24)</td>
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</tbody>
</table>

\(^a\)All morulae and blastocysts were transferred. 
\(^b\)Time after oocyte activation. 
\(^c,d,e\)No significant difference between groups.

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### Table III. Development of oocytes fertilized with round spermatids (oviductal transfer after 24 h in culture)\(^a\)

<table>
<thead>
<tr>
<th>Time of female PN removal(^b)</th>
<th>No. of oocytes cultured</th>
<th>No. (%) of oocytes that developed to 2-cells(^c)</th>
<th>No. (%) of oocytes implanted(^d)</th>
<th>No. (%) of oocytes developed to term(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>56</td>
<td>51 (91)</td>
<td>19 (37)</td>
<td>5 (10)</td>
</tr>
<tr>
<td>8 h</td>
<td>46</td>
<td>45 (98)</td>
<td>15 (33)</td>
<td>8 (18)</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>96 (94)</td>
<td>34 (35)</td>
<td>13 (14)</td>
</tr>
</tbody>
</table>

\(^a\)All 2-cells were transferred. 
\(^b\)Time after oocyte activation. 
\(^c,d,e\)No significant difference between groups.
Perhaps this can also be applied to unfertilized human oocytes after intracytoplasmic sperm injection (ICSI) if spermatozoa undergo PCC due to oocyte activation failure (Flaherty et al., 1995). The technique of removing supernumerary pronuclei from human oocytes has already been established for rescuing polyspermic zygotes (Gordon et al., 1989).

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References

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