Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring

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SUMMARY

Genomic imprinting occurs in both male and female gametes during gametogenesis, but the exact time when imprinting begins and ends is unknown. In the present study we injected nuclei of testicular spermatozoa and round spermatids into mature mouse oocytes to see whether these nuclei are able to participate in syngamy and normal embryonic development. If the injected oocytes develop into normal fertile offspring, imprinting in the male germ cells used must have been completed by the time of injection.

Ninety-two percent of mouse oocytes injected with testicular spermatozoa survived and 94% of these were fertilized normally (extrusion of the second polar body and formation of male and female pronuclei). When 44 two-cell embryos so created were transferred to 5 foster mothers, 24 (54.5%) developed into normal offspring. Unlike testicular spermatozoa, round spermatids could not activate the oocytes, and therefore the oocytes had to be activated artificially either before or after spermatid injection. The highest rate (77%) of normal fertilization was obtained when the oocytes were first activated by electric current, then injected individually with a single spermatid nucleus. When 131 two-cell embryos were transferred to 15 foster mothers, 37 (28.2%) reached full term. All but two grew into healthy adults.

Thus, it would appear that gametic imprinting in mouse spermatogenic cells is completed before spermiogenesis begins. Under the experimental conditions employed, spermatid nuclei were less efficient than testicular sperm nuclei in producing normal offspring, but perhaps this was due to technical rather than inherent problems.

Key words: spermatogenic cell, spermatozoa, spermatid, imprinting, mouse, fertilization

INTRODUCTION

In mammals normal embryonic development requires differential genomic imprinting of both male and female gametes (Monk, 1988; Solter, 1988). Although genomic imprinting of gametes (gametic imprinting) occurs sometime during gametogenesis the exact time when it occurs remains unknown. So far only two genes, Igf2r and Xist have been shown to be gametically imprinted (Brandeis et al., 1993; Stoger et al., 1993; Zuccotti and Monk, 1995). The injection of mature oocytes with nuclei from spermatogenic cells at various stages of differentiation would be a means of determining when the imprinting of male germ cells is completed. Our studies were initiated along this line.

Direct injection of male germ cells into mature oocytes may sound radical, but production of normal offspring following intracytoplasmic sperm injection (ICSI) has been successful in several mammals particularly in humans (Palermo et al., 1992; van Steirteghem et al., 1993a,b; Payne et al., 1994). Apparently we can bypass gamete membrane fusion as long as the nuclei of both male and female gametes have completed their genomic imprinting. Previously we have reported development of normal mice after electrofusion of round spermatid nuclei with mature oocytes (Ogura et al., 1994). However, a rather low success rate raised a possibility of genomic imprinting being complete in only a few of the round spermatids. In this paper we show that this is not the case since simple technical improvements alone have lead to a considerably improved success rate.

MATERIALS AND METHODS

Reagents
All inorganic and organic compounds were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Media
The medium used for culturing oocytes after microsurgery was CZB (Chatot et al., 1989, 1990) supplemented with 5.56 mM D-glucose. The medium for oocyte collection from oviducts, subsequent treatments and micromanipulation was a modified CZB with 20 mM Hepes, a reduced amount of NaHCO₃ (5 mM) and 3 mg ml⁻¹ bovine serum albumin (BSA, fraction V, Calbiochem., La Jolla, CA). This medium was called Hepes-CZB. In the later part of our study we replaced BSA in Hepes-CZB with 0.1 mg ml⁻¹ polyvinyl alcohol (PVA, cold water soluble, Mr 10×10³). PVA kept the wall of the injection pipette less sticky for mineral oil and cell debris over a longer period than BSA. This was beneficial during repeated use of a single pipette.

Preparation of oocytes
B6D2F1 females (6-12 weeks old) were each injected with 7.5 IU pregnant mare serum gonadotropin followed by 7.5 IU human...
Microinjection of testicular spermatozoa and spermatids into oocytes

A single testicular spermatozoon was injected into an oocyte as described previously for injection of mature epididymal spermatozoa (Kimura and Yanagimachi, 1995). A testicular spermatozoon was sucked, tail first, deep into an injection pipette (5 μm I.D. at its tip) (Fig. 2), its head was near the tip of the injection pipette, the pipette was advanced in the ooplasm. After the spermatozoon was pushed forward until its pellucida was drilled by applying several Piezo pulses to the sperm (Kimura and Yanagimachi, 1995). Repeated pipettings released many spermatozoa and spermatogenic cells from tubular fragments. Initially a few spermatozoon displayed sluggish movement, but all were motionless by the end of pipetting. The final suspension contained spermatozoa as well as spermatogenic cells at various stages of development. A droplet (about 3 μl) of this suspension was placed on a plastic Petri dish, covered with mineral oil (Squibb & Sons, Princeton, NJ) and kept for up to 3 hours at 16-17°C before spermatozoa or spermatids were selected for injection into oocytes. In preliminary experiments we used CZB and several other complex media for cell culture to suspend spermatozoa and spermatogenic cells. Since the rates of oocyte activation and subsequent development of zygotes did not differ between the groups using 0.9% NaCl versus other media, we used 0.9% NaCl for suspension of testicular spermatozoa/spermatids prior to injection. For long term storage (>3 hours) of spermatozoa/spermatids, however, more complex cell culture media (e.g., Dulbecco’s PBS) were superior to 0.9% NaCl.

Preparation of testicular spermatozoa and spermatids

A testis was isolated from a mature male mouse (B6D2F1, 8-13 weeks old). After removal of the tunica, seminiferous tubules were placed in 1 ml of cold (4-10°C) 0.9% NaCl containing 1% (w/v) polyvinyl pyrrolidone (PVP, Mr 360×10³, ICN) and cut into minute pieces using a pair of fine scissors. One part of the suspension containing fragments of seminiferous tubules was mixed thoroughly with two parts of cool (4-10°C) PVP-saline (0.9% NaCl containing 12% (w/v) PVP). Repeated pipettings released many spermatozoa and spermatogenic cells from tubular fragments. Initially a few spermatozoa displayed sluggish movement, but all were motionless by the end of pipetting. The final suspension contained spermatozoa as well as spermatogenic cells at various stages of development. A droplet (about 3 μl) of this suspension was placed on a plastic Petri dish, covered with mineral oil (Squibb & Sons, Princeton, NJ) and kept for up to 3 hours at 16-17°C before spermatozoa or spermatids were selected for injection into oocytes. In preliminary experiments we used CZB and several other complex media for cell culture to suspend spermatozoa and spermatogenic cells. Since the rates of oocyte activation and subsequent development of zygotes did not differ between the groups using 0.9% NaCl versus other media, we used 0.9% NaCl for suspension of testicular spermatozoa/spermatids prior to injection. For long term storage (>3 hours) of spermatozoa/spermatogenic cells, however, more complex cell culture media (e.g., Dulbecco’s PBS) were superior to 0.9% NaCl.
necessary for zygote development. Stimulation was achieved by applying a single DC electric pulse (1 kV/cm, 128 microseconds) to a group of spermatid-injected oocytes (usually about 10 at a time) in Hepes-CZB at 25°C.

Since the incidence of normal fertilization following spermatid injection was initially low (Table 1), experiments were conducted to determine whether the electric pulse should be given to the oocytes before or after spermatid injection in order to obtain the highest rate of normal fertilization (for definition of fertilization, see below).

Three experimental protocols are shown diagrammatically in Fig. 4. In protocol A, the oocytes were stimulated first. When stimulated oocytes reached telophase of the second meiosis, 1 hour after incubation at 37°C, each was injected with a spermatid nucleus as described above. In protocols B and C, the oocytes were first injected with spermatid nuclei, then electro-stimulated either 30 minutes (B) or 90 minutes later (C).

**Fig. 4.** Three protocols of spermatid injection. (A) A group of 5-10 oocytes was first electro-stimulated at 25°C. After 60 minutes incubation at 37°C, eggs were injected with a single spermatid each at 16-17°C. It took 10-20 minutes to inject 5-10 eggs. After the last egg was injected, the eggs were kept for additional 20 minutes at 16-17°C, then 10 minutes at 25°C before incubation at 37°C. Eggs were examined for cytological details 5-6 hours after spermatid injection. (B) Oocytes were injected with spermatids at 16-17°C, then electro-activated at 25°C 30 minutes later. (C) Same as above, but the oocytes were electro-activated 90 minutes after spermatid injection.

**RESULTS**

**Oocyte activation following testicular sperm/spermatid injection**

Table 1 summarizes the results of experiments in which a single testicular spermatozoon or round spermatid was injected into individual oocytes. Testicular spermatozoa activated the majority of the oocytes when injected, and no additional stimulation was needed. By 5-7 hours after injection most oocytes had extruded the second polar body and had two large pronuclei (2PN + 1Pb2) (Exp. 1 in Table 1). Spermatids similarly injected, on the contrary, did not activate the oocytes. None of 46 live oocytes examined between 5 and 7 hours after injection were activated, being arrested at metaphase II (Exp. 2 in Table 1). Spermatids had extruded the second polar body and had two large pronuclei (2PN + 1Pb2) (Exp. 1 in Table 1). Spermatids similarly injected, on the contrary, did not activate the oocytes. None of 46 live oocytes examined between 5 and 7 hours after injection were activated, being arrested at metaphase II (Exp. 2 in Table 1). When these oocytes were fixed and examined, the majority (65%) contained spermatid nuclei exhibiting premature chromosome condensation. Of the others 22% contained intact or almost intact spermatid nuclei, 4% contained a highly condensed chromatin mass, and the
remainder had unidentifiable or missing spermatid nuclei. In contrast, spermatid nuclei developed into large male pronuclei when the oocytes were electro-stimulated after injection (Exp. 3 in Table 1).

An egg with two large pronuclei and one second polar body (2PN + 1Pb₂) was classified as ‘fertilized normally.’ Since the rate of normal fertilization following round spermatid injection was considerably lower (37%) than that using testicular spermatozoa (94%) in the above experiments (Table 1), attempts were made to increase the rate of normal fertilization following spermatid injection. In the first experiments, a spermatid nucleus was injected into an oocyte accompanied by the bulk of cytoplasm (Fig. 2) with injection either before or after electrical stimulation as illustrated in Fig. 4. In the second series of experiments the spermatid nucleus was separated from the bulk of cytoplasm before its injection (Fig. 3). The results, summarized in Table 2, show that the highest rate (77%) of normal fertilization (2PN + 1Pb₂; Fig. 5A,B) was obtained when the oocytes were first electro-stimulated, then injected 1 hour later with ‘denuded’ nuclei (protocol A, ‘denuded’). Even under this condition, however, not all the eggs were fertilized normally. Twenty-one percent contained only one large (female) pronucleus (Fig. 6A), together with either a small pronucleus of spermatid origin (Fig. 6B) or a spermatid nucleus that was still almost intact or condensed.

### Development of zygotes

When eggs fertilized by testicular spermatozoa were cultured in vitro, 70% developed into blastocysts (Table 3). Eggs fertilized by spermatids developed well when each contained two large pronuclei and one second polar body. Those with only one large (female) pronucleus seldom developed into blastocysts (Table 3).

The results of embryo transfer (Table 4) indicate that both sperm- and spermatid-injected oocytes can develop into live offspring, although the chance of normal development is higher following injection of spermatid. Except for two that were eaten by a mother soon after birth, all the young born grew into normal adults. Of 35 adults that developed from spermatid-injected oocytes, 7 females and 7 males were randomly selected and mated. All proved to be fertile. The second generation born to these animals was all normal.

We also transferred into each of six foster mothers 6-12 two-cell embryos that had developed from aberrantly fertilized eggs (1PN + 1Pb₂). Four foster mothers gave birth to a total of 5 foster pups in addition to their own pups. One of the foster pups

### Table 2. Fertilization of mouse oocytes by round spermatids, examined 5-6 hours after injection

<table>
<thead>
<tr>
<th>Exp. protocol (see Fig. 4)</th>
<th>State of spermatid nuclei during injection</th>
<th>Total no. of oocytes injected (no. exp.)</th>
<th>Oocytes survived (%)</th>
<th>No. (%) of surviving eggs with*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MetII 2PN+1Pb₂ 1PN+1Pb₂ Others†</td>
</tr>
<tr>
<td>A</td>
<td>‘Undenuded’</td>
<td>53 (5)</td>
<td>51 (96)</td>
<td>7 (14)$     34 (67) 8 (16) 0 (0) 0 (0) 2 (4)</td>
</tr>
<tr>
<td></td>
<td>‘Denuded’</td>
<td>46 (5)</td>
<td>44 (96)</td>
<td>34 (72)$; 14 (28) 6 (14) 3 (7) 0 (0) 0 (0) 1 (2)</td>
</tr>
<tr>
<td>B</td>
<td>‘Undenuded’</td>
<td>51 (5)</td>
<td>44 (86)</td>
<td>12 (27)$; 16 (36) 11 (25) 1 (2) 0 (0) 1 (2) 4 (9)</td>
</tr>
<tr>
<td></td>
<td>‘Denuded’</td>
<td>40 (5)</td>
<td>34 (85)</td>
<td>16 (47)$; 1 (3) 2 (6) 3 (9) 3 (9) 9 (27)</td>
</tr>
<tr>
<td>C</td>
<td>‘Undenuded’</td>
<td>50 (5)</td>
<td>41 (82)</td>
<td>8 (20)$; 6 (15) 11 (27) 10 (24) 1 (2) 5 (12)</td>
</tr>
<tr>
<td></td>
<td>‘Denuded’</td>
<td>44 (5)</td>
<td>41 (93)</td>
<td>7 (17)$; 1 (2) 1 (2) 15 (37) 8 (20) 6 (15)</td>
</tr>
</tbody>
</table>

*Abbreviations: PN, pronucleus; sPN, small pronucleus; Pb₂, second polar body. The number before PN or Pb₂ denotes the number of pronucleus or second polar body in each egg. 2Pb₂, for example, means two second polar bodies instead of the normal one polar body.

†Others include the oocytes without or unidentifiable, nuclear materials.

‡Difference between § and ¶ is significant (P<0.001, $\chi^2$ test).
2401 Sperm injection was alive and looked normal on the day of birth, but was eaten by the mother sometime before the morning of the next day. The remaining foster pups grew into normal adults (1 male and 3 females).

DISCUSSION

The present study demonstrates that both testicular spermatozoa and round spermatids of the mouse can produce zygotes that develop into normal, fertile offspring. Recently, normal fertilization was obtained in humans following oocyte injection with testicular spermatozoa (Schoysman et al., 1993a,b; Silber, 1994; Silber et al., 1994; Devroey et al., 1994). It is important to note that these testicular spermatozoa were collected from the testes of infertile patients lacking or defective in the epididymis and/or the vas deferens. While these testicular spermatozoa may not be wholly identical with those in normal...
testis, it is clear that spermatozoa produced in the testes of mice and men do not need to be exposed to the extra-testicular duct system to support normal development.

It is well established that normal embryonic development in mammals requires differential imprinting of maternal and paternal genomes (Solter, 1988; Monk, 1988; Ueda et al.,

### Table 3. In vitro development of mouse eggs injected with testicular spermatozoa or round spermatids

<table>
<thead>
<tr>
<th>Eggs fertilized by</th>
<th>Fertilized eggs with (protocol)</th>
<th>Total no. of eggs cultured (no. exp.)</th>
<th>No. (%) of eggs developed into</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2PN+1Pb₂</td>
<td>137 (7)</td>
<td>2-cell 135 (99) 4-cell 134 (98) Morula 122 (89) Blastocyst 96 (70)</td>
</tr>
<tr>
<td>Testicular spermatozoa</td>
<td>2PN+1Pb₂ (Protocol A)</td>
<td>73 (6)</td>
<td>2-cell 73 (100) 4-cell 73 (100) Morula 69 (95) Blastocyst 61 (84)</td>
</tr>
<tr>
<td>Spermatid ‘denuded’ nuclei</td>
<td>2PN+1Pb₂ (Protocol B)</td>
<td>82 (9)</td>
<td>2-cell 79 (96) 4-cell 74 (90) Morula 62 (76) Blastocyst 41 (50)</td>
</tr>
<tr>
<td>Spermatid ‘undenuded’ nuclei</td>
<td>1PN+1Pb₂ (Protocol B)</td>
<td>57 (8)</td>
<td>2-cell 56 (100) 4-cell 42 (74) Morula 21 (37) Blastocyst 4 (7)</td>
</tr>
<tr>
<td>Spermatid ‘denuded’ nuclei</td>
<td>1PN+1Pb₂ (Protocol B)</td>
<td>53 (3)</td>
<td>2-cell 53 (100) 4-cell 51 (96) Morula 38 (72) Blastocyst 1 (2)</td>
</tr>
<tr>
<td>Not fertilized*</td>
<td>1PN+1Pb₂</td>
<td>53 (3)</td>
<td>2-cell 53 (100) 4-cell 51 (96) Morula 38 (72) Blastocyst 1 (2)</td>
</tr>
</tbody>
</table>

*Unfertilized oocytes were stimulated with electric current only.

### Table 4. Offspring from eggs fertilized normally (2PN+1Pb₂) after sperm/spermatid injection

<table>
<thead>
<tr>
<th>Embryos developed from eggs fertilized by</th>
<th>No. of recipients</th>
<th>Total no. of foster 2-cell embryos transferred</th>
<th>No. live offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Recipient’s own [albino]</td>
</tr>
<tr>
<td>Testicular spermatozoa</td>
<td>5</td>
<td>44</td>
<td>34</td>
</tr>
<tr>
<td>Spermatids</td>
<td>15†</td>
<td>131</td>
<td>137</td>
</tr>
</tbody>
</table>

*(Foster babies born/2-cell foster embryos transferred) × 100.
†Spermatid nuclei were injected according to experimental protocol B (see Fig. 4).
‡,§The difference between ‡ and § is statistically significant (χ² test, P<0.001).
¶Two cannibalized by a mother soon after birth, so their sexes were not determined.

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Fig. 7. An unactivated oocyte, 1 hour after spermatid injection. This spermatid nucleus (arrow) had undergone premature chromosome condensation; its chromosomes are associated with a spindle. Met II, oocyte’s metaphase II chromosomes.

Fig. 8. An egg with two polar bodies (Pb₂) and two large pronuclei (PN). This egg was injected with a spermatid then electro-activated 90 minutes later. One of two PN-Pb₂ pairs is believed to be of spermatid origin (see Fig. 9 IV).
Imprinting is a mark that differentiates between alleles of paternal and maternal origin and regulates their expression. Recent studies have revealed that specific chromosome regions are imprinted (Cattanach and Jones, 1994), but so far only a few genes have been shown to be involved (Razin and Cedar, 1994). Moreover, as yet gametic imprinting has been proved only for mouse \( Igf2r \) and \( Xist \) genes (Brandeis et al., 1993; Stoger et al., 1993; Zuccotti and Monk, 1995).

Methylation of the cytosine in CpG dinucleotides is one strong candidate for the means of genomic imprinting (Razin and Cedar, 1994). So far, five imprinted genes have been found to be differentially methylated on the paternal and maternal alleles within somatic cells: (1) mouse \( Igf2 \) (Brandies et al., 1993) and (2) mouse \( Xist \) (Norris et al., 1994; Ariel et al., 1995; Zuccotti and Monk, 1995), (3) human \( SNRPN \) from the paternal allele (Glenn et al., 1993), (4) mouse \( Igf2r \) (Stoger et al., 1993), and mouse \( H19 \) from the maternal allele (Bartolomei et al., 1993; Ferguson-Smith et al., 1993).

Although the studies cited above strongly support the notion that methylation represents the primary imprint, the precise chronology of gametic imprinting is not well understood. Injection experiments as reported here do not provide any information about imprinting of particular genes or chromosome regions, but they demonstrate conclusively whether genomic imprinting in male germ cells is complete or incomplete. It is of course possible that different genes are imprinted at different stages of gametogenesis and through different mechanisms. However, it is equally possible that different genes are imprinted simultaneously or within a rather short
time. All we can say at present is that, in the mouse, imprinting of spermatogenic cells is complete before spermiogenesis begins. According to Ariel et al. (1994), the methylation pattern of certain genes changes even after transport of testicular spermatoid to the epididymis. Obviously, these changes are not essential for embryonic development based on the results of our experiments. We are now injecting oocytes with male germ cells at earlier stages of their differentiation, with a view of discovering whether imprinting can occur even within the cytoplasm of maturing oocytes, or whether it is possible only within intact spermatogenic cells.

The incidence of normal fertilization was initially lower with spermatid injection than with testicular spermatoozoa (Table 1). Ogura et al. (1993) electrofused mouse spermatids with oocytes and found that most of the spermatid nuclei did not develop into large pronuclei. We could largely avoid this and increase the incidence of normal fertilization by activating the oocytes first, then injecting ‘denuded’ spermatid nuclei (Table 2, protocol A ‘denuded’).

It is evident that testicular spermatoozoa, like mature epididymal spermatoozoa, contain an oocyte-activating factor. When injected into oocytes, testicular spermatoozoa activated the oocytes and their nuclei transformed into (male) pronuclei. Round spermatids are lacking in the oocyte-activating factor and therefore oocytes injected with round spermatids remained unactivated. Since the nucleus of a spermatid is in the G2 phase of the cell cycle, it undergoes premature chromosome condensation within an unactivated oocyte in the metaphase of the cell cycle. The spermatid nucleus developed into a large pronucleus only when the oocyte was activated by artificial means (e.g. electrical stimulation). Not surprisingly, the timing of spermatid injection and oocyte activation was critical for normal development of pronuclei. When sperm-injected oocytes were left unactivated for an extended period of time, microtubules/spindle associated with prematurely condensed spermatid chromosomes (Fig. 7) (Harrouk and Clarke, 1993). When such oocytes were activated, either some spermatid chromosomes were shed into the pseudo-second polar body (Fig. 8) or two or more spermatid pronuclei were formed, each containing fewer chromosomes than the normal pronucleus. Fig. 9 illustrates the probable behavior (fate) of spermatid nuclei after injection into oocytes.

The reason for the low rate of normal fertilization (2PN + 1Pb2) following injection of cytoplasm-encapsulated spermatid nuclei is not clear, but it may be due in part to the persistence of the spermatid’s cytoplasm around each nucleus for some time after injection. As expected, the zygotes with only one large pronucleus seldom developed into normal blastocysts and into live offspring. In fact, it was rather surprising that any could do so. Some of the living eggs we identified as 1PN + 1Pb2 zygotes in living conditions must have had a minute 1Pb2 zygotes in living conditions must have had a minute 1Pb1 nucleus with a large female pronucleus appears to be possible (see Fig. 6 of Ogura et al., 1993). Ogura et al. (1994), who electrofused round spermatids with mouse oocytes, transferred all 2-cell embryos to foster mothers regardless of the nuclear status of the eggs. Whereas some of them could have been developing parthenogenetically, others could have developed from the eggs with a small spermatid pronucleus. This may explain the low yield of live offspring in the experiments conducted by Ogura et al. (6 pups were born after transferring a total of 346 two-cell embryos to 8 foster mothers). If they had selected and transferred only the embryos developed from the eggs with two large pronuclei and the second polar body (2PN + 1Pb2), the pregnancy rate may have been much higher. Our recommended procedure of intracytoplasmic spermatozoon injection is as follows: (a) activate oocytes first (e.g. by electric pulse); (b) inject a single spermatid nucleus into each oocyte when the eggs have reached telophase II; (c) immediately before injection, free the spermatid nuclei from the bulk of the surrounding cytoplasm. Technically, mechanical injection of spermatids into oocytes, as reported here, is simpler and quicker than electrofusion as employed by Ogura et al. (1994). Recently, Vanderzwalmen et al. (1995) succeeded in fertilizing human oocytes by mechanically injecting spermatids. Sofikitis et al. (1994) obtained live rabbit young after mechanical injection of spermatids into oocytes.

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Sperm injection

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