Production of fertile offspring from genetically infertile male mice

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A number of recessive autosomal genes cause male infertility. Male mice homozygous for the blind-sterile (bs/bs) and quaking-sterile (qk/qk) gene mutations are sterile, because they either do not produce any spermatozoa or produce only a few abnormal spermatozoa. Mice lacking the cyclic AMP responsive-element modulator gene are sterile due to failure of spermiogenesis. All these mice, however, are able to produce fertile offspring when their spermatozoa or round spermatids are injected into oocytes of normal females. This implies that genetic and epigenetic elements necessary for syngamy and embryonic development are established in round spermatids and spermatozoa of these animals, even though their spermatogenic cells are destined to die (bs/bs and qk/qk) or are programmed to undergo apoptosis (cyclic AMP responsive-element modulator–null) without becoming functional spermatozoa.

In all vertebrates, with few possible exceptions, female germ cells become fertilization-competent at the metaphase of the second meiotic division (1, 2). In contrast, male germ cells become fertilization-competent only after the completion of meiosis and their transformation into motile spermatozoa. In mammals, spermatozoa in the testis are virtually immotile. They must undergo maturation in the epididymis and capacitation in the female genital tract before gaining the ability to interact with mature oocytes (2, 3). Spermatozoa become able to fuse with oocytes only after the acrosome reaction, which normally takes place immediately before passing through noncellular coats surrounding individual oocytes (4). An important question is whether all postmeiotic modifications of male germ cells are necessary for syngamy and embryonic development. Are all genes involved in postmeiotic modifications necessary for the development of new individuals?

We found that mouse oocytes fused or injected with round spermatids were able to develop into normal offspring (5, 6). Because secondary, and even late primary, spermatocytes were able to produce normal offspring after injection into oocytes (7–9), we inferred that all genomic and epigenetic elements necessary for embryonic development are established before spermatogenic cells become motile spermatozoa. In other words, all postmeiotic modifications of male germ cells are considered processes solely dedicated for the delivery of male genomes into the oocytes. However, it should be pointed out that all these experiments were performed by using male germ cells from normal fertile male mice. Thereby, a question arises: Would male germ cells that are unable to differentiate into spermatozoa because of genetic causes be able to produce normal offspring? Here, we have used three different mutant mice that display spermiogenic defects at different levels. Mice homozygous for blind-sterile (bs/bs) and quaking-sterile (qk/qk) gene mutations produce a few spermatozoa, but none are fertile (10–12). Male mice homozygous for a mutation in the cyclic AMP responsive-element modulator (CREM) gene are unable to start spermiogenesis, and therefore the most advanced spermatogenic cells in these males are round spermatids (13, 14). We have studied whether spermatozoa or spermatids from these infertile mice are able to produce normal offspring when injected into oocytes.

Materials and Methods

Animals. Females and males of B6D2F1 (B57BL/6 × DBA/2), C57BL/6, and ICR mice were purchased from the National Cancer Institute (Raleigh, NC). Male mice homozygous for the bs/bs and qk/qk mutations were purchased from The Jackson Laboratory. CREM-null mice were generated by homologous recombination and have been described (13). Phenotypes and genotypes of these mutant mice are shown in Fig. 1 and Table 1. All males and females (B6D2F1 and C57BL/6) were 3–6 and 2–3 months old, respectively, when used. Surrogate mothers (ICR) were 3–4 months old. All animals were maintained in temperature- and light-controlled rooms (14 light/10 dark, light on from 5:00 a.m.). The protocol of animal handling and treatment was reviewed and approved by the Animal Care and Use Committee of the University of Hawaii.

Reagents and Media. All inorganic and organic compounds used for the preparation of media were from Sigma, unless otherwise stated. The medium for culturing sperm- or spermatid-injected oocytes was CZB medium (15) supplemented with 5.56 mM D-glucose and 5 mg/ml BSA (fraction V, Calbiochem). The medium for oocyte collection from oviducts, subsequent treatment, and injection of spermatozoa (or spermatids) was modified CZB containing 20 mM Hepes, a reduced amount of NaHCO3 (5 mM), and 0.1 mg/ml polyvinyl alcohol (PVA, cold-water soluble, 30–70 kDa) instead of BSA. This medium was called Hepes–CZB. PVA kept the wall of injection pipettes less sticky over a longer period than BSA, so that adhesion of mineral oil and cell debris was minimized. Incubation atmosphere for CZB and Hepes–CZB was 5% CO2 in air (37°C) and open air (∼25°C), respectively.

Preparation of Oocytes. Females of B6D2F1, C57BL/6, CREM-null (−/−), and normal (+/+ ) mice were each injected with 5 units of equine chorionic gonadotropin followed by injection of 5 units of human chorionic gonadotropin (hCG) 48 h apart. Oocytes surrounded by cumulus oophorus were collected from oviducts ∼15 h after hCG injection. The oocytes for in vitro insemination were used directly, whereas those for sperm (or spermatid) injection were freed from cumulus cells by 5–10-min treatment with 0.1% bovine testicular hyaluronidase (300 units/mg; ICN) in Hepes–CZB. Cumulus-free oocytes were rinsed and kept in CZB for no more than 4 h at 37°C under 5% CO2 in air before injection of spermatozoa or spermatids.

Abbreviations: ICSI, intracytoplasmic sperm injection; ROSI, round spermatid injection; CREM, cyclic AMP responsive-element modulator; bs/bs, blind-sterile; qk/qk, quaking-sterile.

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whereas CREM-null males are infertile due to a defect in spermiogenesis. CREM is a master transcription regulator of several postmeiotic genes (28, 46). Both females and males are phenotypically normal. CREM-null females are fertile, whereas CREM-null males are infertile due to a defect in spermiogenesis.

Homozygous mutation induces deficient myelination of central and peripheral nerves in both male and female (45) and causes a tremor that disappears when mice are at rest. This qk/qk male was undergoing a seizure while being photographed. qk/qk females are fertile. (C) CREM-null male (left) and female mice. CREM is a master transcription regulator of several postmeiotic genes (28, 46). Both females and males are phenotypically normal. CREM-null females are fertile, whereas CREM-null males are infertile due to a defect in spermiogenesis.

**Collection and Examination of Spermatozoa and Spermatids.** Spermatozoa were collected from either the caudae epididymis or testis. They were suspended in CZB medium and examined for motility at room temperature. Those with distinct motility as well as those with vibrating or undulating tails were all recorded as being alive. Viability of spermatozoa was assessed by using the sperm viability test kit (FertiLight, Molecular Probes). The nuclei of live spermatozoa with intact plasma membranes fluoresced bright green, whereas those of dead spermatozoa fluoresced bright red-orange. Round spermatids, collected from the testis (6), were also assessed for viability. Over 500 spermatozoa or spermatids were examined for each sperm sample.

**In Vitro Insemination.** Cauda epididymal spermatozoa of B6D2F1 or qk/qk mouse were allowed to disperse in TYH medium (16) at 37°C for 15 min. An aliquot (200 µl) of homogenous sperm suspension was incubated under mineral oil (Squibb) for 1–1.5 h at 37°C under 5% CO2 in air to allow sperm capacitation. The concentration of spermatozoa at this stage was ~1–2 × 10⁷/ml. Insemination was performed by adding a small volume of the sperm suspension to 200 µl of fresh TYH medium containing cumulus-enclosed oocytes that had been previously placed under mineral oil in a plastic Petri dish (30 × 10 mm; Falcon). The final sperm concentration in fertilization medium was ~10⁷/ml. The oocytes were examined with an inverted microscope 6–8 h after insemination. Those with two distinct pronuclei and a conspicuous second polar body were recorded as normally fertilized (17).

**Intracytoplasmic Sperm Injection (ICSI) and Round Spermatid Injection (ROSI).** ICSI and ROSI oocytes were allowed to develop in CZB medium and were examined at 24-h intervals until the morula/blastocyst stage. To examine their developmental potentials, embryos at either two-cell or morula/blastocyst stages were transferred to the oviducts of ICR (albino) females that had been mated with vasectomized males of the same strain during the previous night. The day of embryo transfer was designated as day 1 of pregnancy. Because the implantation of embryos was governed by the endometrial stage of the uterus, implantation of these embryos occurred around 5 days postcoitum regardless of the developmental stage of embryos at transfer. Surrogate mothers were allowed to deliver and raise pups. Sex of infants was determined 1–2 wk after the birth by examination of external genitalia. Some males born after ICSI or ROSI were killed, and their testes were fixed and examined for histology.

![Fig. 1. Sterile mutant males used in this study. (A) A homozygous bs/bs male with cataracts; bs is an autosomal recessive gene mapped to mouse chromosome 2 adjacent to the agouti gene (43). Blind bs/bs females are fertile. (B) A homozygous qk/qk sterile male; qk is an autosomal recessive gene on chromosome 17. Homozygous mutation induces deficient myelination of central and peripheral nerves in both male and female (45) and causes a tremor that disappears when mice are at rest. This qk/qk male was undergoing a seizure while being photographed. qk/qk females are fertile. (C) CREM-null male (left) and female mice. CREM is a master transcription regulator of several postmeiotic genes (28, 46). Both females and males are phenotypically normal. CREM-null females are fertile, whereas CREM-null males are infertile due to a defect in spermiogenesis.](image)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Genetic background</th>
<th>Phenotype</th>
<th>Fertility</th>
<th>Most advanced stage of spermatogenesis</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>bs</td>
<td>bs/bs</td>
<td>AKR × 129</td>
<td>Blind</td>
<td>Infertile</td>
<td>Deformed sperm</td>
<td>10, 43</td>
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<tr>
<td></td>
<td>bs/+</td>
<td></td>
<td>Normal</td>
<td>Fertile</td>
<td>Normal sperm</td>
<td></td>
</tr>
<tr>
<td>qk</td>
<td>qk/qk</td>
<td>CS7BL/6 × C3H</td>
<td>Tremor, seizure</td>
<td>Infertile</td>
<td>Deformed sperm</td>
<td>12, 44, 45</td>
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<tr>
<td></td>
<td>qk/+</td>
<td></td>
<td>Normal</td>
<td>Fertile</td>
<td>Normal sperm</td>
<td></td>
</tr>
<tr>
<td>CREM</td>
<td>–/–</td>
<td>CS7BL/6</td>
<td>Normal</td>
<td>Infertile</td>
<td>Spermatid</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>+/+</td>
<td></td>
<td>Normal</td>
<td>Fertile</td>
<td>Normal sperm</td>
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</table>
Results

bs/bs Mice and Their Offspring. Homozygous mutant bs/bs mice are blind due to cataracts (Fig. 1A). bs/bs male mice have been reported to have elongated spermatids and deformed spermatoozoa (10), but the bs/bs males we used had neither elongated spermatids nor spermatoozoa. The majority of oocytes were fertilized, and most of transferred mature spermatozoa (not shown). When spermatozoa from a bs/bs male were individually injected into B6D2F1 oocytes, the majority of the oocytes developed arrested at the earliest spermatid stage. (Bar = 20 μm.)

Discussion

A major advance in reproductive biology during the last decade is the development of techniques that allow infertile males to gain fertility by microsurgical injection of spermatoozoa and prespermatoozoal cells into oocytes (19–22). We have previously shown that the majority of BALB/c spermatoozoa with grossly misshapen heads are able to produce normal offspring by ICSI (23). Spermatozoa of mice carrying two complementary haplotypes (f5/f22) are poorly motile and totally infertile in vivo and in vitro (24), yet they are able to produce fertile offspring by ICSI (25). Some transgenic mice are infertile due to poor sperm production but are able to produce live offspring by ROSI or

Table 2. Fertilization and development of B6D2F1 oocytes injected with spermatoozoa of bs/bs and control (bs/+) males

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Experimental series 1</th>
<th>Experimental series 2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total no. of oocytes fertilized (no. exp.)</td>
<td>No. (%) of</td>
</tr>
<tr>
<td>bs/+ Sperm</td>
<td>27 (1)</td>
<td>24 (87.5)</td>
</tr>
<tr>
<td>bs/bs Spermatid</td>
<td>102 (3)</td>
<td>88 (89.8)</td>
</tr>
</tbody>
</table>

Table 3. Fertilization and development of B6D2F1 oocytes injected with sperm and spermatids of qk/qk males

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Experimental series 1</th>
<th>Experimental series 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. oocytes injected (exp. no.)</td>
<td>No. oocytes fertilized</td>
</tr>
<tr>
<td>qk/qk sperm</td>
<td>88 (4)</td>
<td>75</td>
</tr>
<tr>
<td>qk/qk spermatids</td>
<td>130 (2)</td>
<td>125</td>
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</table>
ICSI (26, 27). In this study, we have shown that male mice infertile due to recessive mutations (bs/bs and qk/qk) can be rescued by ICSI and/or ROSI. Round spermatids of CREM-null mice, which are programmed to undergo apoptosis (13, 28), were hereditary nephritic syndrome are barely fertile of oocytes after ROSI have been suboptimal. According to are not really round spermatids. It is also possible that activation limited experience and may result in the selection of the cells that biopsies (36), but this is a difficult task for researchers with problem could be simply technical. Living human round spermatids can be distinguished from other small cells in testicular nuclei and centrosomes of human round spermatids—2. Austin, C. R. (1965)

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