Fertilization, embryonic development, and offspring from mouse eggs injected with round spermatids combined with Ca\(^{2+}\) oscillation-inducing sperm factor

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Round spermatids, precursor male gametes, are known to possess the potential to achieve fertilization and embryonic development when injected into eggs. However, injection of spermatids alone seldom activates eggs in the mouse, as spermatids by themselves cannot induce an increase in intracellular Ca\(^{2+}\), a prerequisite for egg activation. We injected a mouse round spermatid into an egg simultaneously with partially purified sperm factor from differentiated hamster spermatozoa. The combined injection produced repetitive Ca\(^{2+}\) increases (Ca\(^{2+}\) oscillations) lasting for at least 4 h as observed at fertilization, and induced activation in 92\% of eggs. This method provided 75\% fertilization success associated with male and female pronucleus formation and development to 2-cell embryos, while only 7\% of eggs were fertilized by injection of a spermatid alone. Of the 2-cell embryos, ~50\% developed to blastocysts during 5 days of culture in vitro, while no blastocysts were obtained following injection of sperm factor alone. Furthermore, the 2-cell embryos, that were created by spermatids and sperm factor and transplanted into foster mothers, developed into normal offspring, although the percentage was only 22\%. All infants grew into healthy adults carrying normal chromosomes. The sperm factor served as a complementary factor for successful fertilization by round spermatid injection.

Key words: assisted reproductive technology/intracellular calcium oscillation/intracytoplasmic round spermatid injection/mouse egg/sperm factor

Introduction

It has been shown in mice that the nuclei of round spermatids (1n haploid) and secondary spermatocytes (2n haploid) possess the potential to achieve normal fertilization and embryonic development, resulting in normal offspring when injected into eggs (Kimura and Yanagimachi, 1995a,b). Recently, round spermatid injection (ROSI) has been used in therapy for patients with defective spermatogenesis (Tesarik et al., 1995; 1998; Fishel et al., 1997; Aslam et al., 1998; Sofikitis et al., 1998). However, there are debates about the validity of the clinical use of this technology (Devroey, 1998; Silber and Johnson, 1998; Vanderzwalmen et al., 1998). The accumulation of data from experiments in animals will be useful to improve the method and confirm the safety.

In mouse eggs, ROSI produces no increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{\text{\textit{i}}}) (Sato et al., 1998) and is unable to activate eggs (Kimura and Yanagimachi, 1995a,b; Sato et al., 1998). One effective method for assisted egg activation is to apply a high voltage pulse 1 h before or after ROSI (Kimura and Yanagimachi, 1995a,b; Sasagawa and Yanagimachi, 1996) which induces a single transient (~10 min) [Ca\(^{2+}\)]\text{\text{\textit{i}}}, rise (Swann and Ozil, 1994). On the other hand, the Ca\(^{2+}\) response during the in-vitro fertilization (IVF) of mammalian eggs is a long-lasting series of repetitive transient [Ca\(^{2+}\)]\text{\text{\textit{i}}} rises (Ca\(^{2+}\) oscillations) caused by repeated Ca\(^{2+}\) release from the endoplasmic reticulum mainly through inositol 1,4,5-trisphosphate receptors (IP\(_{1,4,5}\)RS) (Miyazaki et al., 1992, 1993; Kline and Kline, 1994; Fissore and Robl, 1994). Correspondingly, Ca\(^{2+}\) oscillations produced by repetitive electric pulses facilitate the pronucleus formation and later embryonic development (Swann and Ozil, 1994; Ozil and Swann, 1995). Combined injection of adenophostin, a potent non-metabolizable agonist of the IP\(_{1,4,5}\)R, with ROSI in mouse eggs has been shown to produce long-lasting Ca\(^{2+}\) oscillations and result in egg activation leading to normal embryonic development and offspring (Sato et al., 1998).

Egg activation can be induced by sperm factor(s). Extracts from the hamster (Swann, 1992), boar (Wu et al., 1997), and human spermatozoa (Sousa et al., 1996; Palermo et al., 1997) induce Ca\(^{2+}\) oscillations when injected into eggs of the mouse as well as the same species. Sperm extract could be a more natural complementary factor for assisted egg activation in ROSI. In the present study, cytosolic proteins from the hamster sperm were partially purified, and after confirming the Ca\(^{2+}\) oscillation-inducing activity, the sperm factor was injected into mouse eggs simultaneously with a whole round spermatid (ROSI plus sperm factor). This method provided successful fertilization and embryonic development in vitro, and the transplanted embryos can result in offspring in foster mothers.
Materials and methods

Preparation of mouse eggs
B6D2F1 female mice (6–10 weeks old) were superovulated by i.p. injection of 5 IU pregnant mare’s serum gonadotrophin (PMSG; Teikoku Hormone Manufacturing, Tokyo, Japan) followed by 5 IU of human chorionic gonadotrophin (HCG; Mochida Pharmaceutical Co, Ltd, Tokyo, Japan) 50 h later. Animals were rapidly killed by cervical dislocation 16–17 h after HCG injection. Mature eggs at metaphase II (MII) were collected from oviducts and freed from cumulus cells by treatment with 0.05% hyaluronidase (P-L Biochemicals, Milwaukee, WI, USA) in M2 medium (Fulton and Whittingham, 1978) for 2–3 min. After washing, 5–10 eggs were immediately transferred to the dish containing M2 medium for ROSI. Other eggs were kept until use in M16 medium (Whittingham, 1971) for up to 3.5 h at 37°C under 5% CO2 in air. Bovine serum albumin (BSA) was added to M2 and M16 media before use (4 mg/ml).

Preparation of hamster sperm factor
Sperm extract was prepared, according to the method of Swann (1990, 1994). The contents of the cauda epididymides isolated from 8–16 golden hamster males (10–14 weeks old) were collected in a glass test tube and diluted by a modified Tyrode’s solution (m-TALP medium; Ohzu and Yanagimachi, 1982) containing 0.1 M Na-pyruvate, 9 mM Na-lactate, 0.05 mM epinephrine, 0.5 mM hypotaurin, and 4 mg/ml BSA. After incubation for 30 min, spermatozoa that swam up in the test tube were collected and washed twice with BSA-free m-TALP by centrifugation (500 × g for 5 min) to remove seminal plasma components and resuspended in an intracellular medium (Swann, 1990) containing 120 mM KCI, 20 mM HEPES/KOH (pH 7.5), 1 mM EDTA, 0.2 phenylmethylsulphonyl fluoride (PMSF), 2 µg/ml pepstatin A (Sigma, St Louis, MO, USA), and 2 µg/ml leupeptin (Sigma). The sperm concentration was adjusted to ~5 × 106 spermatozoa/ml. The sample was sonicated 12 times (for 5 s each) on ice to keep temperature <8°C, and supernatant was obtained after centrifugation (90 000 × g for 90 min at 4°C). Usually 8 ml of supernatant containing 4.0 mg/ml protein was obtained from eight males. The supernatant was concentrated to 1/10 volume (0.8 ml) with a 30 kDa cut-off ultrafilter (30K MicrosepTM; Pall Filtron Corporation, Northborough, MA, USA), and then diluted with 8 ml basal buffer (20 mM HEPES/KOH adjusted to pH 7.5, 1 mM EDTA). The sperm factor was partially purified with Cibacron Blue® F3GA dye affinity chromatography (Swann, 1994) (Econo-Pac® Blue Catridge; Bio-Rad Lab., Hercules, CA, USA). The column was loaded with the supernatant and washed with 10 ml of another buffer (120 mM KCl in basal buffer). Proteins were eluted with 20–30 ml high KCl buffer (1 M KCl in basal buffer) at a flow rate of 1 ml/min at 4°C. The eluate was dialysed twice against low KCl buffer (50 mM KCl in basal buffer plus 200 µM PMSF and 2 µg/ml pepstatin A) for 30 min and concentrated to 100 µl with 30K MicrosepTM. The sample (40 µg/ml protein) was stored at −80°C until use.

Preparation and injection of mouse spermatids
Round spermatids were prepared and injected into eggs, basically according to the method of Kimura and Yanagimachi (1995c). The testis was isolated from a B6D2F1 mouse (8–12 weeks old) and bathed in erythrocyte-lyzing buffer (155 mM NH4Cl, 10 mM KHC03, 2 mM EDTA, pH 7.2). Spermatogenetic cells were isolated (for details, see Sato et al., 1998), suspended in 0.9% NaCl saline, and used within 1.5 h. For ROSI, the cells were placed in a 2 µl drop of 0.9% NaCl containing 2.5% polyvinylpyrrolidone (Sigma) and sperm factor at different concentrations (1.0, 2.1, or 21 mg/ml protein) near a 4 µl drop of M2 medium for eggs on a shallow plastic dish. The drops were covered with paraffin oil. The dish was mounted on the cooled stage (Kitazato Supply, Tokyo, Japan) of an inverted microscope (TMD; Nikon, Tokyo, Japan) equipped with Hoffman modulation contrast optics (Hoffman Modulation Optics Inc, Greenvale, NY, USA). The temperature of the drops was kept at 16–17°C during ROSI to avoid disintegration of eggs (Kimura and Yanagimachi, 1995c). Round spermatids were identified by their small size (~10 µm in diameter) and a centrally located chromatin mass (Ogura et al., 1992). A single spermatid was aspirated into an injection pipette of about 4 µm internal diameter at its tip. Most of the aspirated spermatids were more or less damaged, so the whole spermatid (the nucleus, cytoplasm, and injured plasma membrane) together with sperm factor was injected into an egg. The pipette was connected to a microinjector (IM-4B; Narishige, Tokyo, Japan) and driven by a Piezo electric micromanipulator (PMAS-CT140, Prima Meat Packers, Tsuchiura, Japan), as described previously (Sato et al., 1998). The amount of injected solution was ~4–6 pl (2% of the egg volume). A group of 5–10 eggs were injected with spermatids in 30 min. They were kept at 17, 24, and 37°C for 10 min each, and then transferred to M16 medium in another dish for culture (37°C, 5% CO2 in air).

Measurement of [Ca2+]i
Ca2+ responses of eggs to ROSI plus sperm factor were recorded by a conventional Ca2+ imaging method. Before ROSI, eggs were loaded with the Ca2+-sensitive fluorescent dye fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes Inc, Eugene, OR, USA) by incubation for 5 min at room temperature. Freeze-stored solution of 2 mM fura-2 AM in dimethyl sulfoxide was diluted to 10 µM in M2 medium before use. After ROSI, eggs were transferred to a dish fashioned for Ca2+ imaging (see Nakano et al., 1997). The dish was set on the stage of a phase-contrast inverted microscope (TMD, Nikon) equipped with an image processor (Argus 200; Hamamatsu Photonics, Hamamatsu, Japan). Ca2+ images were sampled at 20 s intervals by applying UV light of 340 nm wavelength for 0.25 s followed by 0.8 s later by 380 nm UV for 0.25 s through a ×40 objective lens (Fluor 40, Nikon). Emission fluorescence (F) was led to a silicon intensified target camera through a 510 ± 10 nm bandpass filter. Data sets were stored on the hard disk of the computer as 8-bit digital images, and processed to calculate the ratio R = F510/F380 later. A calibration curve between R and [Ca2+]i was obtained by measuring R of Ca2+-EDTA-OH [N-(2-hydroxy-ethyl) ethylenedinitrilo-triacetic acid] buffer solutions. Other details have been described previously (Shiraiishi et al., 1995).

In some eggs, Calcium Green Dextran (CGD; molecular weight 1 × 105; Molecular Probes) was used as the Ca2+ indicator, since CGD is not incorporated into the organelle and indicates more faithfully the cytoplasmic [Ca2+]i change. CGD was preinjected into the egg with the solution containing (mM) 0.5 CGD, 140 KCl, 1 MgCl2, and 5 HEPES (pH 7.3). The dye was activated by 488 nm argon laser and emission fluorescence was passed through a band pass filter at 510–535 nm. The value of [Ca2+]i was not calculated because of a single emission light of CGD. Instead, each Ca2+ image was divided by an image just before injection of sperm factor in a pixel-to-pixel manner.

Examination of embryos
After 5 h culture, fertilization of eggs was identified by extrusion of the second polar body and formation of the male and female pronuclei. Eggs with distinct signs of degeneration were discarded. The pronuclei were observed in live eggs with differential interference contrast optics (laser scanning microscopy LSM310, Carl Zeiss, Oberkochen, Tsuchiura, Japan), as described previously (Sato et al., 1998). The temperature of the drops was kept at 17, 24, and 37°C for 10 min each, and then transferred to M16 medium in another dish for culture (37°C, 5% CO2 in air).

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Germany), as described previously (Shirakawa and Miyazaki, 1995; Sato et al., 1998).

Embryo transfer to foster mothers

In some experiments, 2-cell embryos that had been confirmed to have two large pronuclei and one second polar body were transferred to foster mothers, using ICR (albino) female mice (8–13 weeks old) as recipients. The females were mated naturally with males (10–15 weeks old) of the same strain. Pregnancy was confirmed by a vaginal plug. On day 1 (~12 h after mating), between four and nine 2-cell embryos were transferred into the ampullary region of the unilateral oviduct using a glass capillary tube (100–150 μm internal diameter). The pipette was inserted into the oviduct through a hole which was made by a 27 gauge needle in the region slightly above the distended portion of the ampulla. Foster mothers were allowed to deliver and raise foster pups (black eyes and grey/brown/black coats) as well as their own pups (red eyes and white coat). Some of their own pups were removed during lactation.

Examination of chromosomes

Chromosomes were examined by conventional methods (Mandahl, 1992). The spleen was cut into small pieces and treated with collagenase. After culture in Roswell Park Memorial Institute (RPMI) 1640 medium for 15–30 days, cells were arrested at the metaphase by treatment with colcemid (20 ng/ml) and lysed with hypotonic solution. Chromosomes were fixed with Carnoy’s fixative and stained with Giemsa.

Results

Ca2+ oscillations induced by sperm factor

Changes in [Ca2+]i caused by injection of sperm extract or sperm factor alone were recorded by Ca2+ imaging with fura-2 or CGD. As shown in Figure 1A, only a single Ca2+ transient was induced by the sperm extract before partial purification with affinity chromatography, even when injected at a high concentration (40 mg/ml protein) and with a large amount (~10 pl, 5% of the egg volume, examined in eight eggs). The Ca2+ transient reached the peak of 800–1000 nM and lasted for ~5 min. In contrast, when partially purified sperm factor was injected, repetitive [Ca2+]i rises were produced by a small amount (about 0.2 pl) at the concentration of 10–20 mg/ml protein (Figure 1B). The Ca2+ oscillations comprised the first relatively large and long Ca2+ transient and succeeding smaller and shorter ‘Ca2+ spikes’, as have been demonstrated previously (Swann, 1992). The Ca2+ response pattern is usually observed at IVF of mouse eggs (Miyazaki et al., 1993; Nakano et al., 1997; Sato et al., 1998). Thus, the prepared sperm factor was confirmed to possess the Ca2+ oscillation-inducing activity. Injection of a round spermatid alone sometimes caused a [Ca2+]i rise, probably because a small amount of M2 medium containing Ca2+ might have been aspirated into the injection pipette and introduced into the egg. However, no Ca2+ oscillations were produced by a spermatid alone, as shown previously (Sato et al., 1998).

Ca2+ oscillations following simultaneous injection of a round spermatid and sperm factor were recorded in the similar way. The amount of injected solution (0.9% NaCl) was inevitably 4–6 pl for ROSI, so the concentration of sperm factor was set at a low level (2 mg/ml protein). Ca2+ oscillations were produced by this condition in 22 out of 24 eggs. An example is shown in Figure 1C, [Ca2+]i measurement was started 10–18 min after injection, as the egg was left in the dish used for ROSI for 7–15 min to facilitate the recovery from partial damage of the oolemma and the egg was then transferred to another dish for Ca2+ imaging. The record of [Ca2+]i began with Ca2+ spikes (Figure 1C), probably because the first Ca2+ transient had already ceased. Ca2+ spikes at the early stage of Ca2+ oscillations occurred in a high frequency at an interval of ~1 min, and then the interval was gradually prolonged. The peak [Ca2+]i of each spike was lowered progressively from 800–1000 nM, while the duration of 1–2 min remained fairly constant.

Figure 1C also shows Ca2+ oscillations at a later stage: 2–4 h after ROSI plus sperm factor. The Ca2+ response was recorded after the interruption of [Ca2+]i measurement for 90 min during which the egg was kept at 37°C in a CO2 incubator, because continuous long-term irradiation of UV light tended to disturb the generation of Ca2+ spikes (Sato et al., 1998) and sometimes lead to cell lysis of ROSI-treated eggs. In Figure 1C, Ca2+ spikes continued to occur at an interval of ~25 min at 2.5 h after ROSI plus sperm factor, similar to those observed at monospermic fertilization of mouse eggs (Nakano et al., 1997). A Ca2+ spike was generated when [Ca2+]i,
increased gradually and reached a certain level. At the later stage >2.5 h after ROSI plus sperm factor, the amplitude of each Ca\(^{2+}\) spike was reduced progressively during Ca\(^{2+}\) oscillations, and the interval between spikes became shorter, associated with a slight elevation of the basal [Ca\(^{2+}\)] level (Figure 1C). These changes might be partly due to long-term exposure of the egg to UV light. [Ca\(^{2+}\)] measurement that was started hours after ROSI (e.g. Figure 1C) showed that Ca\(^{2+}\) oscillations persisted for at least 4 h (n = 4).

Some eggs were treated with ROSI plus sperm factor at different concentrations. The frequency of Ca\(^{2+}\) oscillations as well as the amplitude and duration of each Ca\(^{2+}\) spike was not greatly affected by increasing the sperm factor concentration 10-fold (21 mg/ml protein) (n = 3). On the other hand, no Ca\(^{2+}\) response was induced, when the concentration was decreased to 1 mg/ml (n = 5). These results suggest a relatively steep dose-response relationship. We used the concentration of 2 mg/ml for observation of development of eggs after ROSI plus sperm factor, considering that it is close to the critical concentration for the induction of long-lasting Ca\(^{2+}\) oscillations.

**Egg activation following injection of spermatid or sperm factor**

Table I summarizes the aspects of egg activation or fertilization examined 5 h after injection of a single spermatid and/or sperm factor. The percentage of survived eggs was 79–92%. Poor repair of the oolemma after withdrawal of the injection pipette was likely to cause cell death. The cell damage seemed to occur a little more often after injection of both spermatid and sperm factor, although the reason was unknown. Mature mammalian eggs are arrested at MII and released from the arrest by [Ca\(^{2+}\)]\(_i\) rises (Kline and Kline, 1992). Egg activation was identified by extrusion of the second polar body (Pb\(_2\)) and formation of the large female pronucleus (PN). Normal egg activation was expressed as 1PN\(+\)1Pb\(_2\). For injection of sperm factor alone, 84% of eggs that survived (36 of 43 eggs) were activated (Table I). For injection of spermatid alone, 51% of eggs (23 of 45 eggs) remained arrested at MII, and 49% of eggs were activated (Table I). An artificial [Ca\(^{2+}\)]\(_i\) rise might have occurred in the eggs that were activated by spermatid alone, as described in the previous section.

<table>
<thead>
<tr>
<th>Material injected</th>
<th>No. of eggs injected</th>
<th>Percentage eggs survived</th>
<th>No. (%) of surviving eggs</th>
<th>No. of eggs examined</th>
<th>No. (%) of eggs developed to 2-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MII</td>
<td>2PN+1Pb(_2)</td>
<td>1PN+1Pb(_2)</td>
</tr>
<tr>
<td>Spermatid with sperm factor</td>
<td>398</td>
<td>331 (89)</td>
<td></td>
<td>12 (4)</td>
<td>249 (75)</td>
</tr>
<tr>
<td>Spermatid alone</td>
<td>53</td>
<td>45 (85)</td>
<td></td>
<td>23 (51)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Sperm factor alone</td>
<td>47</td>
<td>43 (92)</td>
<td></td>
<td>6 (14)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

MII = metaphase of second meiotic division; 1PN = one pronucleus; 2PN = two pronuclei; 1Pb\(_2\) = one second polar body.

*Others include (2PN+0 or 2Pb\(_2\)), (3PN+1 or 2Pb\(_2\)) or (4PN+2Pb\(_2\)).

**Fertilization and cleavage after injection of spermatid plus sperm factor**

Fertilization of eggs was characterized by formation of Pb\(_2\) and two (male and female) pronuclei (2PN), expressed as 2PN+1Pb\(_2\). When a spermatid was injected together with sperm factor, 92% of eggs that survived (307 of 331 eggs) were activated (Table I), and successful fertilization was achieved in 75% of eggs (249 of 331 eggs). In contrast, only three of 45 eggs were fertilized after injection of spermatid alone, even though ~50% of eggs were activated. In some eggs including those three eggs, a spermatid on the way to the maturation process might have been injected. In 19 out of 45 eggs, the spermatid nuclei exhibited either intact chromosomes or prematurely condensed chromosomes. Thus, the sperm factor compensated well for the failure of fertilization by ROSI.

The formation of the male pronucleus was incomplete in 17% of eggs subjected to ROSI plus sperm factor, although the female pronucleus and polar body (1PN+1Pb\(_2\)) were formed. In all, 4% of eggs underwent abnormal fertilization such as 2PN plus 0 or 2 Pb\(_2\), 3PN+1 or 2 Pb\(_2\), or 4PN+2Pb\(_2\). Multiple male pronuclei might be caused by fragmentation of the nucleus of the injected spermatid during injection procedure. Two Pb\(_2\) might result from injection of gametes at earlier stages such as spermatoocytes (Kimura and Yanagimachi, 1995b). The failure of the induction of normal fertilization is thought to be reduced by improving the identification of spermatids and injection techniques.

Development of eggs into the 2-cell stage was examined 24 h after injection. Of eggs that had been identified as fertilized (2PN+1Pb\(_2\)) following ROSI plus sperm factor and were further cultured in vitro, 97% developed into 2-cell embryos (Table I). All three eggs that were fertilized by ROSI alone developed into 2-cell embryos as well. Thus, development advances to the 2-cell stage in almost all eggs, when the formation of the male and female pronuclei is complete. Of eggs that were activated but not fertilized (1PN+1Pb\(_2\)) by ROSI plus sperm factor or sperm factor alone, ~75% also developed to the 2-cell stage: the majority of activated eggs cleaved to two blastomeres, even if the male pronucleus is not formed or absent. In contrast, only 25% of such eggs (1PN+1Pb\(_2\)) injected with spermatid alone underwent cleavage (Table I). Long-lasting Ca\(^{2+}\) oscillations induced by the sperm...
factor may facilitate the development to 2-cell embryos, or extract from mature spermatozoa may contain another factor which supports this development.

**Development of zygotes**

Table II summarizes the aspects of development of the embryos that had been identified as normal 2-cell embryos after ROSI plus sperm factor or sperm factor alone. The table presents the numbers of embryos at various developmental stages at the time of observation on the 5th day (~120 h after injection). Mouse embryos develop to the blastocyst stage at 3.5–4.0 days of gestation and hatching commences at 4.0–4.5 days (Pedersen and Burdsal, 1994). In normal development in vitro after IVF, embryos were at the stage of blastocyst or hatching on the 5th day. For injection of spermatid plus sperm factor, 90 of 175 embryos (51%) were at the stage of blastocyst or hatching. Development of other embryos stopped at various stages before the blastocyst stage, more often at relatively later stages such as the morula stage. In contrast, none of the embryos developed to the blastocyst or hatching stage in the case of injection of sperm factor alone. Development of all those embryos stopped at earlier stages, the most often at the 2-cell stage (Table II). Only one out of 26 embryos developed to the morula stage. Thus, the combined injection of a spermated with sperm factor provided normal embryonic development to blastocysts at the rate of 50%. A problem was that 14% of embryos were dead (suffered from cell lysis) by day 5 (Table II), although they had appeared to be normal 2-cell embryos on day 2.

**Embryonic development in vivo into offspring**

The ability of embryonic development in vivo leading to offspring was examined in some embryos. A total of 18 2-cell embryos that originated from eggs fertilized normally after ROSI plus sperm factor were transferred to three foster mothers. The results indicated that the eggs subjected to ROSI plus sperm factor can develop into live offspring, although the percentage was 22% (Table III). No physical abnormalities were detected in the newborn infants, and all of them grew into normal adults, except one which was eaten by the mother soon after birth. Chromosomes were examined in spleen cells taken from three adult mice. No numerical or morphological chromosome abnormalities were observed.

**Discussion**

The present study demonstrated that partially purified sperm factor which had the Ca$^{2+}$ oscillation-inducing activity served as a good compensatory factor in ROSI for fertilization and embryonic development in the mouse. It has been proposed that cytosolic sperm factor(s) is introduced into the ooplasm upon sperm–egg fusion and induces egg activation (Swann, 1996; Hogben et al., 1998). Oscillin (Parrington et al., 1996) and other proteins (Sette et al., 1997; Kimura et al., 1998) have been raised as candidates of the egg-activating sperm factor. One of the actions of the sperm factor(s) is the induction of Ca$^{2+}$ release from the endoplasmic reticulum (Galione et al., 1997). Intracytoplasmic sperm injection (ICSI) and ROSI provide information about the sperm factor. Ca$^{2+}$ oscillations are induced 15–30 min after ICSI in mouse eggs (Nakano et al., 1997) or several hours after ICSI in human eggs (Tesarak et al., 1994), suggesting that the sperm factor leaks out of the injected spermatozoon and induces repetitive Ca$^{2+}$ release. On the other hand, Ca$^{2+}$ oscillations are not produced by ROSI in mouse eggs (Sato et al., 1998), suggesting that either sperm factors are not expressed in spermatozids, or, if expressed, the factors may be associated with inhibiting molecules. The extract from fully differentiated spermatozoa, therefore, is thought to be a natural complementary factor for egg activation in ROSI.

Recent reports have shown that round spermatid nucleus injection (ROSNI) combined with injection of hamster sperm extract into mouse eggs provided 74% (Sasagawa and Yanagimachi, 1996) or 81% (Sasagawa et al., 1997) success of fertilization, although the sperm factor was not purified and the dose and effect of sperm extract were not assayed in terms of the Ca$^{2+}$ oscillation-inducing activity. We used a low dose of sperm factor close to the critical concentration to induce long-lasting Ca$^{2+}$ oscillations, and the dose (4–6 pl, 2 mg/ml protein) was calculated to be roughly equivalent to 4–6 spermatozoa under the assumption of 100% yield of the effective component. The rate of fertilization (75%) was comparable to that for ROSNI described above and higher

**Table II. In-vitro development of mouse embryos originating from eggs injected with spermaticd and/or sperm factor**

<table>
<thead>
<tr>
<th>Material injected</th>
<th>No. of embryos examined</th>
<th>Day 5 No. (%) of embryos</th>
<th>Recipients</th>
<th>No. of foster 2-cell embryos transferred</th>
<th>Recipient's own (albino)</th>
<th>Foster (black/grey) (%)</th>
<th>Sex of foster offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatid with sperm factor</td>
<td>175</td>
<td>7 (4)</td>
<td>2-cell</td>
<td>4-cell</td>
<td>8-cell</td>
<td>Compaction</td>
<td>Morula</td>
</tr>
<tr>
<td>Sperm factor alone</td>
<td>26</td>
<td>10 (38)</td>
<td></td>
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</table>

**Table III. Offspring originating from eggs fertilized normally (2PN+1Pb$_2$) following injection of round spermatids and sperm factor**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>No. of offspring born</th>
</tr>
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<tbody>
<tr>
<td></td>
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</table>

*One pup was eaten by mother soon after birth, therefore its sex was not determined.*
We thank Doctors H.Shirakawa and T.Awaji for discussion and
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than for ROSI plus adenophostin, a potent agonist of the IP₃R (55%; Sato et al., 1998). About half of the eggs treated with ROSI alone were activated possibly by an artefactual [Ca²⁺]i rise during injection procedure, but the male pronucleus was not formed in most of such eggs. Sperm factor-dependent Ca²⁺ oscillations may facilitate the male pronucleus formation.

The 2-cell embryos were obtained from most of the fertilized eggs after ROSI plus sperm factor and the majority of eggs that were activated by the sperm factor alone. In contrast, only a small fraction of eggs were activated (but not fertilized) by spermatid alone. Sperm factor(s) may support development to cell cleavage. Development beyond the 2-cell stage seems to involve another barrier. Blastocysts were obtained from 50% of the 2-cell embryos after ROSI plus sperm factor, but not at all after injection of sperm factor alone. In the reports of ROSNI plus sperm extract, the rate of blastocysts compared with cultured 2-cell embryos was 54–56% (Sasagawa and Yanagimachi, 1996; Sasagawa et al., 1997), while it was 30% for sperm extract alone (Sasagawa and Yanagimachi, 1996). Some paternal nuclear signal may support development to blastocysts.

Using ROSI plus sperm factor caused transplanted 2-cell embryos to give rise to normal offspring. The rate of successful cases (22%) was relatively low, as were the cases of ROSI plus adenophostin (25%; Sato et al., 1998) and ROSNI plus sperm extract (12%; Sasagawa et al., 1996) in the mouse. Further studies are required to find the reasons for the failure of development to blastocysts and full-grown embryos, including the use of genetically defective mice.

With regard to ROSI in human eggs, Sousa et al. (1996) have shown that Ca²⁺ oscillations occur ~30 min after Ca²⁺ ionophore (A23187) challenge in 76% of eggs which were previously injected with spermatids from fertile men, compared with 80% of eggs treated with ICSI. ROSI using spermatids from patients with complete spermatogenic failure induced Ca²⁺ oscillations in only a small fraction of eggs, suggesting that the sperm factor may be deficient in most of the spermatids of those patients (Aslam et al., 1998). Palermo et al. (1997) have shown that human cytosolic sperm factor injected into human eggs induces Ca²⁺ oscillations and overcomes activation failure of eggs treated with ICSI or ROSI (38 or 22% success of 2PN formation respectively). ROSI has resulted in successful pregnancy (Fishel et al., 1995; Antinori et al., 1997) and birth (Tesarik et al., 1995; 1996; Fishel et al., 1997). The clinical use of ROSI, however, is criticized because of its genetic risks, low pregnancy rate (~10% from reports; Aslam et al., 1998), and inherent technical difficulties, particularly in the identification of round spermatids (Vanderzwalmen et al., 1998). In the tests of patients with non-obstructive azoospermia, spermatzoa in tiny foci or at least elongated spermatids are found, and can be used for ICSI after multiple testicular biopsy (Devroey, 1998; Silber and Johnson, 1998). Additional research efforts in various respects (Sofikitis et al., 1998) are necessary to confirm the safety of the procedure and improve the outcome.

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