# Correction

The authors note that on page 14633, left column, second paragraph, lines 2–5, "The Institutional Review Boards of the Saint Mother Obstetrics and Gynecology Clinic and of the Ministry of Health, Labor, and Welfare of Japan approved this study on December 20, 2009 and July 3, 2011, respectively" should instead appear as "The Institutional Review Board of the Saint Mother Obstetrics and Gynecology Clinic approved this study on December 20, 2009 and approved the submission for the clinical trial registration on July 3, 2011."

# Fourteen babies born after round spermatid injection into human oocytes

Atsushi Tanaka[a,1], Motoi Nagayoshi[a], Youichi Takemoto[a], Izumi Tanaka[a], Hiroshi Kusunoki[b], Seiji Watanabe[c], Keiji Kuroda[d], Satoru Takeda[d], Masahiko Ito[e], and Ryuzo Yanagimachi[f,1]

[a]Saint Mother Obstetrics and Gynecology Clinic, Institute for ART, Fukuoka 807-0825, Japan; [b]Faunal Diversity Sciences, Graduate School of Agriculture, Kobe University, Kobe 657-8501, Japan; [c]Department of Anatomical Science, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan; [d]Department of Obstetrics and Gynecology, Juntendo University School of Medicine, Tokyo 113-0033, Japan; [e]Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan; and [f]Department of Anatomy, Physiology, and Biochemistry, University of Hawaii Medical School, Honolulu, HI 96822

During the human in vitro fertilization procedure in the assisted reproductive technology, intracytoplasmic sperm injection is routinely used to inject a spermatozoon or a less mature elongating spermatid into the oocyte. In some infertile men, round spermatids (haploid male germ cells that have completed meiosis) are the most mature cells visible during testicular biopsy. The microsurgical injection of a round spermatid into an oocyte as a substitute is commonly referred to as round spermatid injection (ROSI). Currently, human ROSI is considered a very inefficient procedure and of no clinical value. Herein, we report the birth and development of 14 children born to 12 women following ROSI of 734 oocytes previously activated by an electric current. The round spermatids came from men who had been diagnosed as not having spermatozoa or elongated spermatids by andrologists at other hospitals after a first Micro-TESE. A key to our success was our ability to identify round spermatids accurately before oocyte injection. As of today, all children born after ROSI in our clinic are without any unusual physical, mental, or epigenetic problems. Thus, for men whose germ cells are unable to develop beyond the round spermatid stage, ROSI can, as a last resort, enable them to have their own genetic offspring.

human | round spermatid | ROSI | azoospermia | male infertility

Azoospermia, a condition in which there are no spermatozoa in the ejaculate, occurs in 1% of men in the general population (1). For men with obstructive azoospermia, microsurgical aspiration of epididymal spermatozoa has been performed, and the pregnancy rate following injection of oocytes with epididymal spermatozoa is comparable to that following injection of ejaculated spermatozoa (2). However, up to 94% (17 of 18) of azoospermic men are diagnosed as having nonobstructive azoospermia (3). For such men, microscopic testicular sperm extraction (micro-TESE) has been widely performed to obtain testicular spermatozoa, which are reportedly found in up to 60% of all such cases (4–7). When neither spermatozoa nor late-stage spermatids are found in the testes, such men are considered sterile and are advised to consider using a sperm donor. However, clinicians should consider another possibility: the injection of round spermatids into oocytes. In our clinic, we have found that about 30% of nonobstructive azoospermic men with neither spermatozoa nor late-stage spermatids did possess round spermatids (8), and that such germ cells can be used as a last resort.

In an earlier investigation, Ogura and Yanagimachi (9) found that hamster round spermatids electro-fused with oocytes formed pronuclei, which could participate in syngamy. Subsequently, they reported the birth of normal mouse offspring following microsurgical injection of round spermatids (ROSI) (10, 11). Male mice homologous for a mutation in the cAMP responsive-element modulator gene (*Crem*) are unable to undergo spermiogenesis, and therefore the most advanced spermatogenic cells present in such males are round spermatids (12). According to Yanagimachi et al. (13), round spermatids collected from the testes of such *Crem*-null males were able to produce fertile offspring following

their injection into mature oocytes. The idea of using spermatids as substitutes for spermatozoa in humans was first proposed by Edwards et al. (14). Since the first ROSI human babies were obtained by Tesarik et al. (15, 16), additional pregnancies have been reported (17–19). However, there have been very few similar reports in recent years, perhaps because many clinical investigators have become pessimistic as to the efficiency, safety, and practical value of ROSI (20–23). Indeed, both the Practice Committee of American Society for Reproductive Medicine and the Practice Committee of Society for Assisted Reproductive Technology (24) considered ROSI as an experimental rather than a recommended practice. In an attempt to take this further, we tried to obtain spermatozoa by culturing human primary spermatocytes in vitro. Although some spermatocytes developed into round spermatids, they did not develop further (25), and attempts to transform round spermatids into spermatozoa in vitro met with limited success (8). Therefore, when we did not found normal-looking spermatozoa or late spermatids in micro-TESE, we used the round spermatids from the patients for the ROSI. Here, we report the details of the ROSI procedure that lead to the birth of 14 babies born between September 2011 and March 2014.

## Results

**Identification of Round Spermatids.** When we failed to find spermatozoa or elongated spermatids but found presumptive round spermatids, all seminiferous tubules thus far collected were enzymatically dissociated and kept frozen until their use for ROSI
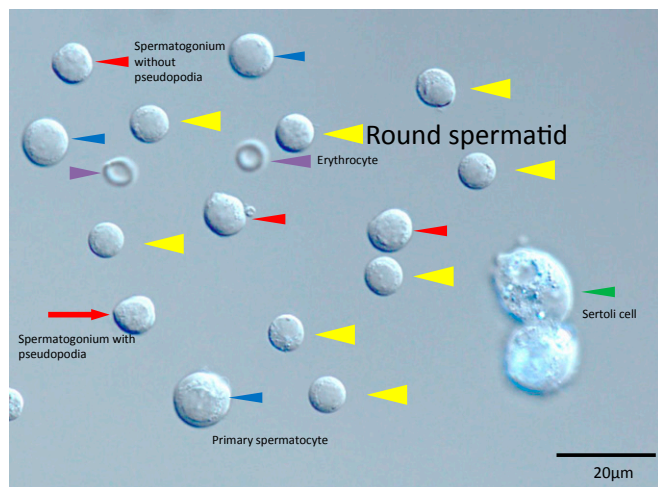
**Fig. 1.** Various types of spermatogenic cells seen after enzymatic dissociation of seminiferous tubules. (Magnification: 400×.) A few erythrocytes (purple arrowheads) with a distinct shape are also seen (we treated dissected seminiferous tubules with erythrocyte-lysing buffer, but some cells remained nonlysed). Spermatogonia with or without pseudopodia (red arrows and red arrowheads), primary spermatocytes (blue arrowheads), round spermatids (yellow arrowheads), and Sertoli cells (green arrowhead) are seen.

(for details, see *Materials and Methods* and *SI Materials and Methods*). Thawed samples contained various types of cells (Fig. 1 and Fig. S1). Under a differential interference microscope, we identified round spermatids by their size and morphology (Fig. 2). FISH and karyotyping of these cells confirmed that our optical identification of round spermatids was fairly accurate (Figs. 2 and 3).

As shown in Fig. 2, round spermatids were the smallest spermatogenic cells (6–8 µm in diameter). Unlike other spermatogenic cells, they did not have distinct nucleoli and the rim of cytoplasm surrounding the nucleus was thinner than that in the spermatogonium. Protruded active pseudopodia were often seen in spermatogonia (26), but not in round spermatids. An acrosome vesicle or cap, definitive evidence for the cell being a spermatid, was seen in less than 10% of the presumptive spermatids we examined. Another important characteristic of the round spermatid was evident in the fact that its cytoplasm could be readily separated from the nucleus when the cell was drawn back and forth within the injection pipette. Thus, although some spermatogonia did resemble round spermatids, pipetting did not separate their cytoplasm from the nucleus as readily as in spermatids. Lymphocytes, about the size of round spermatids, had a "tough" plasma membrane that, unlike that of round spermatids, could not be broken even by vigorous pipetting.

**ROSI and Oocyte Activation.** Fig. 4 shows an oocyte injected with a round spermatid whose plasma membrane ruptured when the cell was sucked into the injection pipette (Fig. 4B). Although ROSI alone could induce oocyte activation, this outcome was not consistent. Therefore, because repetitive intracellular $Ca^{2+}$ oscillations are an essential component of oocyte activation and a prerequisite of normal embryo development (27), we compared the pattern of $Ca^{2+}$ oscillations in oocytes after vigorous ooplasmic aspiration (16), electric stimulation, ROSI, and ROSI plus electric stimulation. As shown in Fig. S2, ROSI combined with electric stimulation (Fig. S2D) was most effective in inducing large, repetitive $Ca^{2+}$ oscillations. Because our previous study had shown that oocytes stimulated with electric current before ROSI developed into cleaving embryos more often than those without such treatment (55% vs. 32%) (Table S1), we subjected all oocytes to electro-stimulation ~10 min before ROSI. An example of ROSI embryo developing in vitro after this stimulation is shown in Fig. S3.
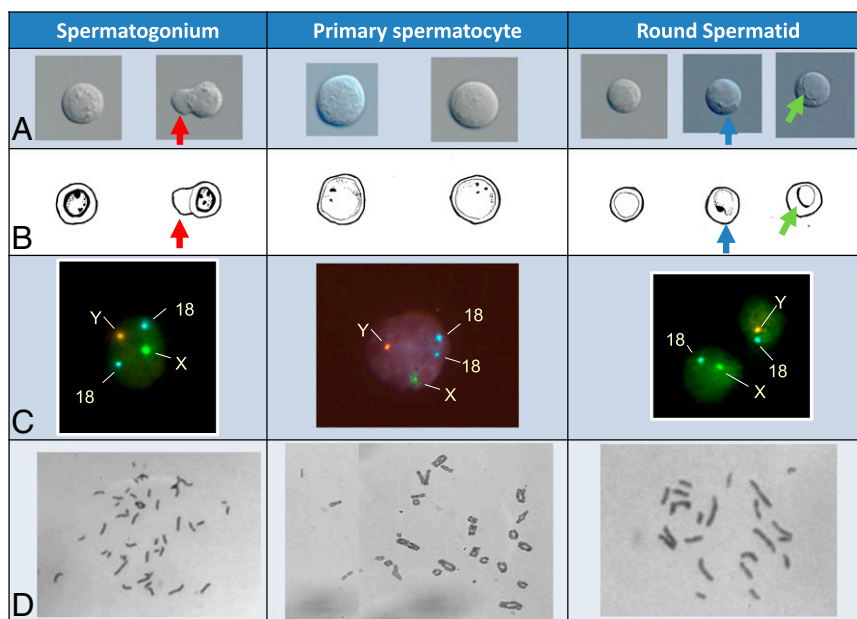


**Fig. 2.** Spermatogonia, primary spermatocytes, and round spermatids, (*A*) seen with interference-contrast optics, and (*B*) their schematic drawings. (Magnification: *A*, 400×.) (*C*) FISH using fluorescent probes specific for chromosomes X, Y, and 18. (Magnification: 400×.) (*D*) Giemsa-stained chromosome spreads. (Magnification: 200×.) A spermatogonium with diploid (46) chromosomes is characterized by the presence of two to three nucleoli adjacent to a clearly visible nuclear envelope. Some spermatogonia have pseudopodia (red arrow in *A*). The spermatogonium shown here has two chromosomes 18, and one X and one Y chromosome. Primary spermatocytes are the largest cells, with two to three small nucleoli and tetrad chromosomes characteristic of meiotic cells. Each cell has two spots for chromosome 18 and one X and one Y. The small round spermatid has an indistinct nuclear membrane, and each has a haploid set of chromosomes (23), one chromosome 18 and either one X or one chromosome Y chromosome. Only about 10% of round spermatids examined showed an acrosome vesicle (blue arrow in *A*) or acrosome cap (green arrow in *A*).

**Fig. 3.** (A) Twelve presumptive round spermatids randomly selected and adherent to a glass slide. (B) The same cells after FISH, each showing a single spot of either chromosome X (green) or Y (orange). (Magnification: A and B, 400×.)

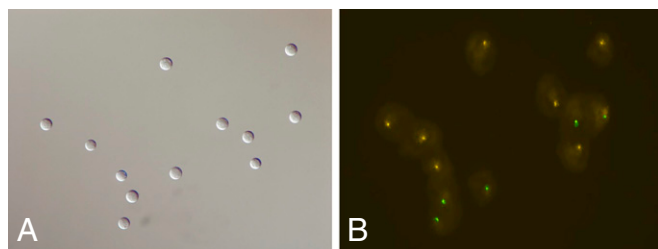**Live Birth of Healthy Babies Following ROSI.** Table 1 summarizes the results of embryo transfer after ROSI. To compare the efficiency of ROSI with that of intracytoplasmic sperm injection (ICSI), part of the data collected in our clinic after TESE and ICSI (28, 29). Currently, ROSI is less efficient than ICSI in obtaining live offspring. Note that in both ROSI and ICSI, embryos that had been kept frozen before transfer to mothers had better chance to develop to term than those that had not been frozen ($P < 0.05$).

Table 2 summarize patients' information, developmental stages of embryos at the time of transfer, karyotypes, and gender of fetuses, and the growth data of babies born after ROSI. As shown in Table 2, all 14 babies were karyotypically normal. The mean (±SD) gestational age and body weight at birth were 38.5 wk (±1.19, n = 12 deliveries) and 2,832.8 g (±497.0, n = 14 babies), respectively. Pathological examination of all miscarriages following ROSI revealed no partial or complete hydatidiform moles. As of today, all children born after ROSI are growing well without any physical or mental problems and with no evidence of Prader–Willi syndrome, Angelman syndrome, or Wiskott–Aldrich syndrome or any other unusual physical and mental aberrations.

### Discussion

**The Use of ROSI in Cases of Spermatogenetic Arrest at the Round Spermatid Stage.** Silber et al. (21) maintain that whenever round spermatids exist in the human testes, there are also mature spermatozoa present. In other words, there is no spermatogenesis arrest at the spermatid stage. Clearly this is not the case for animals. For example, the testes of mutant male mice homologous for the *Crem* gene are devoid of spermatozoa, the most advanced spermatogenic cells in these males being round spermatids. Although these males are, of course, infertile (12, 30), ROSI can help to

overcome this problem (13). According to He et al. (31), human males with *Crem* gene variants are also azoospermic, with the most advanced spermatogenic cells in such men being round spermatids. Moreover, Weinbauer et al. (32) reported the importance of *Crem* protein for the initiation of human spermiogenesis. In mice, *Crem* is an autosomal recessive gene, and therefore when spermatids of *Crem*-null males were injected into oocytes of wild-type (*Crem*^{+/+}) females, all male offspring were fertile (13). This was true for mutant mice lacking such genes as *bs* (blind-sterile) (13), ring finger protein 17 (*RNF17*), CUG triplet repeat, RNA binding protein 1 (*Cugbp1*), and jumonji domain-containing 1a (*Jmjd1a*) (33–36). According to Ayhan et al. (37), in men with mutations of two autosomal recessive genes, *TAF4B* and *ZMYND15*, their spermatogenesis is arrested at the round spermatid stage. It would be interesting to know if their specific type of infertility can be overcome by ROSI.

Table 2 includes histology-based Johnsen's scores (38) of the 12 men who participated in this study. Note that the husband of wife 1 had a Johnsen's score of 1 after initial histological examination of testis biopsies. This meant that neither spermatogenic cells nor Sertoli cells were found in biopsied samples (Fig. S4A). However, later examination of other portions of the samples revealed spermatid-like cells within some of the tubules (Fig. S4B). Intensive searches for spermatozoa and spermatogenic cells in the testis eventually made it possible for us to find spermatids suitable for ROSI. Johnsen's scores (38), which are solely dependent upon histological examination of seminiferous tubules collected from very limited regions of the testis, should be considered as merely representing the predominant pattern of spermatogenesis (39).

**Possible Reasons for Previous Failures of Human ROSI.** In the mouse, identification of round spermatids is not difficult because they exhibit a distinct centrally located chromatin granule observed readily under the interference-contrast microscope used for microsurgical operation of oocytes (40). Round spermatids are the smallest spermatogenic cells in the testis and can be readily distinguished from small blood cell leukocytes. In contrast to the mouse, human spermatids do not show a distinct chromatin granule, at least under an inverted microscope with Hoffman modulation contrast optics. It is very likely that many previous investigators, who attempted ROSI, actually chose the wrong cells in that one cannot expect normal development of oocytes injected with spermatogonia or somatic cells. Nuclei of spermatocytes (tetraploid or diploid) cannot participate in embryo development
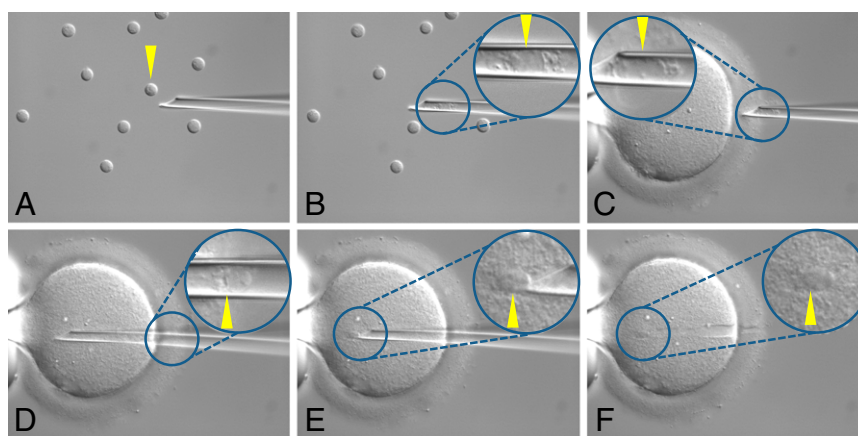


**Fig. 4.** The procedure of ROSI. (A) Immediately before picking up one spermatid. (B) A spermatid aspirated into the injection pipette, the plasma membrane being broken. The white arrowhead indicates the spermatid nucleus. (C–F) Oocytes before, during and after injection of spermatid. Arrowhead indicates the spermatid nucleus. (Magnification: A–F, 400×.)

**Table 1. Clinical outcome of ROSI partners of 76 women in this table had round spermatids as their most advanced spermatogenic cells**

| Assisted fertilization | Cryopreservation of embryos | Total no. of women | Total no. of treatment cycles | Percentage of oocytes fertilized (fert/total) (%) | Percentage Oocytes cleaved (cleaved/total) (%) | Total no. of transferred embryos | No. and percentage of recipients (%) | | | | Total no. of live offspring |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Total transferred cycles | Pregnancy* | Miscarriage | Live offspring delivered | |
| ROSI | No | 58 | 162 | 55.6 (330/594) | 44.9 (267/594) | 152 | 121 | 20 (16.5) | 13 (65.0) | 7 (5.8)[§§] | 9 |
| (present study) | Yes | 28[†] | 42 | 76.4 (107/140) | 71.4 (100/140) | 56 | 42 | 10 (23.8) | 5 (50.0) | 5 (11.9)[¶] | 5 |
| TESE and ICSI | No | 260 | 540 | 44.9 (833/1855) | 43.5 (807/1,855) | 562 | 468 | 134 (28.6) | 27 (20.1) | 107 (22.9)[§] | 121 |
| | Yes | 140 | 295 | 68.2 (825/1210) | 65.3 (790/1,210) | 377 | 290 | 174 (60.0) | 14 (8.0) | 160 (55.2)[§§,¶¶] | 176 |

Partners of 76 women in this table had round spermatids as their most advanced spermatogenic cells. To compare the efficiencies of ROSI and ICSI, part of the data collected in our clinic after TESE and ICSI are included in this table. § vs. §§ and ¶ vs. ¶¶, $P < 0.05$; Fisher's protected least significant difference test.
*No and percentage of recipients with positive germinal sacs detected by ultrasound scanning between 4 and 5 wk of gestation.
[†]Ten of these 28 patients failed to get pregnant previously when they received nonfrozen ROSI embryos.

without first completing meiosis within oocytes, which is very difficult, if not impossible (40–42). An acrosome vesicle or acrosomal cap detected on the nucleus of small round cells indicates that they are round spermatids. However, the absence of such structures does not mean that the cell in question is not an early-stage round spermatid. Even though FISH and karyotyping are a reliable means through which to identify them, we cannot use such invasive techniques for ROSI, which requires live cells. The day may come when we can identify round live spermatids correctly by using an antibody specific to their surface. Computer-assisted identification of live spermatids and other types of spermatogenetic cells may become available in the near future.

Another reason for the failure of many attempts using ROSI could be because of incomplete activation of the recipient oocytes, despite the fact that human round spermatids may contain at least some oocyte-activating ability (43). According to Ogonuki et al. (44), fresh round spermatids injected into mouse oocytes were unable to activate oocytes, whereas spermatids frozen and thawed before injection could do so, suggesting that some endogenous oocyte-activating factor is mobilized by the freeze–thawing routinely used in this study. Although round spermatid injection alone could induce small $Ca^{2+}$ oscillations (Fig. S2C), ROSI with prior

electrical stimulation was most effective in inducing repetitive, large $Ca^{2+}$ oscillations (Fig. S2D). We need further systematic studies to identify the best method to activate human ROSI oocytes. Other oocyte-activating agents to be considered include: $Ca^{2+}$ ionophore (20, 45, 46), phosphatidyl inositol (47, 48), phospholipase Cζ (49–51), 4- (diemethylamino) pyridine (52), and post-acrosomal protein PAWP (53–55).

Interestingly, pregnancy and delivery rates following ROSI were higher when embryos were first kept frozen for some time as opposed to their transfer soon after ROSI (Table 1). Because this was true for ICSI (Table 1), it was perhaps because of better preparation of the uterine endometrium when recipients were allowed to resume their normal ovulation cycle before transfer.

**Safety of ROSI.** The safety of any of the assisted fertilization technologies is a prime concern. Some investigators suspect that the inefficacy of ROSI might be because of incomplete male-specific DNA methylation imprinting in round spermatids, but at least in the mouse, DNA methylation is completed in round spermatids (56) and even before meiosis begins (57). One possible factor might be the inefficient demethylation and remethylation of DNA after introduction of spermatid nuclei into mature oocytes (58).

**Table 2. Clinical data of 14 offspring developed from ROSI oocytes**

| | Parents | | | Data from ROSI to embryo transfer | | | | Data of birth | | | | | Medical check up | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age (years) | | | | | | | | | | | | 1 mo | | 6 mo | | 1 y | | 1.5 y | | 2 y | |
| Case | Husband at TESE | Wife at ROSI | Initial Johnsen's Score* in TESE | Percent Oocytes fertilized (fert/total) (%) | Developmental stage × No. of embryos transfered | Embryo transfered | Karyotype of fetus | Mode of delivery | No. of live offspring | Gender | Gestational weeks | BW | BW | PCD | BW | PCD | BW | PCD | BW | PCD | BW | PCD |
| 1 | 37 | 36 | 1 | 33.3 (2/6) | 8 cell ×2 | Fresh | 46XX | Vaginal | 1 | F | 41 | 2876 | 3728 | N | 7745 | N | ND | ND | 10000 | N | ND | ND |
| 2 | 28 | 28 | 1 | 50.0 (9/18) | 8 cell ×2 | Cryopreservation | 46XX | Vaginal | 1 | F | 39 | 3224 | 4438 | N | ND | ND | ND | ND | 9800 | N | ND | ND |
| 3 | 34 | 37 | 3 | 50.0 (1/2) | Blastocyst ×1 | Fresh | 46XY | C-section | 2[†] | M | 36 | 2340 | 2640 | N | 8340 | N | 9360 | N | 10800 | N | 11750 | N |
| | | | | | | | 46XY | | | M | | 2140 | 2495 | N | 8360 | N | 9490 | N | 11000 | N | 11700 | N |
| 4 | 36 | 32 | 6 | 50.0 (5/10) | 10 cell ×1+ Morula ×1 | Fresh | 46XY | Vaginal | 1 | M | 39 | 3440 | 4115 | N | ND | ND | ND | ND | ND | ND | — | — |
| 5 | 40 | 39 | 4 | 65.0 (13/20) | 11 cell ×1+ Morula ×1 | Cryopreservation | 46XY | C-section | 1 | M | 38 | 2598 | 3490 | N | ND | ND | 8305 | N | — | — | — | — |
| 6 | 28 | 28 | 7 | 50.0 (7/14) | 5 cell ×2 | Fresh | 46XX | Vaginal | 1 | F | 38 | 3115 | 4255 | N | 9240 | N | 9480 | N | — | — | — | — |
| 7 | 34 | 39 | 3 | 75.0 (3/4) | 12 cell ×2 | Fresh | 46XY | Vaginal | 2[‡] | M | 37 | 2666 | 4080 | N | 9790 | N | 9855 | N | — | — | — | — |
| | | | | | | | 46XY | | | M | | 2474 | 3670 | N | 8755 | N | 8945 | N | — | — | — | — |
| 8 | 36 | 35 | 7[§] | 50.0 (2/4) | Morula ×1+ Blastocyst ×1 | Cryopreservation | 46XY | Vaginal | 1 | M | 39 | 3348 | 4596 | N | 9500[¶] | N | — | — | — | — | — | — |
| 9 | 26 | 23 | 6 | 42.1 (8/19) | 8 cell ×2 | Cryopreservation | 46XX | Vaginal | 1 | F | 38 | 2834 | 4145 | N | 7805[‖] | N | — | — | — | — | — | — |
| 10 | 38 | 37 | 7 | 23.1 (3/13) | 10 cell×2 | Fresh | 46XY | Vaginal | 1 | M | 39 | 2776 | 3378 | N | 6105 | N | — | — | — | — | — | — |
| 11 | 41 | 40 | 7 | 93.8 (15/16) | 8 cell×2 | Fresh | 46XY | Vaginal | 1 | M | 39 | 2080 | 2945 | N | — | — | — | — | — | — | — | — |
| 12 | 43 | 34 | 7 | 56.3 (9/16) | Blastocyst ×1 | Cryopreservation | 46XY | Vaginal | 1 | M | 39 | 3748 | 4920** | N | — | — | — | — | — | — | — | — |

BW, body weight (g); F, female; M, male; N, normal; ND, No data; PCD, physical and cognitive development.
*Johnsen (38).
[†]Monozygotic twins.
[‡]Dizygotic twins, **3 mo, [¶]9 mo, [‖]7 mo.
[§]Examination of biopsied sample and re-examination of histology slide revealed the presence of few highly deformed elongated spermatids, but we did not use these cells for this study because they were not tolerant to freeze–thawing the procedure we used routinely for ROSI.

Tricostatin A, an inhibitor of histone deacetylase, which significantly reduces abnormal DNA hypermethylation in somatic cell nuclei transplanted into enucleated oocytes (59), may increase the success rate of human ROSI. Even though all 14 babies born after ROSI are healthy and have no unusual physical or mental problems, because the number of successful case is still small, it might be premature to conclude that conception after ROSI is as safe as in vitro fertilization or ICSI. However, it is likely that the ROSI procedures can be further refined. For example, in most animals, the sperm centrosome plays a crucial role in normal fertilization as the center of the microtubular assembly (sperm aster), which is essential for the approximation and fusion of male and female pronuclei (60, 61). It is also possible that centrosomes in some of the round spermatids we used were "immature." If this proves to be the case, the replacement of an immature centrosome by a mature centrosome, as proposed for ICSI by Nakamura et al. (62), may increase the efficiency of ROSI.

## Materials and Methods

**Ethical Aspects.** This study was conducted with the informed consent of all participating patients. The Institutional Review Boards of the Saint Mother Obstetrics and Gynecology Clinic and of the Ministry of Health, Labor, and Welfare of Japan approved this study on December 20, 2009 and July 3, 2011, respectively. This study was registered and adhered to International Committee of Mdical Journal Editors criteria. The University Hospital Medical Information Network Clinical Trials Registry is UMIN000006117.

**Subjects.** We conducted Micro-TESE on 730 nonobstructive azoospermic men who had already undergone a previous Micro-TESE conducted by andrologists from other institutions and had been found not to have normal spermatogenic cells beyond the round spermatid stage. We found round spermatids in 76 (10.4%) of the 730 men. These 76 men, aged 22–46 y (33.4 ± 5.2), participated in a total of 163 transfer cycles in our clinic between September 2011 and March 2014.

**Collection and Cryopreservation of Spermatogenic Cells from Azoospermic Men.** Testicular tubular biopsies (20–50 mg each) were obtained from not only the surface, but also from deep inside the testis until we found spermatozoa or elongated spermatids. If no such cells were found despite our continuing efforts, but we saw many normal-looking spermatids, all collected tubules were dissociated enzymatically and cryopreserved. Details are provided in *SI Materials and Methods*.

**Thawing of Frozen Testicular Cell Samples and Identification of Round Spermatids Immediately Before ROSI.** Thawing was carried out at 30 °C. Using a differential interference microscope, morphological and physical features of individual cells were examined. Their small size (6–8 μm in diameter), thin rim of cytoplasm around the nucleus, and ready separation of cytoplasm from the nucleus were important identifying features of round spermatids. The accuracy of the visual identification was checked by karyotyping and FISH, which proved to be near 100%. Details are provided in *SI Materials and Methods*.

**Collection and Activation of Oocytes Before ROSI.** Mature oocytes (at metaphase II), collected from ovaries 36 h after hCG injection (63), were maintained for 5–7 h in HFT medium (64) before their activation by either ooplasm aspiration (16) or electronic stimulation (65). ROSI was performed 10 min later. In some experiments, changes in intracellular $Ca^{2+}$ concentration were monitored using a $Ca^{2+}$-sensitive fluorescence indicator. Details are provided in *SI Materials and Methods*.

**ROSI and Embryo Transfer.** A presumptive spermatid, selected on the basis of its morphological and physical features, was injected into an oocyte. One or two oocytes reaching the four-cell to blastocyst stage were transferred to the uterus and pregnancy was assessed 4–5 wk later. Details are provided in *SI Materials and Methods*.

**Statistical Analysis.** Data were analyzed using the Microsoft Excel Add-in software (MacToukei-kaiseki v2.0; Esumi).

1. Willott GM (1982) Frequency of azoospermia. *Forensic Sci Int* 20(3):9–10.
2. Jarvi K, et al. (2010) CUA guideline: The workup of azoospermic males. *Can Urol Assoc J* 4(3):163–167.
3. Patrizio P, Silber S, Ord T, Balmaceda JP, Asch RH (1988) Two births after microsurgical sperm aspiration in congenital absence of vas deferens. *Lancet* 2(8624):1364.
4. Schlegel PN, Girardi SK (1997) Clinical review 87: In vitro fertilization for male factor infertility. *J Clin Endocrinol Metab* 82(3):709–716.
5. Schlegel PN, Su L-M (1997) Physiological consequences of testicular sperm extraction. *Hum Reprod* 12(8):1688–1692.
6. Schlegel PN, et al. (1997) Testicular sperm extraction with intracytoplasmic sperm injection for nonobstructive azoospermia. *Urology* 49:435–440.
7. Ashraf MC, Singh S, Raj D, Ramakrishnan S, Esteves SC (2013) Micro-dissection testicular sperm extraction as an alternative for sperm acquisition in the most difficult cases of Azoospermia: Technique and preliminary results in India. *J Hum Reprod Sci* 6(2):111–123.
8. Tanaka A, et al. (2006) In vitro culture of the immature spermatogenic cells. *Fertil Steril* 86(Suppl):S518–S519.
9. Ogura A, Yanagimachi R (1993) Round spermatid nuclei injected into hamster oocytes from pronuclei and participate in syngamy. *Biol Reprod* 48(2):219–225.
10. Ogura A, Matsuda J, Yanagimachi R (1994) Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc Natl Acad Sci USA* 91(16):7460–7462.
11. Kimura Y, Yanagimachi R (1995) Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 121(8):2397–2405.
12. Nantel F, et al. (1996) Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature* 380(6570):159–162.
13. Yanagimachi R, et al. (2004) Production of fertile offspring from genetically infertile male mice. *Proc Natl Acad Sci USA* 101(6):1691–1695.
14. Edwards RG, Tarin JJ, Dean N, Hirsch A, Tan SL (1994) Are spermatid injections into human oocytes now mandatory? *Hum Reprod* 9(12):2217–2219.
15. Tesarik J, Mendoza C (1996) Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. *Hum Reprod* 11(4):772–779.
16. Tesarik J, et al. (1996) Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum Reprod* 11(4):780–783.
17. Gianaroli L, et al. (1999) Birth of a healthy infant after conception with round spermatids isolated from cryopreserved testicular tissue. *Fertil Steril* 72(3):539–541.
18. Vanderzwalmen P, et al. (1997) Intracytoplasmic injection of spermatids retrieved from testicular tissue: Influence of testicular pathology, type of selected spermatids and oocyte activation. *Hum Reprod* 12(6):1203–1213.
19. Barak Y, et al. (1998) Pregnancy and birth after transfer of embryos that developed from single-nucleated zygotes obtained by injection of round spermatids into oocytes. *Fertil Steril* 70(1):67–70.
20. Van Der Zwalmen P, et al. (1995) Fertilization of an oocyte microinseminatred with a spermatid in an in-vitro fertilization programme. *Hum Reprod* 10(3):502–503.
21. Silber SJ, Johnson L, Verheyen G, Van Steirteghem A (2000) Round spermatid injection. *Fertil Steril* 73(5):897–900.
22. Silber SJ, Johnson L (1998) Are spermatid injections of any clinical value? ROSNI and ROSI revisited. Round spermatid nucleus injection and round spermatid injection. *Hum Reprod* 13(3):509–515.
23. Vanderzwalmen P, et al. (1998) Is there a future for spermatid injections? *Hum Reprod* 13(Suppl 4):71–84.
24. Practice Committee of American Society for Reproductive Medicine; Practice Committee of Society for Assisted Reproductive Technology (2008) Round spermatid nucleus injection (ROSNI). *Fertil Steril* 90(5, Suppl):S199–S201.
25. Tanaka A, et al. (2003) Completion of meiosis in human primary spermatocytes through in vitro coculture with Vero cells. *Fertil Steril* 79(Suppl 1):795–801.
26. Tanaka A, et al. (2008) Isolated spermatogonia protrude active pseudopodia in vitro. *Fertil Steril* 90(2):453–455.
27. Ozil JP, Banrezes B, Tóth S, Pan H, Schultz RM (2006) $Ca^{2+}$ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev Biol* 300(2):534–544.
28. Tanaka A, Nagayoshi M, Awarta S, Himeno I, Tanaka I (2008) Improvement of a photodynamic system for observation of seminiferous tubules in microdissection-testicular sperm extraction (MD-TESE). *Fertil Steril* 90:S106.
29. Yamaguchi T, et al. (2014) Comparative study of obstructive azoospermia with MESE or MD-TESE. *Obstet Gynecol Pract* 63(6):881–885.
30. Blendy JA, Kaestner KH, Weinbauer GF, Nieschlag E, Schütz G (1996) Severe impairment of spermatogenesis in mice lacking the *CREM* gene. *Nature* 380(6570):162–165.
31. He XJ, et al. (2014) CREM variants rs4934540 and rs2295415 conferred susceptibility to nonobstructive azoospermia risk in the Chinese population. *Biol Reprod* 91(2):52.
32. Weinbauer GF, Behr R, Bergmann M, Nieschlag E (1998) Testicular cAMP responsive element modulator (CREM) protein is expressed in round spermatids but is absent or reduced in men with round spermatid maturation arrest. *Mol Hum Reprod* 4(1):9–15.

33. Pan J, et al. (2005) RNF17, a component of the mammalian germ cell nuage, is essential for spermiogenesis. *Development* 132(18):4029–4039.
34. Kress C, Gautier-Courteille C, Osborne HB, Babinet C, Paillard L (2007) Inactivation of CUG-BP1/CELF1 causes growth, viability, and spermatogenesis defects in mice. *Mol Cell Biol* 27(3):1146–1157.
35. van der Weyden L, et al. (2006) Loss of TSLC1 causes male infertility due to a defect at the spermatid stage of spermatogenesis. *Mol Cell Biol* 26(9):3595–3609.
36. Liu Z, et al. (2010) Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. *J Biol Chem* 285(4):2758–2770.
37. Ayhan Ö, et al. (2014) Truncating mutations in TAF4B and ZMYND15 causing recessive azoospermia. *J Med Genet* 51(4):239–244.
38. Johnsen SG (1970) Testicular biopsy score count—A method for registration of spermatogenesis in human testes: Normal values and results in 335 hypogonadal males. *Hormones* 1(1):2–25.
39. Berookhim BM, Palermo GD, Zaninovic N, Rosenwaks Z, Schlegel PN (2014) Microdissection testicular sperm extraction in men with Sertoli cell-only testicular histology. *Fertil Steril* 102(5):1282–1286.
40. Kimura Y, Yanagimachi R (1995) Development of normal mice from oocytes injected with secondary spermatocyte nuclei. *Biol Reprod* 53(4):855–862.
41. Kimura Y, Tateno H, Handel MA, Yanagimachi R (1998) Factors affecting meiotic and developmental competence of primary spermatocyte nuclei injected into mouse oocytes. *Biol Reprod* 59(4):871–877.
42. Ogura A, et al. (1998) Development of normal mice from metaphase I oocytes fertilized with primary spermatocytes. *Proc Natl Acad Sci USA* 95(10):5611–5615.
43. Yazawa H, Yanagida K, Sato A (2007) Human round spermatids from azoospermic men exhibit oocyte-activation and $Ca^{2+}$ oscillation-inducing activities. *Zygote* 15(4):337–346.
44. Ogonuki N, Inoue K, Ogura A (2011) Birth of normal mice following round spermatid injection without artificial oocyte activation. *J Reprod Dev* 57(4):534–538.
45. Steinhardt RA, Epel D, Carroll EJ, Jr, Yanagimachi R (1974) Is calcium ionophore a universal activator for unfertilised eggs? *Nature* 252(5478):41–43.
46. Sousa M, Mendoza C, Barros A, Tesarik J (1996) Calcium responses of human oocytes after intracytoplasmic injection of leukocytes, spermatocytes and round spermatids. *Mol Hum Reprod* 2(11):853–857.
47. Miyazaki S (1988) Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *J Cell Biol* 106(2):345–353.
48. Amano T, Mori T, Watanabe T (2004) Activation and development of porcine oocytes matured in vitro following injection of inositol 1,4,5-trisphosphate. *Anim Reprod Sci* 80(1-2):101–112.
49. Nomikos M, Kashir J, Swann K, Lai FA (2013) Sperm PLCζ: From structure to $Ca^{2+}$ oscillations, egg activation and therapeutic potential. *FEBS Lett* 587(22):3609–3616.
50. Ito M, Shikano T, Kuroda K, Miyazaki S (2008) Relationship between nuclear sequestration of PLCzeta and termination of PLCzeta-induced $Ca^{2+}$ oscillations in mouse eggs. *Cell Calcium* 44(4):400–410.
51. Ito M, et al. (2008) Difference in $Ca^{2+}$ oscillation-inducing activity and nuclear translocation ability of PLCZ1, an egg-activating sperm factor candidate, between mouse, rat, human, and medaka fish. *Biol Reprod* 78(6):1081–1090.
52. Tachibana M, et al. (2013) Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell* 153(6):1228–1238.
53. Kimura Y, et al. (1998) Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. *Biol Reprod* 58(6):1407–1415.
54. Wu AT, et al. (2007) PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. *J Biol Chem* 282(16):12164–12175.
55. Aarabi M, et al. (2014) Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J* 28(10):4434–4440.
56. Shamanski FL, Kimura Y, Lavoir M-C, Pedersen RA, Yanagimachi R (1999) Status of genomic imprinting in mouse spermatids. *Hum Reprod* 14(4):1050–1056.
57. Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM (2007) Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. *Dev Biol* 307(2):368–379.
58. Kishigami S, et al. (2006) Epigenetic abnormalities of the mouse paternal zygotic genome associated with microinsemination of round spermatids. *Dev Biol* 289(1):195–205.
59. Kishigami S, et al. (2006) Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. *Biochem Biophys Res Commun* 340(1):183–189.
60. Simerly C, et al. (1995) The paternal inheritance of the centrosome, the cell's microtubule-organizing center, in humans, and the implications for infertility. *Nat Med* 1(1):47–52.
61. Palermo GD, Colombero LT, Rosenwaks Z (1997) The human sperm centrosome is responsible for normal syngamy and early embryonic development. *Rev Reprod* 2(1):19–27.
62. Nakamura S, et al. (2005) A trial to restore defective human sperm centrosomal function. *Hum Reprod* 20(7):1933–1937.
63. Tan SL (1994) Luteinizing hormone-releasing hormone agonists for ovarian stimulation in assisted reproduction. *Curr Opin Obstet Gynecol* 6(2):166–172.
64. Trounson AO, Mohr LR, Wood C, Leeton JF (1982) Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. *J Reprod Fertil* 64(2):285–294.
65. Levron J, Cohen J, Willadsen S (1995) Highly effective method of human oocyte activation. *Zygote* 3(2):157–161.
66. Verheyen G, et al. (1995) Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Hum Reprod* 10(11):2956–2959.
67. Tanaka A, et al. (2009) Metaphase II karyoplast transfer from human in-vitro matured oocytes to enucleated mature oocytes. *Reprod Biomed Online* 19(4):514–520.

# Supporting Information

## Tanaka et al. 10.1073/pnas.1517466112

### SI Materials and Methods

**Collection of Spermatogenic Cells from Azoospermic Men.** We performed semen analysis at least twice for each man. When no spermatozoa were found, we performed hormone assays (FSH, LH, and testosterone measurements) and palpation. All males involved in this study had high blood FSH (more than 10 mIU/mL) and almost half of them possessed small soft testes.

Collection of seminiferous tubules was performed under general anesthesia supplemented with local anesthesia. The operation began with the larger testis. A vertical incision was made in the tunica albuginea to expose a mass of seminiferous tubules. The outer surfaces of the seminiferous tubules were carefully examined under a microscope (10–32× magnification) and a mass (20–50 mg) of tubules of large diameter was isolated using forceps. We inferred that thick and more opaque seminiferous tubules were more likely to be the sites of active spermatogenesis. A dissected tubular mass was handed to an embryologist who examined it in human tubal fluid (HTF) with 10% serum protein substitute (SPS) (SAGE In Vitro Fertilization; Cooper Surgical) under a dissecting microscope. The thickest regions of the tubules were cut, and cells were released from tubules with needles (27 G × 3/4 inches; Terumo). On occasion, 0.25% trypsin and 0.02% EDTA were included in the medium to facilitate their release, following which they were examined using a differential interference microscope to identify the cell types. The presence or absence of spermatozoa and developmental stages of spermatogenic cells were reported to the physician within 2 min. Most of the first biopsy sample was left in a dish, but a small portion was fixed with Bouin's fluid for subsequent histological evaluation (Johnsen's score) (38). Whenever normal-looking spermatozoa were found in the first biopsy sample, several more samples were collected from nearby regions of the testis. Small drops of cryoprotectant medium, each containing one or a few spermatozoa, were verified for subsequent ICSI, because sperm collection and ICSI could not be performed on the same day in most cases. When we failed to find spermatozoa in the first biopsy, we collected up to 10 more samples, not only from the surface, but also from deep inside the testis. If no spermatozoa were found, we then collected more samples (up to 10) from the other testis. Searches continued until normal-looking spermatozoa were found within the biopsied samples. When neither these nor normal elongated spermatids were found, but there were often many normal-looking round spermatids, we pooled all biopsied samples in one single dish. After rinsing in erythrocyte-lysing buffer (66), these cell suspensions were transferred to Dulbecco's phosphate buffer (D-PBS; Sigma-Aldrich) containing both 0.125% collagenase (Type IV-S; Sigma-Aldrich) and 0.001% DNase I (Sigma-Aldrich) and then vortexed to disperse the cells. DNase was used to digest viscous DNA released from the nuclei of dead cells with damaged plasma membranes. All procedures were performed at 25–32 °C. Cell suspensions were then filtered through 30- and 15-μm meshes (NRS-030 and NRS-013; Nippon Rikagaku Kikai) to collect round spermatids and other testicular cells, which were then cryopreserved to await treatment cycles.

**Cryopreservation of Spermatogenic Cells.** Testicular cells collected as described above were suspended in a freezing medium (D-PBS) supplemented with 0.6 M ethylene glycol, 0.125 M sucrose and antibiotics, drawn in 0.25-mL Cassou straws (IMV Technologies) and cooled to 4 °C before sealing with tamping polyvinyl alcohol powder (Fujihira Industry). Straws were placed in the −7 °C cooling chamber of a programmable alcohol-bath-freezer (ET-U5; Chino). A few minutes later, the straws were automatically seeded, held at the same temperature (−7 °C) for 20 min, then cooled to −30 °C at a speed of −0.3 °C/min before plunging into −196 °C liquid nitrogen, where frozen cells were stored until use.

**Identification of Round Spermatids Immediately Before ROSI.** Thawing was carried out by exposing straws in air for 5 s, and then steeping in tepid water (30 °C) until ice in the straw had melted. The cell suspension in the straw was then transferred to a test tube and the cryoprotectant in the medium was diluted stepwise by adding HTF with 10% (vol/vol) SPS before centrifugation at 70 × g for 5 min at 4 °C. Finally, by using a sterile calibrated pipette (Drummond Scientific), thawed spermatogenic cells were transferred to a microdroplet of HTF with 10% SPS in a sterile plastic culture dish (60-mm IVF Round Dish; Becton Dickinson). The fluid in the dish was previously covered with sterile mineral oil (M-8410l; Sigma-Aldrich).

Correct identification of round spermatids is the key to successful ROSI (Fig. 1). Although round spermatids can be distinguished from other spermatogenic cells by cytogenetic methods (FISH and karyotyping) (Fig. 2), noninvasive methods obviously must be used to distinguish round spermatids for clinical use. We accomplished this on the basis of their morphological and physical features. Round spermatids (6–8 μm in diameter; slightly smaller than erythrocytes) were the smallest spermatogenic cells being much smaller than spermatocytes (10–12 μm) and slightly smaller than most spermatogonia (8–10 μm). Differential interference microscopy revealed two to three nucleoli within the nuclei of spermatogonia and spermatocytes, but not in round spermatids. The rim of cytoplasm surrounding the nucleus of round spermatids is thinner than that of the spermatogonia; protruded active pseudopodia were often seen in spermatogonia (26) but not in round spermatids. The presence of an acrosomal vesicle or cap identified the cell as a round spermatid, but we found such features in less than 10% of presumptive spermatids (Fig. 2). Another important characteristic of the round spermatid was seen in the fact that its cytoplasm could be readily separated from the nucleus if sucked back and forth within the injection pipette unlike that of spermatogonia. Lymphocytes, about the size of round spermatids, also had a "tough" plasma membrane, which could not be broken, even by vigorous pipetting. Round spermatids, whose nuclei fragmented as they were sucked into the pipette, were considered dead and were not used for ROSI. Cells that met round spermatid criteria were confirmed as such by karyotyping and FISH with almost 100% accuracy (Fig. 3). In cases where we were not certain about the presence or absence of round spermatids in a biopsied sample, we used a portion of the corresponding frozen sample for FISH.

**Collection and Activation of Oocytes Before ROSI.** Mature oocytes (at metaphase II) surrounded by cumulus cells were aspirated from the ovaries of 76 women (mean age. $34.5 \pm 4.5$ y old; range, 22–45 y) 36 h following 5,000 IU hCG undergoing controlled ovarian stimulation according to the long protocol of GnRH agonist administration (63). Oocyte-cumulus complexes were maintained in HTF medium with 10% SPS for 5–6 h (64) before they were freed from cumulus cells by 1-min treatment with hyaluronidase (300–1,000 U/mL, type VIII; Sigma-Aldrich).

Because our previous study showed that oocytes activated before ROSI developed into cleaving embryos at a higher rate than those without activation treatment (32% vs. 55%) (Table S1),

we routinely activate oocytes ∼10 min before the ROSI procedure. To determine the best protocol for activation, we used the oocytes that were immature (in the germinal vesicle stage) at the time of oocyte retrieval from ovaries and then allowed to mature in vitro (67).

We compared two methods of oocyte activation: vigorous ooplasm aspiration and the application of electric current. For the first method, an oocyte in HTF medium with 10% SPS was held by a pipette attached to a micromanipulator (MO0202 U; Narishige). Then, cytoplasm (about 0.1 pL) was vigorously aspirated from the ooplasm during ICSI, as originally reported by Tesarik and Mendoza (15). For the second method, oocytes were activated by electric current (65). For this, oocytes were placed in 295-mM mannitol solution with 0.1 mM $CaCl_2$ and 0.05 mM $MgCl_2$, and stimulated with an alternating current of 5 V/cm at 1 MHz for 10 s followed by a single 1.2-kV/cm pulse of direct current for 99 ms using an electro-cell fusion generator (LF201; Nepagne). After electrical stimulation, oocytes were returned to the HTF medium with 10% SPS to examine whether meiosis II had been completed.

To examine changes in intracellular $Ca^{2+}$ concentration, we first loaded in vitro matured oocytes with plasma membrane-permeable $Ca^{2+}$ sensitive fluorescence indicator (Fluo-8H AM; ATT Bioquest). Changes in the intracellular $Ca^{2+}$ concentrations of these oocytes were recorded (50, 51) at 37 °C for 70 min under the atmosphere of 5% $CO_2$ in air. $Ca^{2+}$ images were analyzed using the NIS-Elements AR software (Nikon).

**ROSI and Embryo Transfer.** A presumptive spermatid, selected based on the criteria described earlier, was drawn into an injection pipette sucked back and forth to rupture its plasma membrane. The nucleus, along with a small amount of cytoplasm, was then injected into an oocyte (Fig. 4). This was performed ∼10 min after electric stimulation of the oocyte, ROSI oocytes were then cultured using sequential culture media system (G-1 plus, G-2 plus, and OVOIL; Vitrolife) at 37 °C in 6% $CO_2$, 5% $O_2$, and 89% $N_2$. Some embryos reaching the four-cell stage, or more advanced stages of development, were cryopreserved using the Cryotop Vitrification Kit (Kitazato). Vitrification and thawing of embryos was performed following the manufacturer's instructions. One or two unfrozen or frozen-thawed embryos at the five-cell to blastocyst stage were transferred to the uterus. Clinical pregnancy was assessed 4–5 wk after embryo transfer by the presence or absence of an intrauterine gestational sac on vaginal ultrasound. The birth rate was expressed as the number of live newborns per transfer cycle.
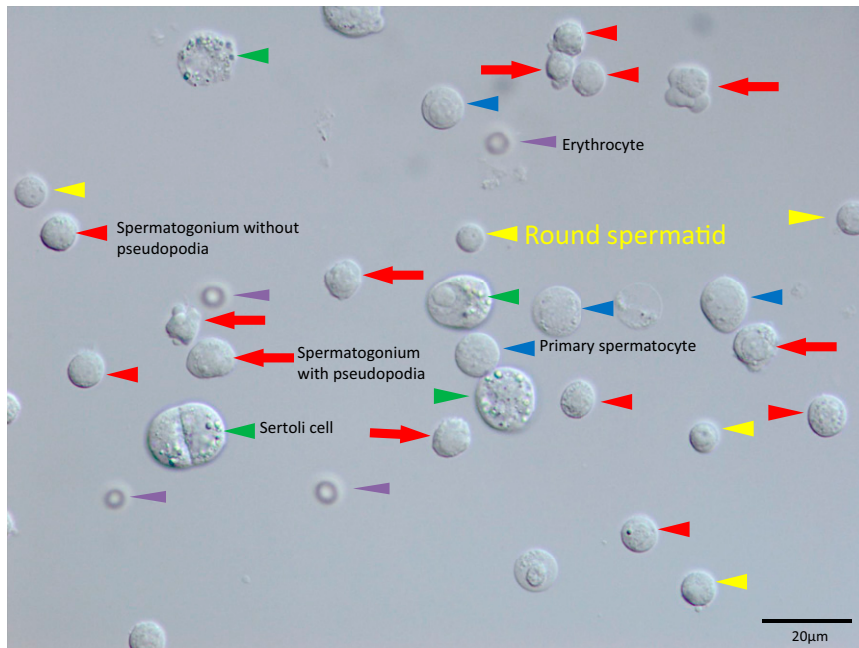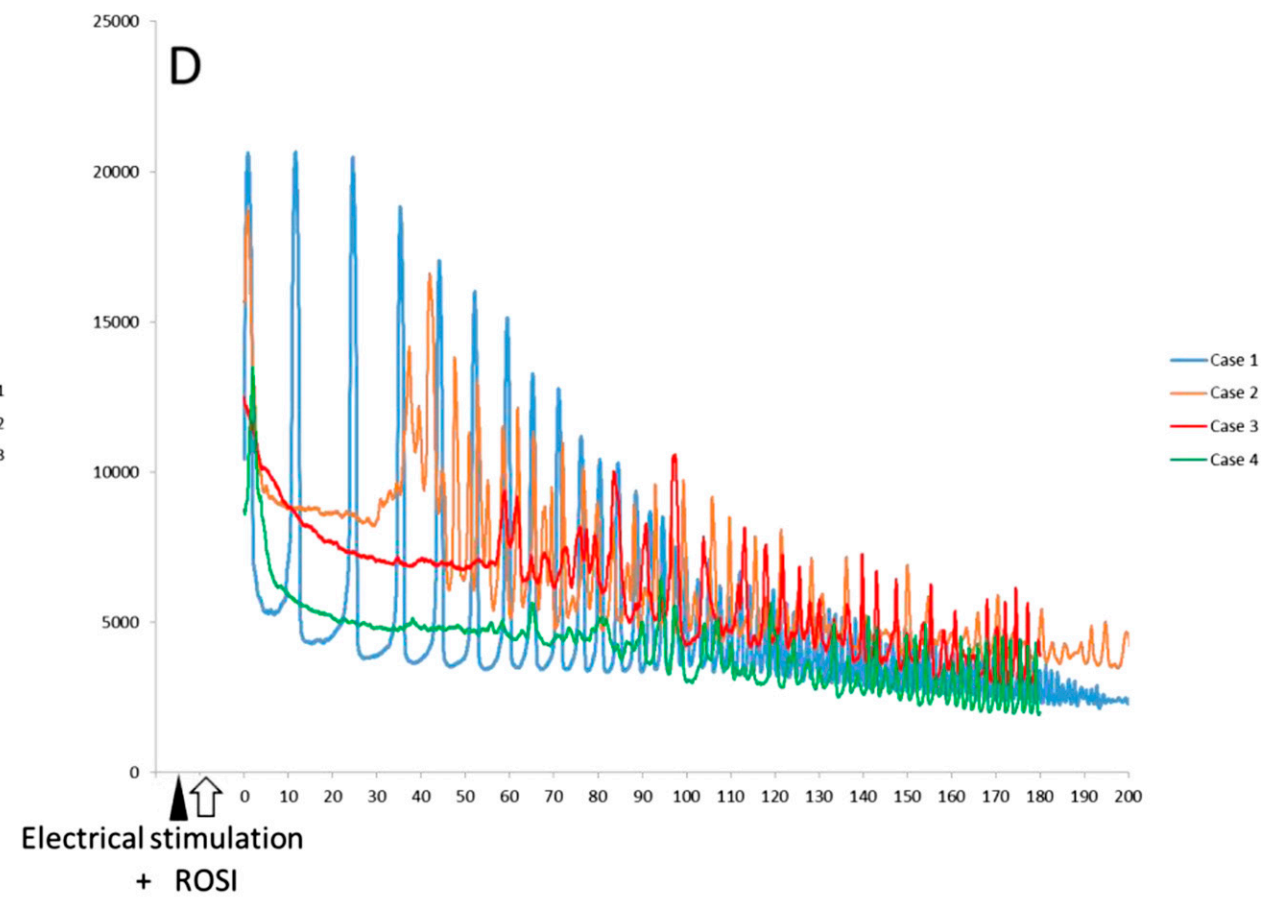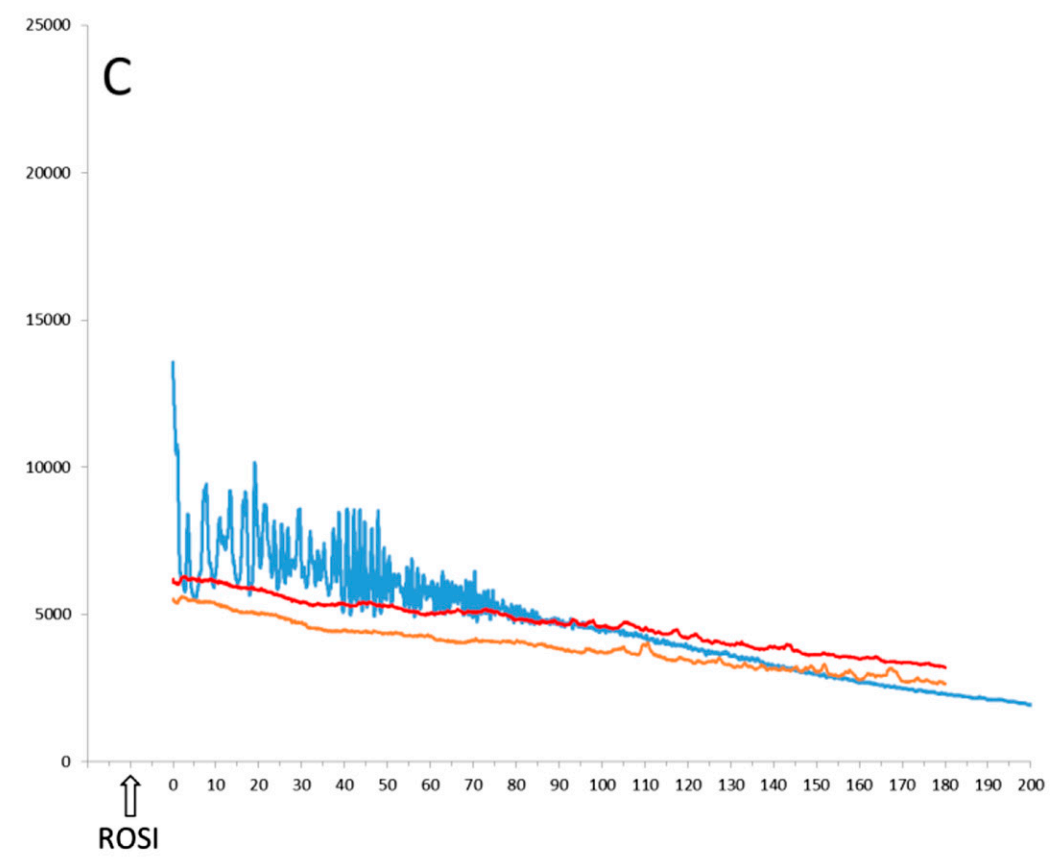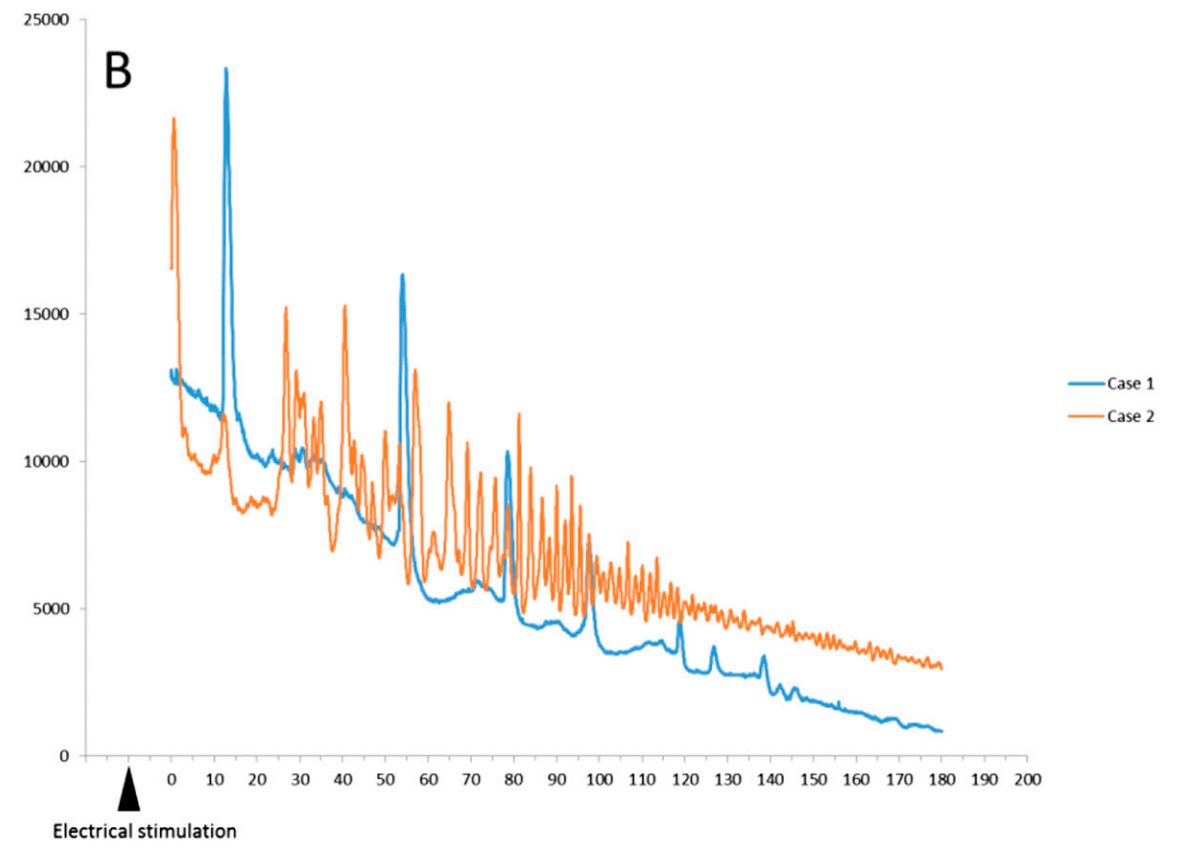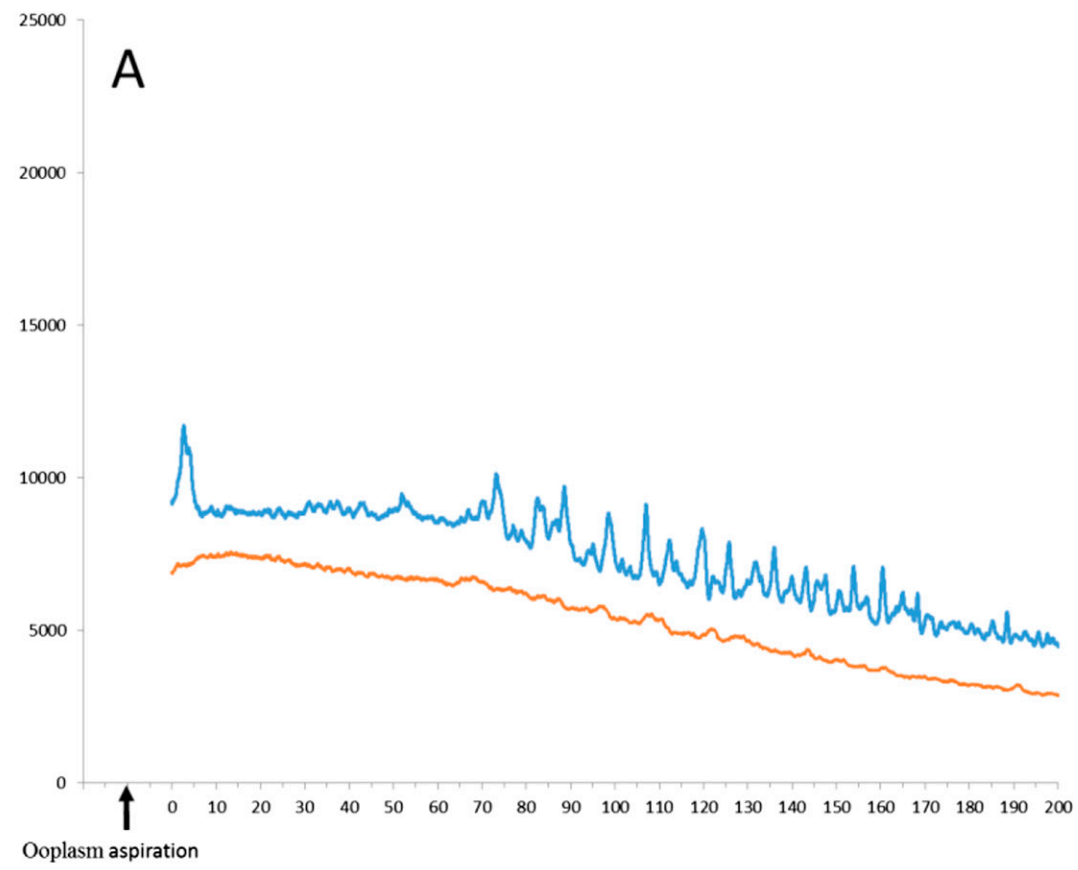


**Fig. S1.** More examples of various cell types obtained after enzymatic dissociation of seminiferous tubules. Round spermatids are pointed by yellow arrowheads. (Magnification: 400×.)

**Fig. S2.** Intracellular Ca²⁺ oscillations within in vitro matured oocytes after (*A*) ooplasm aspiration, (*B*) electrical stimulation, (*C*) ROSI alone, and (*D*) electric stimulation plus ROSI. Note that neither ooplasm aspiration nor electric stimulation alone induced repetitive Ca²⁺ oscillations consistently. In contrast, electric stimulation plus ROSI (*D*) induced consistent large, repetitive Ca²⁺ oscillations.
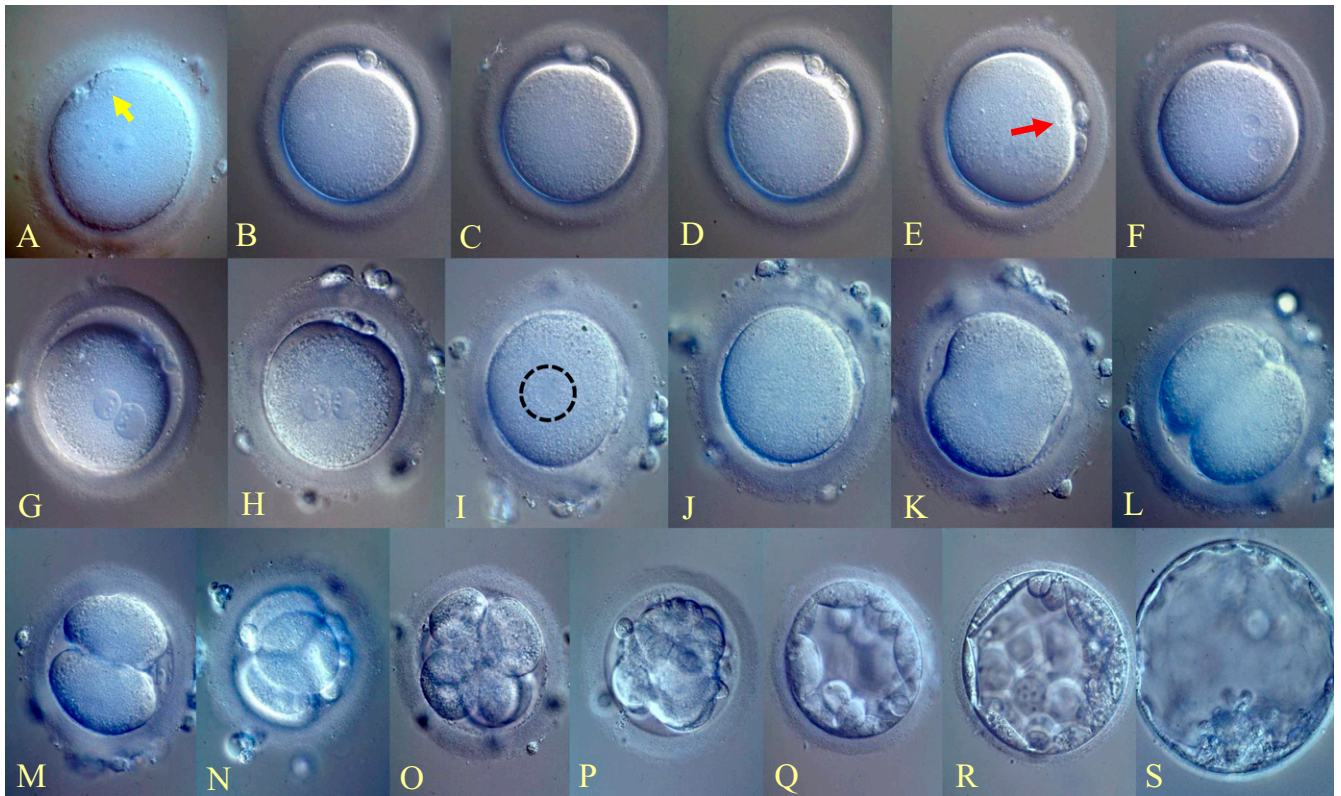
**Fig. S3.** The preimplantation development of electro-activated ROSI oocytes. (*A* and *B*) metaphase II (yellow arrow), (*C*) anaphase II, (*D*) telophase II, (*E*) appearance of a small female pronucleus (red arrow), (*F–H*) female and male pronucleus, (*I*) syngamy (dotted line), (*J*) disappearance of syngamy, (*K*) starting cell division, (*L* and *M*) two-cell, (*N*) four-cell, (*O*) eight-cell, (*P*) compacted morula, (*Q*) early blastocyst, (*R*) blastocyst, and (*S*) expanded blastocyst. (Magnification: *A–S*, 400×.)
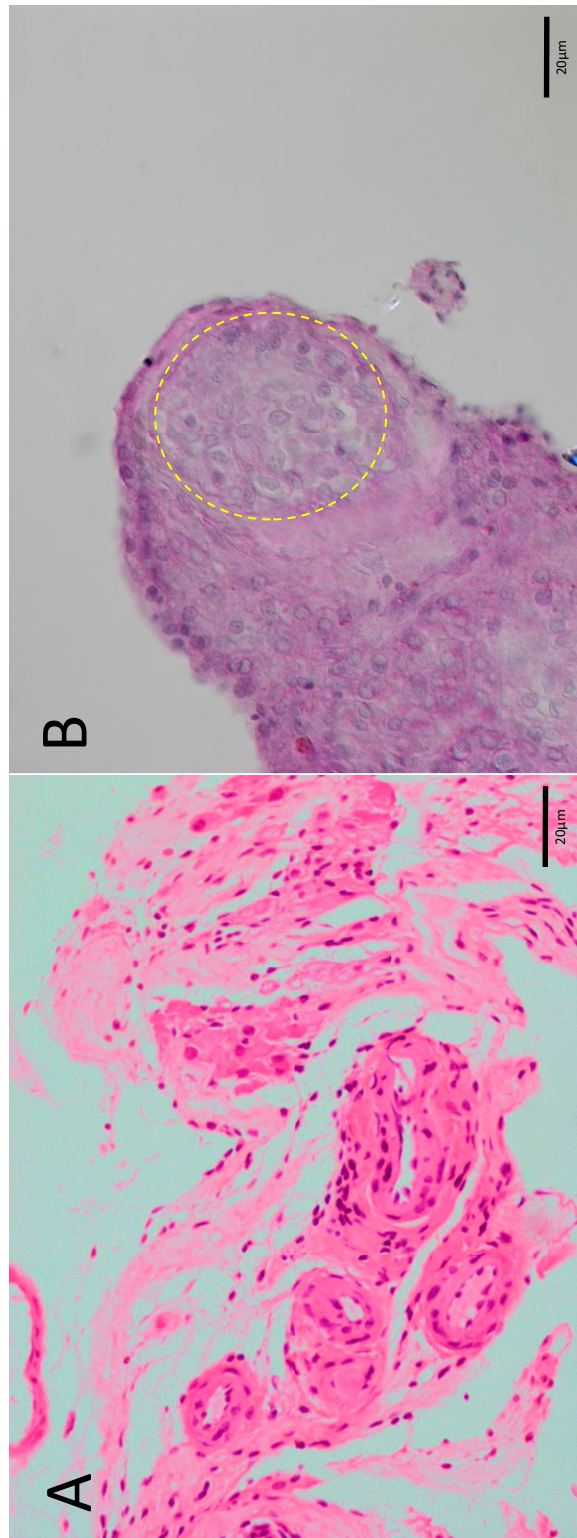
**Fig. S4.** Histological sections of seminiferous tubules of patient 1 (Table 2). (*A*) H&E-stained section, showing seminiferous tubes with neither Sertoli cells nor spermatogenic cells; based on this, a pathologist rated the Johnsen score of this patient as 1 (Table 2). (*B*) Later examination of other seminiferous tubules of this man revealed the presence of spermatogenenic cell-like cells in some other seminiferous tubes (PAS and hematoxylin-stained). See cells within a dotted circle (basement membrane of a seminiferous tubule). (Magnification: *A* and *B*, 400×.)

**Table S1. Comparative study of oocyte cultivation with at without electrical stimulation**

| Activation method | Oocytes | No. fertilized (%) 1 or 2 PN | No. developed (%) |
|---|---|---|---|
| None* | 58 | 19 (32.8) | 19 (32.8) |
| Electrical stimulation | 42 | 27 (64.3) | 23 (54.8) |

*ROSI alone.