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

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SUMMARY

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

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

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

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

INTRODUCTION

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

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

MATERIALS AND METHODS

Reagents

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

Media

The medium used for culturing oocytes after microsurgery was CZB (Chatot et al., 1989, 1990) supplemented with 5.56 mM D-glucose. The medium for oocyte collection from oviducts, subsequent treatments and micromanipulation was a modified CZB with 20 mM Hepes, a reduced amount of $NaHCO_3$ (5 mM) and 3 mg ml^{-1} bovine serum albumin (BSA, fraction V, Calbiochem., La Jolla, CA). This medium was called Hepes-CZB. In the later part of our study we replaced BSA in Hepes-CZB with 0.1 mg ml^{-1} polyvinyl alcohol (PVA, cold water soluble, M_r 10×10^3). PVA kept the wall of the injection pipette less sticky for mineral oil and cell debris over a longer period than BSA. This was beneficial during repeated use of a single pipette.

Preparation of oocytes

B6D2F1 females (6-12 weeks old) were each injected with 7.5 IU pregnant mare serum gonadotropin followed by 7.5 IU human

chorionic gonadotropin (hCG) 48 hours later. Oocytes were collected from oviducts about 16 hours after hCG injection. They were freed from the cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (300 USP units mg^{-1}; ICN Biochemicals, Costa Mesa, CA) in Hepes-CZB. The oocytes were rinsed and kept in CZB for up to 3.5 hours at 37°C under 5% CO_2 in air.

Preparation of testicular spermatozoa and spermatids

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

Microinjection of testicular spermatozoa and spermatids into oocytes

A single testicular spermatozoon was injected into an oocyte as described previously for injection of mature epididymal spermatozoa (Kimura and Yanagimachi, 1995). A testicular spermatozoon was sucked, tail first, deep into an injection pipette (4.5-5 μm I.D. at the tip) which had been attached to a Piezo electric pipette-driving unit (Model PMM-10, Prima Meat Packers, Tsuchiura, Japan). Meanwhile, a mature unfertilized oocyte was held by a holding pipette and its zona pellucida was drilled by applying several Piezo pulses to the sperm injection pipette. After the spermatozoon was pushed forward until its head was near the tip of the injection pipette, the pipette was advanced mechanically until its tip almost reached the opposite side of the oocyte's cortex. The oolemma was broken by applying 1 or 2 Piezo pulses and the entire spermatozoon was expelled into the ooplasm before the pipette was gently withdrawn. All procedures were performed at 16-17ºC. Usually it took 10 to 20 minutes to inject spermatozoa into a group of 5 to 10 oocytes. Injected oocytes were kept at 16-17°C for about 20 minutes, and were then kept at room temperature (25°C) for about 10 minutes before further incubation at 37°C.

Round spermatids were easily recognized by their small size and a centrally located chromatin mass (Ogura and Yanagimachi, 1993) (Fig. 1). When a single spermatid (around 10 μm in diameter) was sucked into an injection pipette (5 μm I.D. at its tip) (Fig. 2), its smooth cell surface gradually became 'rough' in most instances. Perhaps the delicate plasma membrane of the spermatid is damaged by the sharp edge of the pipette tip and/or friction against its inner wall. The whole spermatid, with or without 'disintegrating' plasma membrane, was immediately injected into an oocyte as described above. In a series of experiments, a spermatid was drawn in and out of an injection pipette (4 μm I.D.) repeatedly until the plasma membrane was completely ruptured and the spermatid nucleus became almost completely separated from the cytoplasm (Fig. 3). The nucleus and the bulk of the cytoplasm were then injected into an oocyte. For spermatid injection we selected oocytes with completely or extensively degenerate first polar bodies. In this way true second polar body and 'pseudo-second' polar body of spermatid origin could be distinguished from the first polar body.

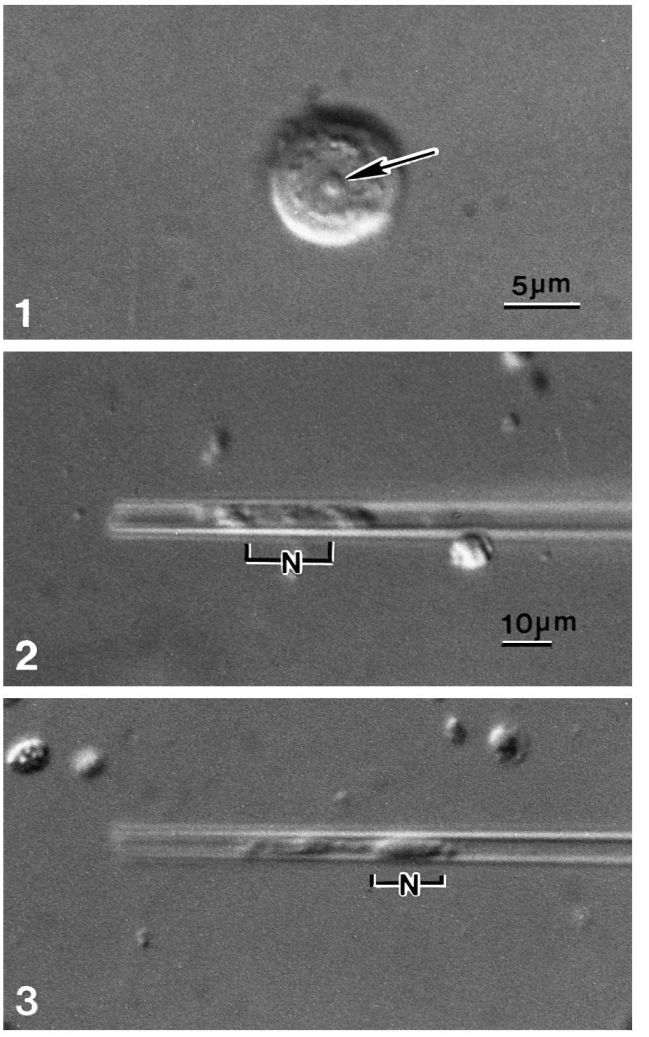

Fig. 1. A round spermatid. An arrow indicates a centrally located chromatin mass within the nucleus.
Fig. 2. A spermatid drawn into an injection pipette, showing spermatid nucleus (N) surrounded by cytoplasm.
Fig. 3. Same as above, but the nucleus (N) was separated from the bulk of cytoplasm by drawing in and out of a pipette repeatedly.

When the spermatozoon with a long tail was injected, the introduction of a fairly large quantity (about 6 pl) of medium into the oocyte was unavoidable. Therefore in such cases as much medium as possible was retrieved before pipette withdrawal. During spermatid injection, by contrast, only 1-2 pl of medium was introduced, obviating the need for any medium retrieval before pipette withdrawal.

Electrical activation of oocytes injected with spermatids

Mouse oocytes injected with mature epididymal spermatozoa were activated without further treatment. Since the introduction of the medium alone did not activate the oocytes, it must be the spermatozoa that do this (Kimura and Yanagimachi, 1995). In contrast to spermatozoa, spermatids were unable to activate oocytes, and therefore pre- or postinjection stimulation of the oocytes was

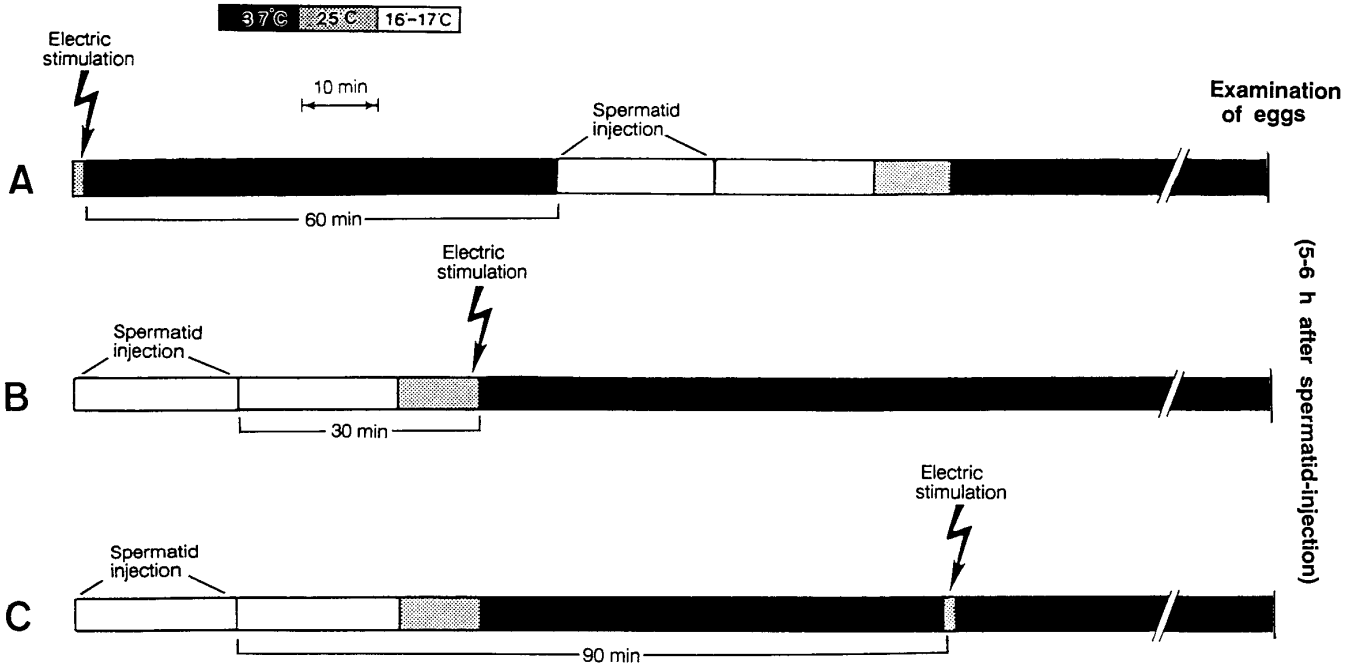

Fig. 4. Three protocols of spermatid injection. (A) A group of 5-10 oocytes was first electro-stimulated at 25°C. After 60 minutes incubation at 37°C, eggs were injected with a single spermatid each at 16-17°C. It took 10-20 minutes to inject 5-10 eggs. After the last egg was injected, the eggs were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

necessary for zygote development. Stimulation was achieved by applying a single DC electric pulse (1 kV/cm, 128 μseconds) to a group of spermatid-injected oocytes (usually about 10 at a time) in Hepes-CZB at 25°C.

Since the incidence of normal fertilization following spermatid injection was initially low (Table 1), experiments were conducted to determine whether the electric pulse should be given to the oocytes before or after spermatid injection in order to obtain the highest rate of normal fertilization (for definition of fertilization, see below). Three experimental protocols are shown diagrammatically in Fig. 4. In protocol A, the oocytes were stimulated first. When stimulated oocytes reached telophase of the second meiosis, 1 hour after incubation at 37ºC, each was injected with a spermatid nucleus as described above. In protocols B and C, the oocytes were first injected with spermatid nuclei, then electro-stimulated either 30 minutes (B) or 90 minutes later (C).

Culture and examination of zygotes

After injection, oocytes were incubated in CZB medium under mineral oil in a plastic Petri dish at 37°C under 5% CO_2 in air for 5-7 hours, then examined using an inverted microscope with an ×10 objective. Oocytes with two large pronuclei and one second polar body (2PN + 1Pb_2) were considered 'fertilized normally.' Eggs with distinct signs of degeneration were discarded. The eggs injected with spermatids often had one large pronucleus and the second polar body (1PN + 1Pb_2). Sometimes a minute pronucleus-like structure was visible within a live egg. Some of these eggs were fixed and stained (Yanagida et al., 1991) for examination of cytological details.

The eggs with two distinct pronuclei and the second polar body as well as those with a single pronucleus and the second polar body (with or without a minute pronucleus-like structure) were cultured continuously in CZB for up to 120 hours after sperm/spermatid injection.

Embryo transfer to foster mothers

Two-cell embryos that had developed from normally fertilized (2PN + 1 Pb_2) or aberrantly fertilized eggs (1PN + 1Pb_2) were selected at random. On average, 9 embryos (range 5-12) were transferred into the oviducts of each recipient female (Swiss-Webster, albino) as described previously (Kimura and Yanagimachi, 1995). Mothers were allowed to deliver and raise their own pups (red eyes and white coat) as well as foster pups (black eyes and grey/brown/black coat). Some of their own pups were removed during lactation, but all foster pups were allowed to develop and mature. In the experiments using spermatids, about half of the oocytes were each injected with 'undenuded' nuclei, and half were injected with 'denuded' nuclei. Since there was no significant difference in the results between these two experiment groups, data obtained were combined in tabulation.

RESULTS

Oocyte activation following testicular sperm/spermatid injection

Table 1 summarizes the results of experiments in which a single testicular spermatozoon or round spermatid was injected into individual oocytes. Testicular spermatozoa activated the majority of the oocytes when injected, and no additional stimulation was needed. By 5-7 hours after injection most oocytes had extruded the second polar body and had two large pronuclei (2PN + 1Pb_2) (Exp. 1 in Table 1). Spermatids similarly injected, on the contrary, did not activate the oocytes. None of 46 live oocytes examined between 5 and 7 hours after injection were activated, being arrested at metaphase II (Exp. 2 in Table 1). When these oocytes were fixed and examined, the majority (65%) contained spermatid nuclei exhibiting premature chromosome condensation. Of the others 22% contained intact or almost intact spermatid nuclei, 4% contained a highly condensed chromatin mass, and the

Table 1. Fertilization of mouse oocytes by testicular spermatozoa and round spermatids: examined in living condition 5-7 hours after injection

Exp.	Type of cell injected (no. exp.)	Total no. of oocytes injected	Post-inject. application of electric stimulation†	Oocytes survived (%)	No. (%) of surviving eggs with*			
					MetII	2PN+1Pb$_2$	1PN+1Pb$_2$	Others‡
1	Testicular spermatozoa (9)	191	–	176 (92)	5 (3)	165 (94)§	1 (0.5)	5 (3)
2	Spermatid (3)	50	–	46 (92)	46 (100)	0	0	0
3	Spermatid (15)	462	+	395 (85)	12 (3)	145 (37)¶	184 (47)	54 (14)

*Abbreviations: MetII, metaphase of second meiotic division; 1PN, one pronucleus; 2PN, two pronuclei; 1Pb$_2$, one second polar body.
†(+) Oocytes were electro-stimulated about 30 minutes after spermatid injection; (–) not stimulated.
‡Others include eggs with 1-4 pronuclei without second polar body and those with condensed or slightly decondensed sperm heads.
§,¶Difference between § and ¶ is significant (P<0.001, χ^2 test).

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

Exp. protocol (see Fig. 4)	State of spermatid nuclei during injection	Total no. of oocytes injected (no. exp.)	Oocytes survived (%)	No. (%) of surviving eggs with*					
				2PN+1Pb$_2$	1PN+sPN+1Pb$_2$	1PN+1Pb$_2$+condensed or intact spermatid nucleus	2PN+2Pb$_2$	3PN+1 or 2Pb$_2$	Others†
A	‘Undenuded’	53 (5)	51 (96)	7 (14)§	34 (67)	8 (16)	0 (0)	0 (0)	2 (4)
	‘Denuded’	46 (5)	44 (96)	34 (77)‡	6 (14)	3 (7)	0 (0)	0 (0)	1 (2)
B	‘Undenuded’	51 (5)	44 (86)	12 (27)§	16 (36)	11 (25)	1 (2)	0 (0)	4 (9)
	‘Denuded’	40 (5)	34 (85)	16 (47)§	1 (3)	2 (6)	3 (9)	3 (9)	9 (27)
C	‘Undenuded’	50 (5)	41 (82)	8 (20)§	6 (15)	11 (27)	10 (24)	1 (2)	5 (12)
	‘Denuded’	44 (5)	41 (93)	7 (17)§	1 (2)	1 (2)	15 (37)	8 (20)	6 (15)

*Abbreviations: PN, pronucleus; sPN, small pronucleus; Pb$_2$, second polar body. The number before PN or Pb$_2$ denotes the number of pronucleus or second polar body in each egg. 2Pb$_2$, for example, means two second polar bodies instead of the normal one polar body.
†Others include the oocytes without or unidentifiable, nuclear materials.
‡,§Difference is significant between ‡ and § (χ^2 test, P<0.01).

remainder had unidentifiable or missing spermatid nuclei. In contrast, spermatid nuclei developed into large male pronuclei when the oocytes were electro-stimulated after injection (Exp. 3 in Table 1).

An egg with two large pronuclei and one second polar body (2PN + 1Pb$_2$) was classified as ‘fertilized normally.’ Since the rate of normal fertilization following round spermatid injection was considerably lower (37%) than that using testicular spermatozoa (94%) in the above experiments (Table 1), attempts were made to increase the rate of normal fertilization following spermatid injection. In the first experiments, a spermatid nucleus was injected into an oocyte accompanied by the bulk of cytoplasm (Fig. 2) with injection either before or after electrical stimulation as illustrated in Fig. 4. In the second series of experiments the spermatid nucleus was separated from the bulk of cytoplasm before its injection (Fig. 3). The results, summarized in Table 2, show that the highest rate (77%) of normal fertilization (2PN + 1Pb$_2$; Fig. 5A,B) was obtained when the oocytes were first electro-stimulated, then injected 1 hour later with ‘denuded’ nuclei (protocol A, ‘denuded’). Even under this condition, however, not all the eggs were fertilized normally. Twenty-one percent contained only one large (female) pronucleus (Fig. 6A), together with either a small pronucleus of spermatid origin (Fig. 6B) or a spermatid nucleus that was still almost intact or condensed.

Development of zygotes

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

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

We also transferred into each of six foster mothers 6-12 two-cell embryos that had developed from aberrantly fertilized eggs (1PN +1Pb$_2$). Four foster mothers gave birth to a total of 5 foster pups in addition to their own pups. One of the foster pups

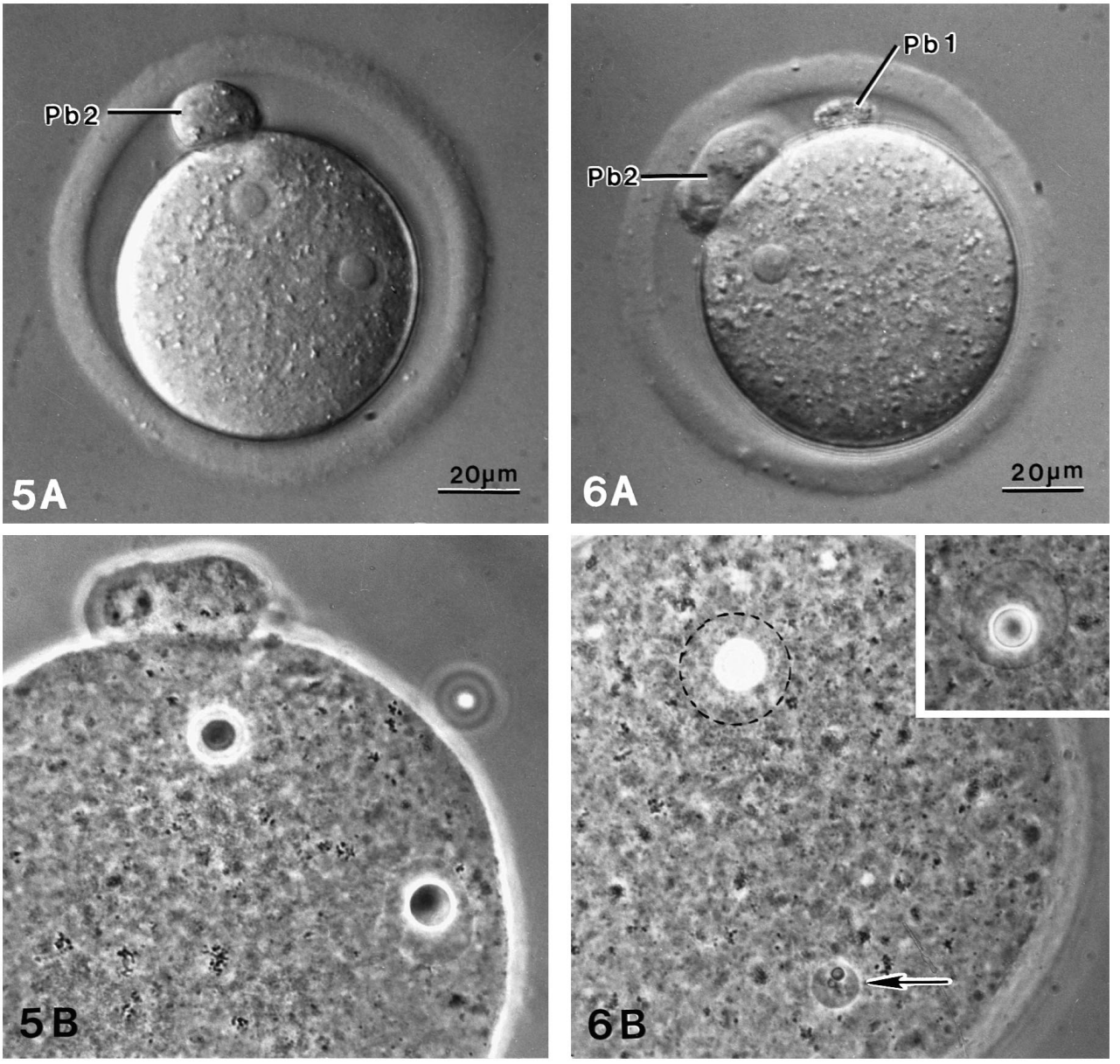

Fig. 5. (A) An egg fertilized normally after spermatid injection, with the second polar body (Pb_2) and two large pronuclei; photographed with the interference-contrast microscope. The first polar body of this egg had disintegrated and disappeared prior to spermatid injection. The pronucleus closer to the polar body is believed to be the female pronucleus. (B) Same as A, but flattened under a coverslip, fixed and stained before being photographed with the phase-contract microscope.

Fig. 6. (A) An egg with one large pronucleus and two polar bodies, photographed with the interference-contrast microscope. Pb_1, the degenerate first polar body; Pb_2, the second polar body. (B) Same as A, but flattened under a coverslip, fixed and stained before being photographed with the phase-contrast microscope. A small pronucleus of spermatid origin (arrow) is visible. Inset – the egg pronucleus which was out of focus in the area indicated by a dotted circle.

was alive and looked normal on the day of birth, but was eaten by the mother sometime before the morning of the next day. The remaining foster pups grew into normal adults (1 male and 3 females).

DISCUSSION

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

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

Eggs fertilized by	Fertilized eggs with (protocol)	Total no. of eggs cultured (no. exp.)	No. (%) of eggs developed into			
			2-cell	4-cell	Morula	Blastocyst
Testicular spermatozoa	2PN+1Pb$_2$	137 (7)	135 (99)	134 (98)	122 (89)	96 (70)
Spermatid 'denuded' nuclei	2PN+1Pb$_2$ (Protocol A)	73 (6)	73 (100)	73 (100)	69 (95)	61 (84)
Spermatid 'undenuded' nuclei	2PN+1Pb$_2$ (Protocol B)	82 (9)	79 (96)	74 (90)	62 (76)	41 (50)
Spermatid 'denuded' nuclei	1PN+1Pb$_2$ (Protocol B)	57 (8)	56 (100)	42 (74)	21 (37)	4 (7)
Not fertilized*	1PN+1Pb$_2$	53 (3)	53 (100)	51 (96)	38 (72)	1 (2)

*Unfertilized oocytes were stimulated with electric current only.

Table 4. Offspring from eggs fertilized normally (2PN+1Pb$_2$) after sperm/spermatid injection

Embryos developed from eggs fertilized by	No. of recipients	Total no. of foster 2-cell embryos transferred	No. of live offspring		
			Recipient's own [albino]	Foster [black/gray] (%)*	Sex of foster offspring
Testicular spermatozoa	5	44	34	24 (54.5)‡	15M, 9F
Spermatids	15†	131	137	37 (28.2)§	16M, 19F, 2?¶

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

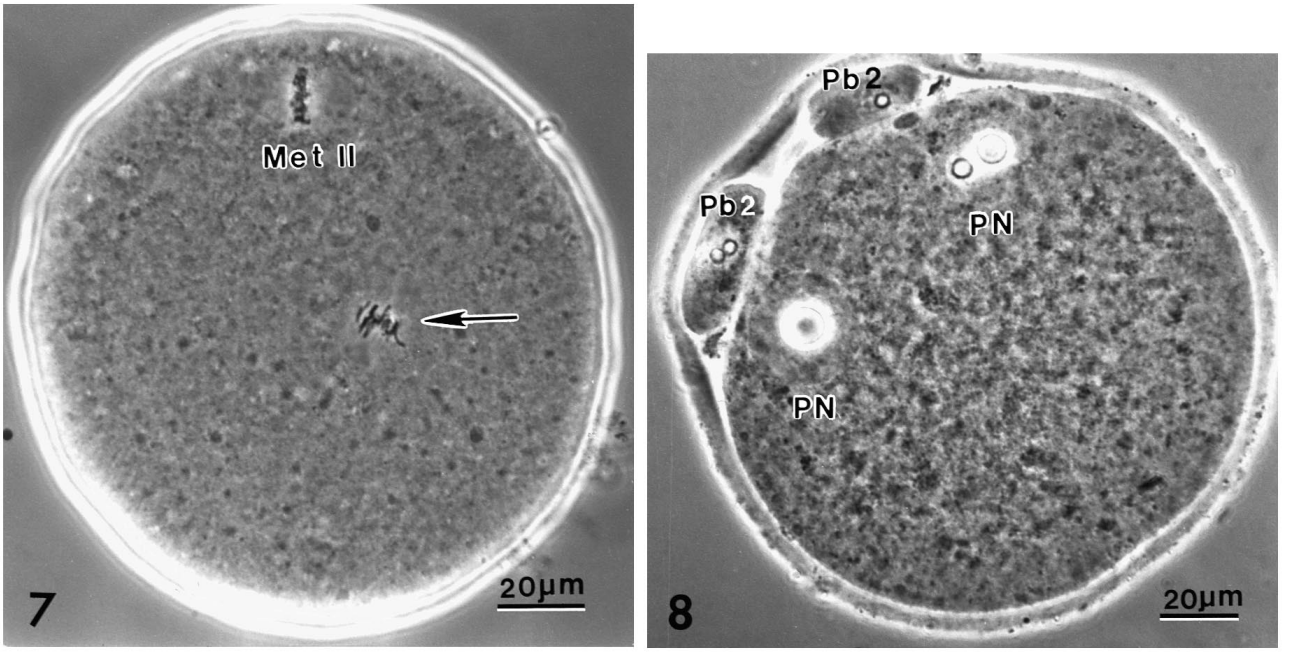

Fig. 7. An unactivated oocyte, 1 hour after spermatid injection. This spermatid nucleus (arrow) had undergone premature chromosome condensation; its chromosomes are associated with a spindle. Met II, oocyte's metaphase II chromosomes.
Fig. 8. An egg with two polar bodies (Pb$_2$) and two large pronuclei (PN). This egg was injected with a spermatid then electro-activated 90 minutes later. One of two PN-Pb$_2$ pairs is believed to be of spermatid origin (see Fig. 9 IV).

testis, it is clear that spermatozoa produced in the testes of mice and men do not need to be exposed to the extra-testicular duct system to support normal development.

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

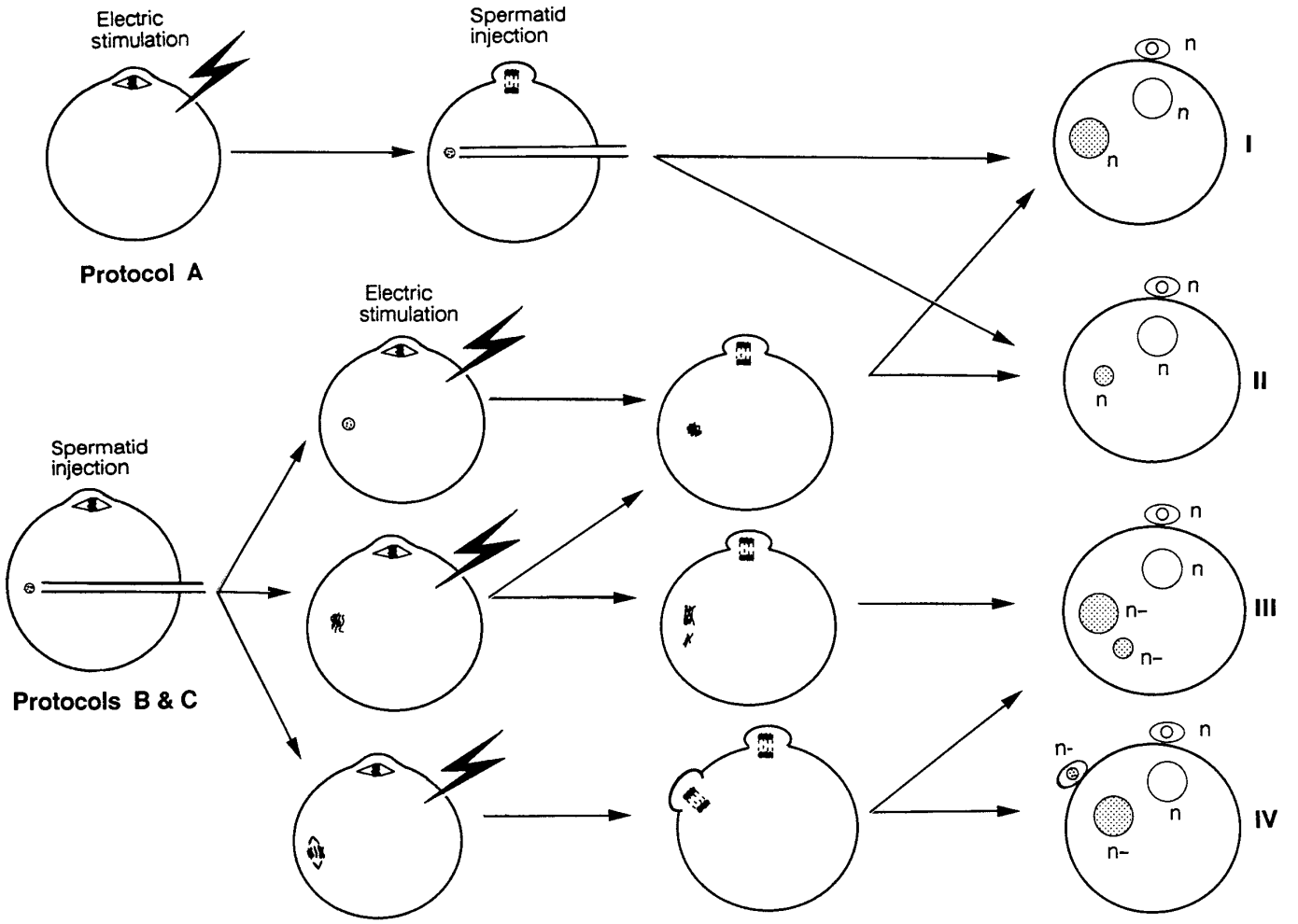

Fig. 9. Diagrams illustrating possible pathways leading to the production of various forms of zygotes after spermatid injection (zygotes with 'intact' spermatid nuclei or highly condensed spermatid nuclei are not included in this diagram). When oocytes were electro-activated prior to spermatid injection (protocol A), some became zygotes with two large pronuclei and one second polar body (2PN + 1Pb$_2$) (I), while in others the spermatid nucleus failed to develop into a large pronucleus (II). The incidence of normal fertilization (I) was high when spermatid nuclei had been 'denuded' immediately before injection. When spermatid-injected oocytes were left unactivated for 30-90 minutes (protocols B and C), many spermatid nuclei underwent premature chromosome condensation, and the chromosomes sometimes spread. Association of microtubules to chromosomes increased with time. When such oocytes were electro-activated, three or more pronuclei (III) or two second polar bodies (IV) were formed. Spermatid pronuclei and the pseudo second polar bodies thus formed must have fewer chromosomes (n–) than the normal haploid number (n) of chromosomes. If the spermatid nucleus did not undergo premature chromosome condensation or prematurely condensed spermatid chromosomes did not disperse and were not associated with microtubules, the nucleus transformed into a large (I) or small (II) spermatid pronucleus.

1992; Razin and Kafri, 1994). Imprinting is a mark that differentiates between alleles of paternal and maternal origin and regulates their expression. Recent studies have revealed that specific chromosome regions are imprinted (Cattanach and Jones, 1994), but so far only a few genes have been shown to be involved (Razin and Cedar, 1994). Moreover, as yet gametic imprinting has been proved only for mouse *Igf2r* and *Xist* genes (Brandeis et al., 1993; Stoger et al., 1993; Zuccotti and Monk, 1995).

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

Although the studies cited above strongly support the notion that methylation represents the primary imprint, the precise chronology of gametic imprinting is not well understood. Injection experiments as reported here do not provide any information about imprinting of particular genes or chromosome regions, but they demonstrate conclusively whether genomic imprinting in male germ cells is complete or incomplete. It is of course possible that different genes are imprinted at different stages of gametogenesis and through different mechanisms. However, it is equally possible that different genes are imprinted simultaneously or within a rather short

time. All we can say at present is that, in the mouse, imprinting of spermatogenic cells is complete before spermiogenesis begins. According to Ariel et al. (1994), the methylation pattern of certain genes changes even after transport of testicular spermatozoa to the epididymis. Obviously, these changes are not essential for embryonic development based on the results of our experiments. We are now injecting oocytes with male germ cells at earlier stages of their differentiation, with a view of discovering whether imprinting can occur even within the cytoplasm of maturing oocytes, or whether it is possible only within intact spermatogenic cells.

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

It is evident that testicular spermatozoa, like mature epididymal spermatozoa, contain an oocyte-activating factor. When injected into oocytes, testicular spermatozoa activated the oocytes and their nuclei transformed into (male) pronuclei. Round spermatids are lacking in the oocyte-activating factor and therefore oocytes injected with round spermatids remained unactivated. Since the nucleus of a spermatid is in the G_2 phase of the cell cycle, it undergoes premature chromosome condensation within an unactivated oocyte in the metaphase of the cell cycle. The spermatid nucleus developed into a large pronucleus only when the oocyte was activated by artificial means (e.g. electrical stimulation). Not surprisingly, the timing of spermatid injection and oocyte activation was critical for normal development of pronuclei. When sperm-injected oocytes were left unactivated for an extended period of time, microtubules/spindle associated with prematurely condensed spermatid chromosomes (Fig. 7) (Harrouk and Clarke, 1993). When such oocytes were activated, either some spermatid chromosomes were shed into the pseudo-second polar body (Fig. 8) or two or more spermatid pronuclei were formed, each containing fewer chromosomes than the normal pronucleus. Fig. 9 illustrates the probable behavior (fate) of spermatid nuclei after injection into oocytes.

The reason for the low rate of normal fertilization (2PN + $1Pb_2$) following injection of cytoplasm-encapsulated spermatid nuclei is not clear, but it may be due in part to the persistence of the spermatid's cytoplasm around each nucleus for some time after injection. As expected, the zygotes with only one large pronucleus seldom developed into normal blastocysts and into live offspring. In fact, it was rather surprising that any could do so. Some of the living eggs we identified as 1PN + $1Pb_2$ zygotes in living conditions must have had a minute pronucleus of spermatid origin. It is unknown at present whether such a small spermatid pronucleus remains small or becomes larger, but meeting/union of a small spermatid pronucleus with a large female pronucleus appears to be possible (see Fig. 6 of Ogura et al., 1993). Ogura et al. (1994), who electrofused round spermatids with mouse oocytes, transferred all 2-cell embryos to foster mothers regardless of the nuclear status of the eggs. Whereas some of them could have been developing parthenogenetically, others could have developed from the eggs with a small spermatid pronucleus. This may explain the low yield of live offspring in the experiments conducted by Ogura et al. (6 pups were born after transferring a total of 346 two-cell embryos to 8 foster mothers). If they had selected and transferred only the embryos developed from the eggs with two large pronuclei and the second polar body (2PN + $1Pb_2$), the pregnancy rate may have been much higher. Our recommended procedure of intracytoplasmic spermatid injection is as follows: (a) activate oocytes first (e.g. by electric pulse); (2) inject a single spermatid nucleus into each oocyte when the eggs have reached telophase II; (3) immediately before injection, free the spermatid nuclei from the bulk of the surrounding cytoplasm. Technically, mechanical injection of spermatids into oocytes, as reported here, is simpler and quicker than electrofusion as employed by Ogura et al. (1994). Recently, Vanderzwalmen et al. (1995) succeeded in fertilizing human oocytes by mechanically injecting spermatids. Sofikitis et al. (1994) obtained live rabbit young after mechanical injection of spermatids into oocytes.

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