Full-Term Development of Mouse Eggs Transplanted With Male Pronuclei Derived From Round Spermatids: The Effect of Synchronization Between Male and Female Pronucleus on Embryonic Development

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ABSTRACT Pronucleus transplanted mice have been produced, but their donor male pronuclei were derived from mature sperm and were completely synchronous with female pronuclei because both male and female pronuclei came from the same fertilized oocyte. The present study firstly produced male pronuclei by introducing round spermatids into enucleated mouse oocytes, then transferred the male pronuclei into mouse oocytes at three activation stages and finally compared the effect of three kinds of oocytes on the development of reconstructed embryos. Our results indicate that, in enucleated oocytes, mouse round spermatid nuclei can transform to male pronuclei in a higher proportion, and the synchronization between male and female pronucleus does not significantly influence the early cleavage but the later and full-term development of reconstructed embryos. *Mol. Reprod. Dev. 71: 439–443, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: mouse; round spermatid; pronucleus transplantation; embryonic development

INTRODUCTION

The microfertilization technique has enabled us to use immature male germ cells (spermatogenic cells) as substitute gametes. Normal offspring have been born after microfertilization with round spermatids in mouse (Ogura et al., 1994; Kimura and Yanagimachi, 1995a; Liu et al., 1997), rabbit (Sofikitis et al., 1994), human (Tesarik et al., 1995), and hamster (Haigo et al., 2004), and with secondary spermatocytes in mouse (Kimura and Yanagimachi, 1995b). Although pronucleus transplanted mice have also been produced (McGrath and Solter, 1983), the donor pronuclei in McGrath and Solter's study were derived from mature sperm, and the male and female pronuclei from an in vitro fertilized oocyte were entirely transplanted into another fertilized oocyte which was pre-enucleated, therefore the male and female pronuclei were completely synchronous. Previous investigations (Barton et al., 1984; Surani et al., 1986) in mice additionally demonstrated that maternal genome was relatively more important for the development of early embryos while the paternal genome was crucial for the proliferation of extraembryonic tissues, and only if the pronucleus in recipient oocyte was of parental type opposite to the donor pronucleus, the reconstructed egg could develop to term.

In a previous study, we have demonstrated that the male pronucleus from a polyspermy mouse oocyte could integrate with female pronucleus and mouse reconstructed eggs underwent normal mitosis. Moreover, some embryos could develop to the blastocyst stage and seven offsprings were born after embryo transfer (our unpublished data). We hereby designed an experiment in order to determine whether mouse round spermatids could effectively transit to male pronuclei in enucleated oocytes and support embryo development after transferring the round spermatid-derived male pronucleus into mouse oocytes. A much more interesting question is whether the male pronuclei derived from mouse round spermatid can combine with the oocytes at three activation stages, herein namely asynchronous (metaphase II), semi-synchronous (pre-activated for two hours), or synchronous (haploid parthenogenetic) oocytes, to support the full-term development of mouse embryos. Therefore, we also tested whether the synchronization between male and female pronucleus was absolutely necessary to the full-term development of mouse embryos.

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MATERIALS AND METHODS

Animals

Kunming female (a white strain) and C57BL/6 male (a black strain) mice, 2–4 months old, were used in this study. They were kept in an air-conditioned room (23°C, 50% relative humidity) under 14-hr light and 10-hr dark cycles.

Preparation of Spermatids

To collect spermatogenic cells, the seminiferous tubules of a C57BL/6 male mouse were minced as described previously (Kimura and Yanagimachi, 1995a; Liu et al., 1997), and the cells were suspended in 0.9% NaCl solution. The round spermatids used in this study were immature haploid cells that had a decondensed nucleus. One aliquot of spermatogenic cell suspension was mixed with ~10 aliquots of HEPES–CZB medium containing 12% (w/v) polyvinylpyrrolidone (PVP, Mr 360 kDa; Sigma) in a micro-manipulation chamber.

Collection of Oocytes

Mature oocytes were collected from the oviducts of Kunming female mice that had been induced to superovulate with 7.5IU pregnant mare serum gonadotropin (PMSG, Tianjin Animal Hormone Factory), followed by 7.5IU human chorionic gonadotropin (hCG, Ningbo Animal Hormone Factory) 48 hr later. Oocytes were collected from oviducts approximately 13–14 hr after hCG injection, placed in CZB medium (Chatot et al., 1989), and treated with 0.1% hyaluronidase in order to disperse cumulus cells. The oocytes were then placed in CZB medium with 5 mg/ml BSA, covered with paraffin oil, and stored at 37°C (5% CO_2/air).

Preparation of Male Pronucleus

Preparation of haploid male pronucleus was undertaken by intracytoplasmic injection with piezo-driven micromanipulator (Kimura and Yanagimachi, 1995a; Zhou et al., 2000). Oocytes were firstly enucleated with an 8–10 μm (I.D.) blunt glass pipette, then activated by Ca^{2+}-free CZB medium containing Sr^{2+} for 20 min (Kishigami et al., 2004), and finally injected with mouse round spermatids within 2 hr (Fig. 1). After culturing in fresh CZB medium for about 4 hr, the formation of mouse male pronucleus was determined.

Transplantation of Male Pronucleus Into Mouse Oocytes at Three Activation Stages: The Design for Male Pronucleus Transplantation (shown as in Fig. 1)

Experiment 1 (Exp 1): metaphase II mouse oocytes as recipients. Pre-constructed male pronuclei were introduced into the unactivated (asynchronous) oocytes by intracytoplasmic injection with an 10–12 μm (I.D.) blunt glass pipette as reported previously, the reconstructed oocytes were then activated with 10 mM Sr^{2+} in Ca^{2+}-free CZB medium (Bos-Mikich et al., 1995) for 4 hr, and finally cultured in fresh CZB medium at 37°C (5% CO_2/air).

Experiment 2 (Exp 2): pre-activated metaphase II mouse oocytes (semi-synchronous) as recipients. The oocytes were pre-activated with 10 mM Sr^{2+} for 2 hr, then the pre-constructed male pronuclei

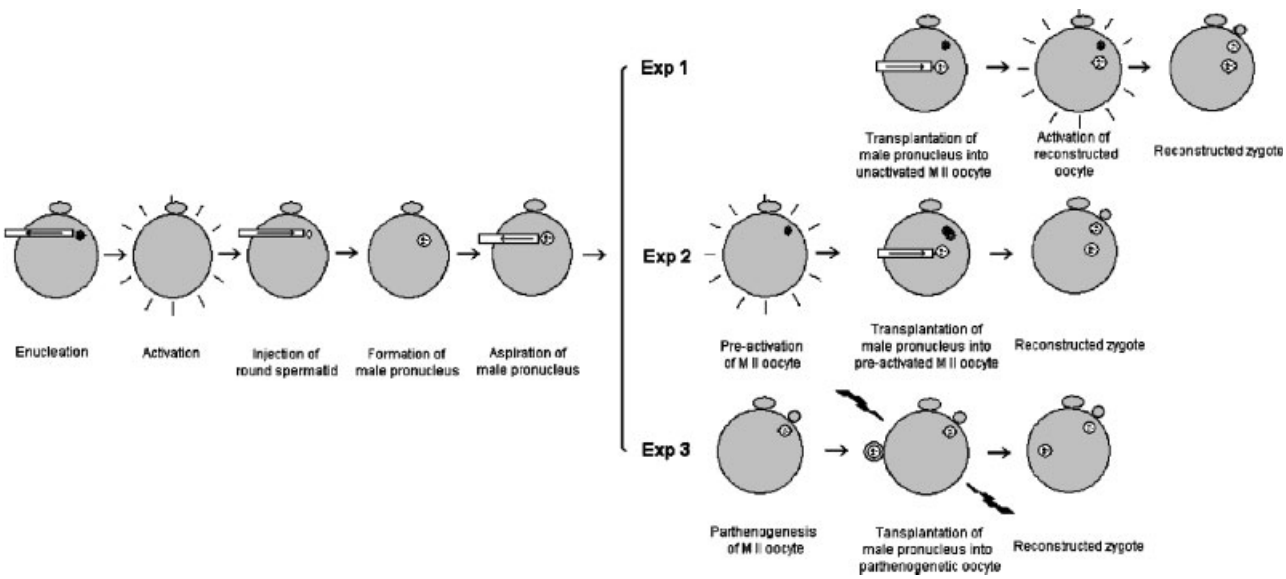

Fig. 1. Schemes of male pronucleus transplantation into different recipient oocytes. When preparing the haploid male pronucleus, the oocytes were firstly enucleated, then activated, and finally injected with round spermatids. After formation of the male pronuclei, they were transplanted into different recipient oocytes at three activation stages (as shown in Exp 1, 2, and 3). In Exp 1, pre-constructed male pronuclei were introduced into the unactivated oocytes and then the reconstructed oocytes were activated with Sr^{2+} for 4 hr; in Exp 2, the oocytes were pre-activated with Sr^{2+} for 2 hr, then the pre-constructed male pronuclei were introduced into the oocytes; in Exp 3, the oocytes were activated with Sr^{2+} for 4 hr, then the pre-constructed male pronuclei were introduced into the haploid parthenogenetic oocytes by electrofusion.

were introduced into them, and finally the transplanted mouse oocytes were cultured in CZB medium.

Experiment 3(Exp 3): haploid parthenogenetic mouse oocytes (synchronous) as recipients. The oocytes were activated with Sr^{2+} for 4 hr. The pre-constructed male pronuclei were then introduced into the haploid parthenogenetic oocytes by electrofusion as reported previously with a little modification (Ogura et al., 1997). By applying an electric pulse (1–2 kV/cm, 20 msec) in fusion medium (300 mM mannitol, 0.1 mM $MgSO_4$, and 0.5 mg/ml polyvinyl alcohol), the oocyte–pronucleus pairs fused. The reconstructed mouse oocytes that fused with male pronuclei were further cultured in fresh CZB medium.

Embryo Transfer

Embryos reaching the morula/blastocyst stage 96 hr after activation were transferred into the uteri of day 3 pseudo-pregnant Kunming White females (day 1 was the day after mating with vasectomized males). Forty-eight hours after culture, 4- to 8-cell embryos were transferred into the oviducts of day 1 pseudopregnant females. The uteri of pseudo-pregnant females were examined for fetuses and implantation sites on day 19 or 20.

Statistical Analysis

Data were analyzed by using Chi-square (χ^2) test. Difference was significant when P value was less than 0.05.

RESULTS

Formation of Male Pronucleus (MP) After Transplantation of Mouse Round Spermatids Into Enucleated Mouse Oocytes

Round spermatid nuclei introduced into metaphase II oocytes could transform to male pronuclei in enucleated oocytes and the percentage of male pronucleus formation was 81% (1812/2237, as shown in Table 1). The shape and size of MP (Fig. 2A) in enucleated oocytes were also similar to those in round spermatid injection (ROSI) oocytes. These results suggest that most mouse round spermatids in the enucleated oocytes can transform to normal MPs.

TABLE 1. Formation of Male Pronuclei Derived From Mouse Round Spermatids

Number of oocytes	Number of enucleated oocytes (%)	Number of ROSI oocytes (%)	Number of ROSI oocytes with male pronucleus (%)
2,644	2,380 (90.01)	2,237 (93.99)	1,812 (81.00)

Percentages of 2PN and Embryonic Development of Pronucleus-Transplanted Mouse Eggs in Exp 1, Exp 2, and Exp 3, Respectively

Percentage of 2PN formation in Exp 1 was not significantly different from that in Exp 2 and Exp 3. As shown in Table 2, most embryos (81.07% vs. 84.92% vs. 90.65%, $P > 0.05$) developed to two-cell stage after cultured for 24 hr, and the development rates of 4–8-cell embryos among three experiments were not significantly different (67.96% vs. 65.92% vs. 71.03%, $P > 0.05$), while the rates of blastocyst formation were 16.02%, 29.05%, and 41%, respectively ($P < 0.05$). It suggests that the synchronization between male and female pronuclei does not significantly influence the 2PN formation and early cleavage but later development of mouse reconstructed embryos.

Birth of Pups After Embryo Transfer

In Exp 1, none of 17 foster females gave birth to a pup. Nevertheless, in Exp 2, one of 19 fosters gave birth to four pups, including two males (one black and one agouti) and two females (all black); one of 20 fosters gave birth to two offspring (both black females) in Exp 3. Moreover, all pups had black eyes and pigmented coat (Fig. 2H).

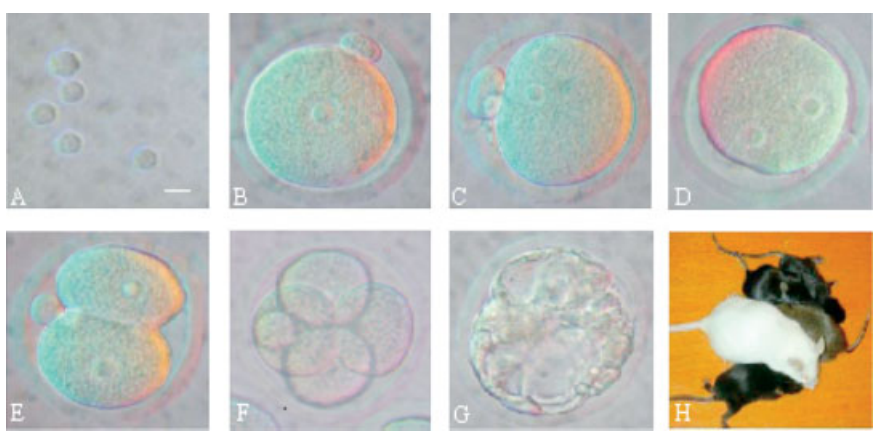

Fig. 2. Morphology of mouse round spermatids (**A**), male proucleus (**B**) derived from round spermatid and parthenogenetic female pronucleus (**C**), and full-term development of male pronucleus-transplanted mice (**D**, zygote with a male and a female pronucleus; **E**, 2-cell embryo; **F**, 4-cell embryo; **G**, blastocyst; **H**, the foster and pups). Scale bar = 10 μm.

TABLE 2. Development of Mouse Reconstructed Oocytes After Male Pronucleus Transplantation Into Different Oocyte Recipients

Type of recipient oocytes	Site of embryo transfer	No. of eggs with two pronuclei	No. (%) of embryos that developed to			No. of recipient females	No. of pregnant recipients (pups)
			2-cell	4–8-cell	Morula/ blastocysts		
Unactivated	Uterus	206	167(81.07)[a]	140(67.96)[a]	33(16.02)[a]	3	0(0)
	Fallopian tube	387	314	263	–	14	
Pre-activated	Uterus	179	152(84.92)[a]	118(65.92)[a]	52(29.05)[b]	5	1(4)
	Fallopian tube	432	367	285	–	14	
Parthenogenetic	Uterus	107	97(90.65)[a]	76(71.03)[a]	44(41)[b]	4	1(2)
	Fallopian tube	472	428	335	–	16	

Values with different superscripts within a column differ significantly ($P < 0.05$).

Donors of round spermatid nuclei were C57BL/6 (black coat) and foster mothers were inbred Kunming mice (white coat), which had never been exposed to pigmented males, therefore, the offspring should be derived from the reconstructed oocytes which were introduced with C57BL/6 male pronuclei. All pups presently grew normally.

DISCUSSION

During normal fertilization, the formation of male pronucleus involves in three basic processes including the breakdown of sperm nuclear envelope, the decondensation of chromatin, and the development of pronucleus envelope (Longo, 1997); the related study demonstrated that in anucleate oocytes before germinal vesicle breakdown (GVBD), the first and second steps were independent of, and the last step was dependent on germinal vesicle karyoplasms (Balakier and Tarkowski, 1980). Previous observations (Iwamatsu and Chang, 1972; Barros and Munoz, 1973; Niwa and Chang, 1975; Binor and Wolf, 1979) also suggested that the ability of intact oocytes to fertilize increased from the GV stage onwards and reached its maximum at metaphase II, and the fertilization rate of enucleated oocytes was positively correlated with the progression of maturation, i.e., with the stage at which the oocytes were enucleated. In order to ensure that the work shall be done in the right way, we selected enucleated metaphase II mouse oocytes as recipients of round spermatid nuclei, and pre-activated the enucleated oocytes with Ca^{2+}-free CZB containing 10 mM Sr^{2+} for 20 min before round spermatid injection. The percentage of male pronucleus was more than 81%, similar to the fertilization rate of ROSI eggs pre-activated with Sr^{2+} (our unpublished data), but higher than that of eggs which were post-activated with Sr^{2+}. This study demonstrates that mouse round spermatids become competent like mature sperm to be transformed to pronuclei in enucleated MII oocytes, and that pre-activation with Sr^{2+} for 20 min possibly benefits for the formation of male pronuclei and the development of embryos.

After fertilization, the male and female pronucleus generally migrates towards each other, resulting in the apposition of the 2PN. Once adjacent, the pronuclei become associated in a characteristic fashion to establish the genome of the embryo and conclude the process of fertilization (Longo, 1997). Therefore, during in vivo or in vitro fertilization, the formation and development of male and female pronuclei is almost completely synchronous. In the present study, three schemes were designed to determine whether different oocyte recipients at three activation stages influenced the development of male pronucleus-transplanted embryos. We found that, although the percentages of PN formation were not significantly different, those of blastocyst formation were significantly different among three schemes (16.02%, 29.05%, and 41% in Exp 1, 2, and 3 respectively, as shown in Table 2). When unactivated M II oocytes were used as male pronucleus recipients, no pups were produced, while pre-activated and haploid gynogenetic oocytes were used as recipients, four and two healthy offspring were born, respectively. Our previous study about spindle rotation in mouse parthenogenetic oocytes (Zhu et al., 2003) have demonstrated that at 2 hr after activation, the oocytes are about to exit from anaphase II and enter telophase II while the daughter chromatids are drawn to the spindle poles; at 2–6 hr, oocytes exit from telophase II, the second polar body (Pb2) extrudes and follows by the formation of female pronucleus in which the nuclear material is linear chromatins. However, when unactivated metaphase II oocytes were used as recipients, the female pronucleus formed at about 4 hr after oocyte activation, which means the male pronucleus formation was ahead of female pronucleus at least 4 hr, therefore the male and female pronuclei could not normally associate with each other, and the reconstructed eggs could not normaly develop. The later development of reconstructed zygotes in Exp 2 was inferior to that in Exp 3, but there was not significant statistical difference between them ($P > 0.05$), and both groups brought about normal pups. It suggests that both pre-activated and haploid parthenogenetic oocytes have the ability to accept the male pronuclei derived from round spermatids and to integrate with the paternal chromosomes, but the unactivated oocytes have not this ability. Therefore, the

synchronization between male and female pronuclei seems essential to the later development of the reconstructed oocytes and the birth of pups.

Both foster mothers and recipients of male pronuclei are inbred Kunming females (white coat) and the donors of round spermatid are C57BL/6 male (black coat). Therefore, the phenotype of their pups should be agouti or black coat. The recipient Kunming females have never been exposed to pigmented males, so the offspring should be derived from the oocytes transplanted with male pronuclei. A total of six pups, one agouti and five blacks are produced in the present study, indicating that the phenotype of offspring is expressed in a natural manner without genetic mutation.

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REFERENCES

Balakier H, Tarkowski AK. 1980. The role of germinal vesicle karyoplasm in the development of male pronucleus in the mouse. Exp Cell Res 128:79–85.

Barros C, Munoz G. 1973. Sperm–egg interaction in immature hamster oocytes. J Exp Zool 186:73–78.

Barton SC, Surani MA, Norris ML. 1984. Role of paternal and maternal genomes in mouse development. Nature 311:374–376.

Binor Z, Wolf DP. 1979. In-vitro maturation and penetration of mouse primary oocytes after removal of the zona pellucida. J Reprod Fertil 56:309–314.

Bos-Mikich A, Swann K, Whittingham DG. 1995. Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. Mol Reprod Dev 41:84–90.

Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 86:679–688.

Haigo K, Yamauchi Y, Yazama F, Yanagimachi R, Horiuchi T. 2004. Full-term development of hamster embryos produced by injection of round spermatids into oocytes. Biol Reprod 71:194–198.

Iwamatsu T, Chang MC. 1972. Sperm penetration in vitro of mouse oocytes at various times during maturation. J Reprod Fertil 31:237–247.

Kimura Y, Yanagimachi R. 1995a. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 121:2397–2405.

Kimura Y, Yanagimachi R. 1995b. Development of normal mice from oocytes injected with secondary spermatocyte nuclei. Biol Reprod 53:855–862.

Kishigami S, Wakayama S, Nguyen VT, Wakayama T. 2004. Similar time restriction for intracytoplasmic sperm injection and round spermatid injection into activated oocytes for efficient offspring production. Biol Reprod 70:1863–1869.

Liu L, Sun QY, Duan CW, Liu H, Song XF, Qian JF, Chen DY. 1997. Subzonal fertilization of mouse round spermatids. Sci China C Life Sci 40:152–158.

Longo FJ. 1997. Fertilization. 2nd edn. Cambridge: Chapman & Hall, The Univ Press.

McGrath J, Solter D. 1983. Nuclear transplantation in mouse embryos. J Exp Zool 228:355–362.

Niwa K, Chang MC. 1975. Temporal relationship between insemination and sperm penetration in immature rats, with special reference to the effect of incubation of spermatozoa. Fertil Steril 26:1266–1272.

Ogura A, Matsuda J, Yanagimachi R. 1994. Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci USA 91:7460–7462.

Ogura A, Wakayama T, Suzuki O, Shin TY, Matsuda J, Kobayashi Y. 1997. Chromosomes of mouse primary spermatocytes undergo meiotic divisions after incorporation into homologous immature oocytes. Zygote 5:177–182.

Sofikitis NV, Miyagawa I, Agapitos E, Pasyianos P, Toda T, Hellstrom WJ, Kawamura H. 1994. Reproductive capacity of the nucleus of the male gamete after completion of meiosis. J Assist Reprod Genet 11:335–341.

Surani MA, Barton SC, Norris ML. 1986. Nuclear transplantation in the mouse: Heritable differences between parental genomes after activation of the embryonic genome. Cell 45:127–136.

Tesarik J, Mendoza C, Testart J. 1995. Viable embryos from injection of round spermatids into oocytes. N Engl J Med 333:525.

Zhou Q, Boulanger L, Renard JP. 2000. A simplified method for the reconstruction of fully competent mouse zygotes from adult somatic donor nuclei. Cloning 2:35–44.

Zhu ZY, Chen DY, Li JS, Lian L, Lei L, Han ZM, Sun QY. 2003. Rotation of meiotic spindle is controlled by microfilaments in mouse oocytes. Biol Reprod 68:943–946.