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Fate of genetically marked mitochondrial DNA from spermatocytes microinjected into mouse zygotes

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Summary

Cytoplasts from single spermatocytes of NZB/BinJ mice were separated from the nuclei and individually microinjected into B6D2F1 (C57BL/6 × DNBA/2J) hybrid embryos at the pronuclear stage (20 h after hCG injection). Of 363 zygotes injected, 311 (86%) survived and developed. From these experiments, we transferred 222 embryos into 20 pseudopregnant recipients. Eighteen (90%) became pregnant and 82 pups were born (37% of transfers). Mitochondrial DNA (mt DNA) from the NZB/BinJ strain lacks a *Rsa*I restriction site and can thus be distinguished from the host embryo following PCR amplification. We were unable to detect the transferred mtDNA in blastocysts on day 4–5 after injection. Nor could we detect NZB/BinJ mtDNA in placentae, nor in tissues from mice born to host mothers following the transfer of blastocysts that developed from injected zygotes. Rejection of paternal mitochondria by the embryo normally occurs at the 4- to 8-cell stage in mice and is apparently dependent on mutual recognition between the mitochondria and the nuclear genome. We conclude that this mechanism has probably already developed by the time the germ cells have become committed to meiosis.

Key words: Embryo, Mitochondrial DNA, Spermatocyte, Zygote

Introduction

Whether paternally derived mitochondrial DNA (mtDNA) can survive in the embryo is a question that is currently intriguing many biologists (Cummins, 1998*b*). It is also relevant to the subject of cloning, as it has implications for the synergistic control of mitochondrial function by nuclear and mitochondrially encoded genes (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Wakayama *et al.*, 1998). Kaneda *et al.* (1995) showed that in mice the spermatozoon's mtDNA disappears at an early embryonic stage in intraspecific but not interspecific crosses. This suggests that there must be a high degree of interaction between nuclear and mitochondrial factors that stringently controls the elimination of the paternal contribution. Moreover, recent results from the same group (Shitara *et al.*, 1998) showed that paternally derived mtDNA from *Mus spretus* × *Mus musculus* crosses was not evenly distributed to all tissues in the F1 hybrids. Nor was it transmitted to the following generations through the female germ line when the F1 mice were backcrossed. This calls into question Gyllensten *et al.*'s (1991) original estimate of a background 'leakage' rate of male mtDNA of around 1 in 1000, suggesting that this may have been an artefact derived from a stable heteroplasmic lineage. Independent results from a number of animal models summarised elsewhere (Cummins, 1997, 1998*a*, *b*) show that the sperm's mitochondria are eliminated rather abruptly by proteolysis during early embryogenesis. In mouse embryos we observed that fluorescently labelled mitochondria of microinjected spermatozoa and spermatids disappeared during the second mitotic cycle (4- to 8-cell transition) (Cummins *et al.*, 1997, 1998).

Mature sperm and late spermatid mitochondria show a number of quantitative and qualitative changes

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compared with earlier germ cell forms and with somatic cells (Cummins, 1998*b*). It is therefore possible that factors responsible for the recognition and elimination of the sperm mitochondria by the early embryo will develop late in spermiogenesis. To examine this point we microinjected cytoplasm from pachytene spermatocytes into zygotes, using a genetally marked mouse strain that allows one to distinguish between donor and recipient mtDNA (Jenuth *et al.*, 1996, 1997). This question has relevance for human assisted reproduction, where immature germ cells are now being used by microinjection to treat infertility in men with spermatogenetic arrest, as this could possibly lead to inherited mitochondrial disorders (Cummins, 1997, 1998*a*; Barak *et al.*, 1998; Sousa *et al.*, 1998).

Materials and methods

Reagents

Polyvinyl alcohol (PVA, cold-water-soluble, molecular weight *c.* 10 000) and polyvinylpyrrolidone (PVP, molecular weight *c.* 360 000) were purchased from Sigma Chemical Co. (St Louis, MO). Bovine testicular hyaluronidase (200 UPS U/mg) was obtained from ICN Biochemicals (Costa Mesa, CA). Bovine serum albumin (BSA fraction V) was purchased from Calbiochem (La Jolla, CA) and mineral oil from Squibb (Princeton, NJ). All other reagents were obtained from Sigma Scientific unless otherwise stated.

Media

The medium used for the culture of mouse oocytes after microsurgery was CZB medium (Chatot *et al.*, 1989, 1990) supplemented with 5.56 mM D-glucose. The medium for collection of oocytes from oviducts and subsequent oocyte treatments, including micromanipulation, was a modified HEPES-CZB (Kimura & Yanagimachi, 1995*a–c*) containing 20 mM HEPES-HCl, a reduced amount of $NaHCO_3$ (5 mM), and 0.1 mg/ml PVA instead of BSA. The medium used for parthenogenetic activation of mouse oocytes was Ca^{2+}-free CZB containing 10 mM $SrCl_2$ (Sr^{2+}-CZB). CZB and Sr^{2+}-CZB were used under 5% CO_2 in air, and HEPES-CZB was used under air.

Male mutant mice

NZB/BinJ (NZB) male mice were purchased from the Jackson Laboratory (600 Main Street, Bar Harbor, ME 04609). These carry a mutation at position 3961 in the ND2 gene that eliminates an *Rsa*I cleavage site normally present in the mouse mitochondrial genome (Jenuth *et al.*, 1996).

Collection of unfertilised oocytes

B6D2F1 (C57BL/6 × DBA/2J) hybrid females (6–12 weeks old; from the National Cancer Institute, USA) were injected with 5 IU PMSG followed by 5 IU hCG 48 h later. Oocytes were collected from oviducts about 15 h after the injection of hCG, and were freed from the cumulus cells by treatment with 0.1% bovine testicular hyaluronidase in HEPES-CZB. The oocytes were rinsed and kept in CZB medium for up to 4 h at 37 °C in an atmosphere of 5% CO_2 in air before micromanipulation.

Collection of fertilised oocytes

B6D2F1 females (6–12 weeks old) were injected with 5 IU PMSG followed by 5 IU hCG 48 h later. After hCG injection, each female was placed in a cage with a B6D2F1 male of proven fertility and left overnight for mating (checked by the presence of a vaginal plug the next morning). Fertilised oocytes with two distinct pronuclei were collected from oviducts 20 h after hCG injection. Loosely packed cumulus cells were removed by treatment with 0.1% bovine testicular hyaluronidase in HEPES-CZB.

Preparation of primary spermatocytes and round spermatids

Testes were isolated from the NZB male mice and placed in HEPES-CZB. After removal of the tunica, seminiferous tubules were macerated using a pair of fine scissors. One part of the suspension, containing fragments of seminiferous tubules was mixed thoroughly with HEPES-CZB medium containing 12% (w/v) PVP. A drop of this cell suspension was transferred into the micromanipulation chamber on the microscope stage and kept under mineral oil for up to 2 h at room temperature before injection.

Pathytene spermatocytes and round spermatids were easily distinguished from other spermatogenetic cells on the grounds of size and morphology (Kimura & Yanagimachi, 1995*a*, *c*).

Intracytoplasmic injection of cytoplasm of primary spermatocytes

A drop of medium containing some testicular cell suspension was transferred into the micromanipulation chamber on the microscope stage. In the drop, a single pachytene spermatocyte was drawn into the injection pipette and moved back and forth until the plasma membrane was broken. The cytoplasm was aspirated free of the nucleus and immediately injected into fertilised oocytes with two distinct pronuclei. The whole procedure was performed in HEPES-CZB within 2 h of preparation of the testicular cell suspension. The

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injected oocytes were transferred into CZB medium under mineral oil and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The procedure is summarised in Fig. 1.

Round spermatid injection into unfertilised oocytes

Round (haploid) spermatid injection into oocytes was carried out by a modification of a technique described by Kimura & Yanagimachi (1995*a*). Before microinjection, the oocytes were parthogenetically activated by incubation in Sr^{2+}-CZB medium for 1 h. They were thoroughly rinsed with CZB before being transferred onto the microscope stage.

Only round spermatids were selected, and they were injected into oocytes immediately after denudation. Most of the injected oocytes were at telophase of the second meiotic division. The injected oocytes were transferred into CZB medium under mineral oil and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Examination of oocytes and embryos

After injection, those zygotes that survived were selected for continuous culture for up to 96 h to assess embryonic development.

Embryo transfer

The embryos that developed to blastocysts at 72 h after injection were selected randomly and transferred to the oviducts or uteri of Swiss albino females, mated the previous night to vasectomised males of the same strain. On average about 10 embryos were transferred to each recipient female. Recipient females were allowed to deliver and raise their own pups. The sex of the pups was determined by examining their external genitalia.

Extraction of mtDNA

To extract DNA from tissues (heart, liver, kidney, ovary, testis and muscle), pups were killed at the age of 1–2 weeks. Tissues were macerated with fine scissors and homogenised individually before being treated with 100 µl lysis buffer (10 mM Tris-HCl pH 7.6; 10 mM EDTA; 2% sodium doececyl sulphate; 50 mM NaCl; 1 mg/ml proteinase K) and incubated at 56 °C for 4 h. Then 100 µl of 4 M NaCl was added to the lysate and DNA was precipitated by adding 2.2 ml of 100% ethanol. DNA was removed from the solution using a sterile glass rod and kept with 100 ml of distilled water in an Eppendorf tube at 4 °C until shipping to the laboratory in Australia. Placentae were also retained from some litters and extracted as above.

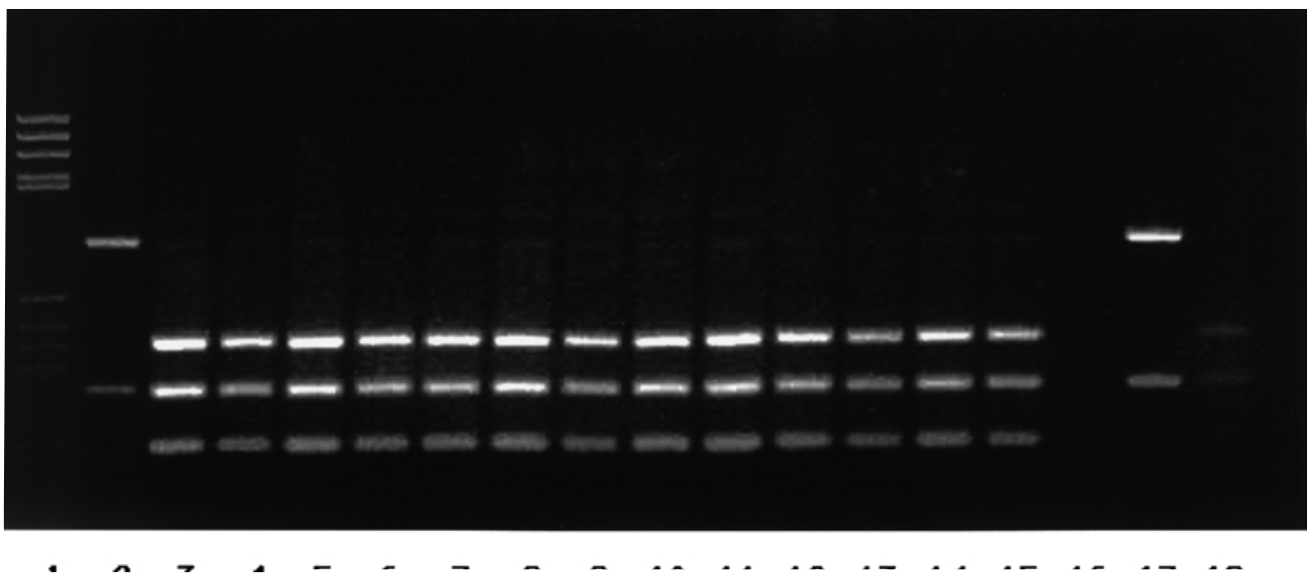


Figure 1 These gels show representative results of *Rsa*I digests of PCR products from the extracted mouse tissues. Lane 1, molecular weight markers (Boehringer Mannheim). Lane 2, heart muscle from an NZB/BinJ mouse showing two bands at 170 bp and 338 bp. Lane 3, mouse A1 heart showing three bands at 119, 170 and 219 bp due to an *Rsa*I sensitive site in the normal mouse mitochondrial genome at position 3961. Lane 4, mouse A1 liver. Lane 5, mouse A1 kidney. Lane 6, mouse A1 muscle. Lane 7, mouse A1 testis. Lane 8, mouse A2 heart. Lane 9, mouse A2 kidney. Lane 10, mouse A2 liver. Lane 11, mouse A2 ovary. Lane 12, Mouse B1 kidney. Lane 13, mouse B1 liver. Lane 14, 3/3 placenta from newborn pups. Lane 15, 3/3 placenta from newborn pups. Lane 16, blank control. Lane 17, heart tissue from NZB/BinJ: nested PCR–*Rsa*I digest. Lane 18, pooled blastocysts (n = 60) from Chelex-treated nested PCR–*Rsa*I digest. Only two faint bands can be seen due to the small amounts of DNA that could be extracted, but there is no evidence of the 338 bp band that would characterise a NZB/BinJ mitochondrial genotype.

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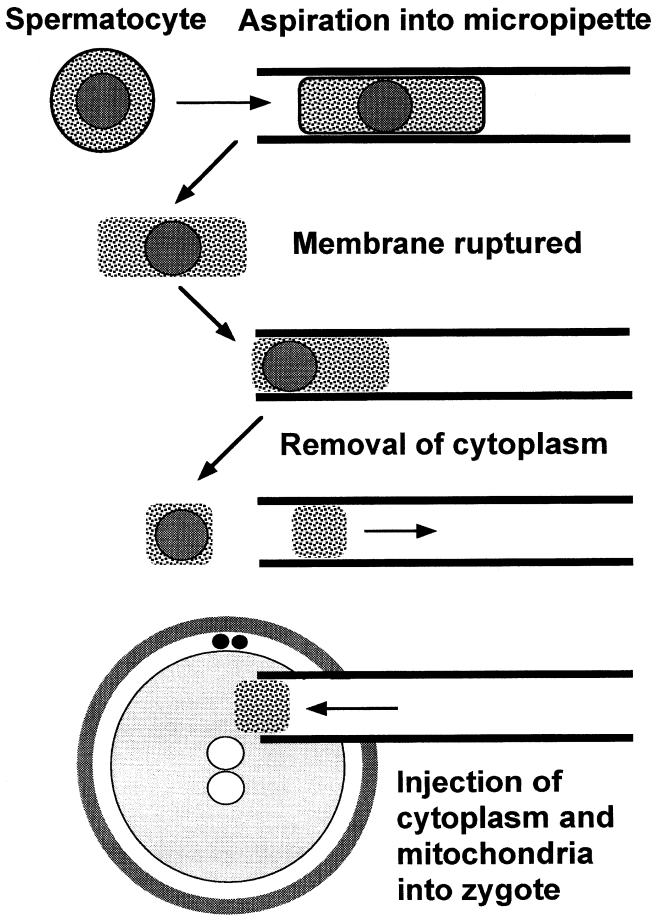


Figure 2 Cartoon summarising the essential steps in carrying out cytoplasmic transfer to the 2-pronuclear zygote.

Embryos were placed either singly or in groups in Eppendorf tubes and stored frozen in about 5 µl distilled water at –50 °C. Just before shipping they were thawed and 100 µl lysis buffer was added to each tube.

DNA extraction from tissues

In Australia, an aliquot of NaCl was added to each Eppendorf tube to achieve a final concentration of 1.5 M. The samples were mixed by inversion and centrifuged at 200 *g*. The addition of 2 volumes of ice-cold absolute ethanol precipitated the DNA, which was then recovered with a sterile disposable loop. The DNA was then dissolved and stored in 200 µl of sterile water.

Mitochondrial genotype analysis

BALB mice contain a *Rsa*I enzyme restriction site at position 3961 of the *ND2* gene of mitochondrial DNA. This site is absent in the NZB/BinJ strain (Loveland *et al.*, 1990; Jenuth *et al.*, 1996, 1997). PCR amplification of the region containing the *Rsa*I restriction site was carried out using the following primers: 3571–3591F: 5′-GAGCATCTTATCCACGCTTCC-3′ and 4079–4059R: 5′-CTGCTTCAGTTGATCGTGGGT-3′ (Gibco, BRL) at 0.4 µM, 2.5 mM $MgCl_2$, 100 µM dNTPs and 0.7 units Tth+ in a 1× reaction buffer (Biotech International) using a PCT-100 programmable thermal cycler (MJ Research). Cycling conditions were as follows: (1) 94 °C for 5 min; (2) 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and (3) a final extension at 72 °C for 5 min.

A 15 µl aliquot of the final product was digested overnight with *Rsa*I (New England Biolabs), then electrophoresed on a 2.5% agarose gel and visualised by ethidium bromide staining under ultraviolet light.

Oocyte isolation and analysis

Oocytes were suspended in lysis buffer at the University of Hawaii and sent by courier to the laboratory in Australia. Chelex 100 (Biorad, Richmond, CA) was added to a final concentration of 5% to precipitate proteins. The samples were boiled for 20 min before being centrifuged briefly. A nested PCR was applied to amplify the mtDNA. The first-round primers were as follows: 3509–3529F: 5′-TCTACTCAACTAACTTCATAA-3′ and 4194–4174R: 5′-GGATAAGACCGTTGTTTGTT-3′. The reaction conditions were the same as for mitochondrial genotype analysis except that 5 µl of oocyte DNA was used. PCR cycling conditions were as follows: (1) 94 °C for 5 min; (2) 15 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min; and (3) a final extension of 72 °C for 5 min.

A 1 µl aliquot was then used for the second round of amplification using conditions as described above. A 15 µl sample of the final product was then digested and analysed as described above.

Results

The results of transferring NZB spermatocyte cytoplasm to B6D2F1 zygotes are summarised in Tables 1 and 2. We carried out six experiments using testes from four NZB males. A total of 363 zygotes was injected, of which 311 (86%) survived the micromanipulation procedure. From these, 222 embryos were transferred into 20 pseudopregnant recipients. Eighteen of the recipients (90%) became pregnant and 82 pups were born (37% of the embryos transferred).

PCR results

We were unable to detect any NZB/BinJ mtDNA in any of the tissues derived from microinjected zygotes at the blastocyst stage or following birth. We estimate

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Table 1 Embryo transfer following injection of zygotes with NZB spermatocyte cytoplasm

Male	Eggs injected	Eggs surviving injection	Embryos transferred	Stage transferred	Females pregnant	No. of pups
1	31	22 (71%)	8	Blastocyst	0/1	0
2a	45	34 (76%)	33	Blastocyst	2/3 (67%)	5 (15%[a])
2b	45	39 (87%)	39	2-cell	2/3 (67%)	6 (15%[a])
3a	72	62 (86%)	58	21 8-cell, 37 morulae	4/4 (100%)	19 (33%[a])
3b	87	76 (87%)	54	39 4-cell, 9 morulae, 6 blastocysts	5/5 (100%)	26 (48[a])
4	83	78 (94%)	58	30 2-cell, 12 morulae, 16 blastocysts	4/4 (100%)	26 (45%[a])

[a]Percentage of embryos transferred that were born alive.

Table 2 Results of embryo culture for examination of embryonic mtDNA

Male	Eggs injected	Eggs surviving injection	Stage reached
5a; spermatocyte injection	89	83 (93%)	65 blastocysts (78%)
5b; spermatid injection	114	112 (98%)	20 blastocysts (18%)

that we should have been able to detect levels down to between 1–5% of the total. A representative gel is shown in Fig. 1.

Discussion

We cannot be certain that *no* transferred mitochondria survived in the embryos because the PCR technique tends to amplify selectively the majority DNA species present, and the transferred mitochondria may have been below our level of sensitivity for detection. However, these results appear to be consistent with what we have observed for mature sperm and for spermatid injection, where the mitochondria disappear at the 4- to 8-cell stage (Cummins *et al.*, 1997, 1998).

Somatic cell mitochondria can be shown to survive and proliferate in mouse oocytes (Pinkert *et al.*, 1997). Heteroplasmic mouse embryos can also be generated by a variety of permutations of nuclear transfer and cytoplasmic fusion (Jenuth *et al.*, 1996, 1997; Meirelles & Smith, 1997, 1998). However, sperm mitochondria do not survive long in cell lines (Manfredi *et al.*, 1997), suggesting a low inherent viable potential. During spermatogenesis the mitochondria undergo marked differentiation from a 'somatic type' to a 'germ-cell type', then to those of the mature spermatozoon following meiosis (Hecht *et al.*, 1984; Cummins, 1998*b*). The nuclear expression of mitochondrial transcription factor A is down-regulated at this time (Larsson *et al.*, 1997), although the sperm mitochondria apparently retain some potential capacity for normal transcription (Alcivar *et al.*, 1989).

In the embryo there is uncoupling of mitochondrial function from replication. While transcription may start at the 2-cell stage, in the mouse embryo replication does not commence until the general increase in cell mass at the egg-cylinder stage at 5–6 days (Pikó & Taylor, 1987; Pikó, 1992; Cummins, 1998*b*). The recognition and elimination of the paternal mitochondria by the embryo depends on some as yet unidentified nuclear encoded factor that marks the sperm for lysis (Kaneda *et al.*, 1995). This probably occurs through a species-specific interaction between the nuclear and mitochondrial genomes, as paternal mtDNA can persist sporadically in interspecific hybrids into the first generation (Shitara *et al.*, 1998). Our results here indicate that this factor or factors may have developed by the time the germ cells have become committed to meiosis. Further study is needed to characterise this unique early recognition system.

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