Mouse zygotes as recipients in embryo cloning

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Abstract

Zygotes have not been recognized as nuclear recipients since enucleated zygotes receiving nuclei from beyond two-cell stage embryos are not able to form blastocysts. In the present study, a new technique of zygote enucleation is presented, which consists in selectively removing the nuclear membrane with genetic material of pronuclei, but leaving other pronuclear components in the cytoplasm. With selective enucleation it is possible – after transfer of eight-cell stage nuclei – to obtain 70.5 and 7.8% of preimplantation and full-term development respectively. Origin of cloned mice from introduced nuclei was confirmed by the coat colour and glucose phosphate isomerase (GPI) isozyme of the donor. We suggest that some pronuclear factors – taken away from the zygotes in the karyoplasts upon classical enucleation – are needed to reprogram the introduced nuclei.

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Introduction

The first evidence that embryonic nuclei introduced into zygotes can persist in developing embryos was achieved almost 30 years ago when [Modliński \(1978\)](#) micro-surgically introduced mouse eight-cell stage nuclei of the CBA/H T6T6 strain into intact zygotes and showed the presence of two T6 marker chromosomes in resulting tetraploid blastocysts. Three years later, [Illmensee & Hoppe \(1981\)](#) reported successful mouse cloning via microsurgical injection of inner cell mass (ICM) nuclei into microsurgically enucleated zygotes. However, this result has never been repeated. [McGrath & Solter \(1984\)](#) transferred mouse embryonic nuclei from the preimplantation stages into enucleated zygotes and drew a conclusion of the ‘inability of mouse blastomere nuclei transferred in enucleated zygotes to support development *in vitro*’. Nevertheless, attempts followed to clone mammals from zygotes reconstituted either with embryonic or embryonic stem (ES) cell nuclei (mouse, [Robl et al. 1986](#), [Howlett et al. 1987](#), [Tsunoda et al. 1987](#), [Smith et al. 1988](#), [Cheong et al. 1992](#), [Wakayama et al. 2000](#); rat, [Kono et al. 1988](#); rabbit, [Modliński & Smorąg 1991](#); pig, [Prather et al. 1989](#); cattle, [Prather & First 1990](#); rhesus, [Meng et al. 1997](#)). In all cases, the reconstituted zygotes were able to develop only when early two-cell stage nuclei had been transferred. The developmental abilities of more advanced embryonic nuclei were severely limited; in the overwhelming majority of cases they were not able to support

development of reconstituted zygotes beyond the first two cleavage divisions. These failures to produce mammals from reconstituted zygotes culminated in the final conclusion of [Wakayama et al. \(2000\)](#) – following [Solter's \(1999\)](#) reply to [Illmensee's letter \(1999\)](#) – ‘that there is no firm reason for *Hineininterpretierung* of the claim of mouse cloning using zygotes’.

However, it should be indicated that in all cloning experiments since 1983, zygotes have been enucleated using the method of [McGrath & Solter \(1983\)](#), in which karyoplasts containing intact pronuclei are removed (CE, complete enucleation). Thus, we have developed an alternative method of enucleation of interphase cells based on the technique described earlier ([Modliński 1975](#)), which allows the removal of the pronuclear envelope of with attached chromatin and to leave the pronuclear contents in the zygote's cytoplasm (SE, selective enucleation).

Materials and Methods

All inorganic and organic compounds were purchased from Sigma unless otherwise stated.

Collection of zygotes and embryos

Mature (C57Bl10×CBA/H) F1, DBA/2 and CBA/H T6T6 mice approximately 3 months old originated from our own colony. They were kept in a temperature-controlled room with a 12 h light:12 h darkness cycle (lights on

from 0600 to 1800 h). Food (Labofeed H, Kcynia, Poland; metabolic energy of 13.0 MJ/kg) and water were available *ad libitum*. Donor females were killed by cervical dislocation. Recipient females and males that were subjected to vasectomy were anaesthetized by i.p. injection of 0.15–0.20 ml (depending on body weight) of 0.75% pentobarbital (Vetbutal, Biovet Puławy, Poland).

Female (C57Bl×CBA/H) F1 mice were superovulated by injection of 7.5 IU pregnant mare serum gonadotrophin (Folligon, Intervet, Holland) followed by 7.5 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet, Holland) 48–52 h later and mated with F1 males. Zygotes were collected from the oviducts 18–20 h after hCG injection and were devoid of cumulus cells by treatment with hyaluronidase (150 IU/ml PBS), washed three times in M2 medium and then cultured in KSOM medium (KCl-enriched simplex optimized medium; Specialty Media, Phillipsburg, NJ, USA) at 37 °C (5% CO₂ in air) until both pronuclei became clearly visible. Prior to enucleation, zygotes were preincubated for 20–30 min in M2 medium with the addition of cytochalasin B (CB, 5 µg/ml) and nocodazole (0.25 µg/ml).

Eight-cell embryos collected from spontaneously ovulated DBA/2 and CBA/H T6T6 females mated with DBA/2 and CBA/H T6T6 males respectively were used as donors of nuclei. Embryos were flushed from oviducts and tubo-uterine junctions around noon on the third day after mating (vaginal plug, day 1). Zonae pellucidae were removed from eight-cell embryos by treatment with 0.5% pronase in PBS for 3–5 min (Mintz 1962). After rinsing the embryos in three changes of M2 medium, they were transferred to Dulbecco's salt solution, which was devoid of Ca and Mg ions, for 15 min. After this treatment, the embryos were pipetted with a flame-polished narrow-bore pipette in M2 medium to disaggregate them into single blastomeres. Before micromanipulation, the isolated blastomeres were incubated in M2 medium supplemented with CB (5 µg/ml) for 20–30 min.

The experiments were performed according to the rules of the Polish Governmental Act for Animal Care and were approved (No. 33/2003) by the III Local Ethics Committee for Animal Care at Warsaw Agricultural University.

Selective enucleation (SE) of zygotes

Micromanipulations were performed under inverted Leitz Fluovolt microscope equipped with Nomarski DIC and Leitz mechanical micromanipulators. Preincubated zygotes were placed in the drop of the same incubation medium under paraffin oil in a micromanipulation chamber. A conical pipette with the diameter of 1–2 µm at its tip was used for enucleation. This pipette was introduced in the vicinity of an early/mid-pronucleus and, by applying strong negative pressure the nuclear envelope was adhered to the tip of the pipette (Fig. 1). Upon withdrawing the pipette from the zygote,

Figure 1 Selective enucleation of a mouse zygote: (A) a conical pipette with a diameter of 1–2 µm at its tip approaches the zygote; two apposed pronuclei – each with a nucleolus – visible in the zygote cytoplasm; (B) puncturing zona pellucida to enter the cytoplasm; (C) applying negative pressure to the nuclear envelope of the lower pronucleus; (D) withdrawing the pipette from the zygote and, simultaneously, pulling out the nuclear envelope; (E) the pronucleus tears open, releasing its contents into the cytoplasm; (F) the nuclear envelope, with chromatin attached to it, is removed from the zygote; nucleolus remains in the cytoplasm. The second pronucleus will be later removed from this zygote in the same way.

the pronucleus tears open due to increase in its internal pressure and the nuclear envelope, with the attached chromatin network (see Fig. 2 for Hoechst stained pronuclei), is removed. The liquid pronuclear contents and nucleoli remain in the cytoplasm. The second pronucleus is removed in the same way.

Complete enucleation (CE) of zygotes

Complete enucleation was performed using the standard enucleation technique of McGrath & Solter (1983).

Zygote–blastomere fusion

Enucleated zygotes were placed with isolated blastomeres in M2 medium+CB (5 µg/ml) and a single blastomere was introduced under the *zona pellucida* into the perivitelline space. The pairs of cells were washed three times in CB-free M2 medium and exposed to electric pulses (Kubiak & Tarkowski 1985). Electrofusion was performed in 0.3 M mannitol supplemented with 0.1 mM MgSO₄ and 0.05 mM CaCl₂ using Electrocell Manipulator ECM 2001 (BTX Gentronics, San Diego, CA,

Figure 2 Selective enucleation of a mouse zygote stained with Hoechst stain (4 µg/ml) to follow DNA removal. (A) and (B) Two pronuclei (arrowheads) and the nuclei of polar bodies (arrows) are visible; an asterisk (*) points to an unessential artefact; (C) and (D) after removal of the first pronucleus, nucleoli (arrow) and no residual fluorescence are visible in the cytoplasm; the pronucleus attached to the tip of the micropipette is highly fluorescent (arrowhead); (E)–(H) after removal of the second pronucleus no residual fluorescence remains in the cytoplasm; the pronucleus attached to the tip of the micropipette is highly fluorescent (arrowhead).

USA) in the BTX 453 fusion chamber. Two direct current (DC) pulses (1.2 kV/cm) of 55 µs each were applied. Treated pairs were rinsed three times in M2 medium, incubated in M2 at 37.5 °C and monitored for fusions.

Culture in vitro and in vivo

The fused pairs were washed three times in the prewarmed KSOM medium and placed in KSOM for culture (37.5 °C, 5% CO₂ in air). Reconstituted zygotes were either cultured *in vitro* for 5 days or cultured 2–4 days to be transferred (as two- to eight-cell embryos) into pseudopregnant Swiss albino females mated with proved vasectomized Swiss albino males.

Electrophoretic and karyological analysis

Blood samples were frozen in small amount of redistilled water and samples of tissues/organs were frozen in Tris–glycine buffer; all were stored at –20 °C. Before electrophoresis, the samples were thawed and frozen thrice, and supernatant was applied to the plates. Electrophoresis was performed on cellulose acetate plates (Titan III H, Helena Biosciences, Gateshead, UK) as described by Buehr & McLaren (1985), with minor modifications.

Chromosome preparations were made of blastocysts using air-drying method (Tarkowski 1966) and stained with Giemsa stain.

Results

The efficiency of selective enucleation (Figs 1 and 2) is given in Table 1. Out of 268 zygotes reconstructed using DBA2 blastomeres, 240 (89.5%) survived the double enucleation. In zygotes reconstructed with CBA/H T6T6 blastomere nuclei, the efficiency was 82.8%. As it is shown in Fig. 2, during selective enucleation the chromatin (DNA) is removed along with pronuclear envelope. In the overwhelming majority of cases all nucleoli were left in the zygote cytoplasm. In a small proportion of SE zygotes, one or two smallest nucleoli were removed along with the pronuclear envelope.

Table 1 Enucleation of mouse zygotes and their reconstruction with eight-cell blastomere nuclei.

Blastomere strain of mice	Enucleation	No. of enucleated zygotes	No. of surviving zygotes (%)	No. of zygote/blastomere pairs	No. of fused pairs
DBA2	SE ^a	118	103	89	85
DBA2	SE ^b	150	137	133	129
Total		268	240 (89.5)	222 (82.8)	214 (79.8)
CBA/H T6T6	SE ^a	28	22	20	20
CBA/H T6T6	SE ^b	65	55	53	51
Total		93	77 (82.8)	73 (78.5)	71 (76.3)
CBA/H T6T6	CE	49	47 (95.9)	47(95.9)	42 (85.7)

SE, selective enucleation; CE, complete enucleation.

^aFor blastocysts. ^bFor transfer to recipients.

Table 2 *In vitro* development of enucleated zygotes reconstituted with 1/8 blastomeres.

Strain of blastomeres	Type of enucleation	No. of development up to (%)					
		Two-cell day 2	Four-cell day 3	Eight-cell day 4	Morula day 5	Blastocyst day 5	Total
DBA2	SE ^a	81/85 (95.3)	64/85 (75.3)	61/85 (71.7)	20/85 (23.5)	40/85 (47)	85
DBA2	SE ^b	38/40 (95)	39/89 (43.8)	33/89 (37.1)	n.d.	n.d.	129
CBA/H T6T6	SE ^a	n.o.	n.o.	n.o.	10/20 (50)	6/20 (30)	20
CBA/H T6T6	SE ^b	12/12 (100)	20/39 (51.3)	18/39 (46.2)	n.d.	n.d.	51
CBA/H T6T6	CE ^a	22/42 (52.3)	1/42 (2.4)	0/42	0/42	0/42	42

SE, selective enucleation; CE, complete enucleation; n.d., not done; n.o., no observation.

^aFor blastocysts. ^bFor transfer to recipients.

Nucleoli which remained in the cytoplasm usually integrated immediately into a single structure.

Out of 222 selectively enucleated zygotes injected with eight-cell DBA2 blastomeres, 214 (96.4%) fused. Out of them, 85 were cultured *in vitro* for 5 days and another 129 SE reconstituted zygotes were cultured *in vitro* for differing duration (up to 4 days) to be transferred to recipient females for postimplantation development.

Out of 73 selectively enucleated zygotes injected with an eight-cell CBA/H T6T6 blastomere, 71 (97.3%) fused. Out of them, 20 were cultured *in vitro* for 5 days and another 51 SE reconstituted zygotes were cultured *in vitro* for differing duration (up to 4 days) to be transferred to recipient females for postimplantation development. Of the 47 completely enucleated zygotes injected with eight-cell CBA/H T6T6 blastomeres, 42 (89.4%) fused. All of them were cultured *in vitro* for 5 days. Out of 85 SE zygotes reconstituted with DBA2 blastomeres and cultured *in vitro* for 5 days, 20 and 40 developed to compacted morula and blastocyst stages respectively (Table 2). Dynamics of cleavage in this group is also shown in Table 2. Out of 20 SE zygotes reconstituted with CBA/H T6T6 blastomeres and cultured *in vitro* for 5 days, ten and six developed to compacted morula and blastocyst stages respectively (Table 2).

Three groups of zygotes were fused with CBA/H T6T6 eight-cell blastomeres, carrying a small marker T6 chromosome, thus providing a tool to karyologically prove the origin of developing reconstituted embryos. The same strain of blastomeres was used for reconstitution of completely enucleated zygotes. Indeed, when CBA/H T6T6 nuclei were injected into selectively enucleated zygotes, the blastocysts carrying two T6 marker chromosomes were produced (Fig. 3). No development beyond four-cell stage was obtained after transfer of CBA/H T6T6 eight-cell nuclei into 42 completely enucleated zygotes.

Out of 129 embryos developed from SE zygotes reconstituted with DBA2 nuclei and cultured *in vitro* to be later transferred to foster mothers, 77 (38 two-cell, 6 four-cell and 33 eight-cell embryos) were transferred into nine recipients at the first day of pseudopregnancy

(Table 3). Four of them became pregnant and six young (7.8%) were obtained. Since the first born pup was eaten by the recipient, the remaining five (two females and three males) were delivered by Caesarean section and fed by foster mothers. Their coat colour indicated the DBA/2 origin (Fig. 4) which was also confirmed by the electrophoretic separation of glucose phosphate isomerase (GPI) isozymes (Fig. 5). One male, upon sexual maturation, proved to be fertile. Out of 32 embryos developed from SE zygotes reconstituted with CBA/H T6T6 nuclei and transferred to four recipient females, only one implantation site was found upon Caesarean section at day 20.

Discussion

Full-term development was obtained after the transfer of embryonic nuclei into telophase I and anaphase II

Figure 3 Chromosome spread from a cloned mouse blastocyst. F1 (C57BL/6×CBA/H) zygote was reconstructed with CBA/H T6T6 1/8 blastomere nucleus. The smallest 2 of the 40 chromosomes present are T6 marker chromosomes.

Table 3 *In vivo* development of enucleated zygotes reconstituted with 1/8 blastomeres.

Strain of mice	Enucleation	No. of recipients	No. of transferred embryos	No. of pregnant recipients	No. of young born (%)
DBA2	SE	9	77	4	6 (7.8)
CBA/H T6T6	SE	4	32	1	0 ^a

SE, selective enucleation.

^aOne empty implantation site was found upon Caesarean section at day 20.

oocytes (Kono *et al.* 1992) and into two-cell blastomeres (mouse, Tsunoda *et al.* 1987, Kono & Tsunoda 1989, Kono *et al.* 1991, rat, Roh *et al.* 2003), as well as after the transfer of somatic nuclei into telophase II oocytes (cow, Bordignon & Smith 1998, Liu *et al.* 2000; goat, Baguishi *et al.* 1999) and two-cell embryos (rabbit, Skrzyszowska *et al.* 2006). However, in almost all cloning procedures performed during the last 20 years, metaphase II oocytes were the cytoplasts of choice.

Zygotes are not recognized as nuclear recipients on the basis that enucleated zygotes receiving nuclei from beyond two-cell stage are unable to progress through more than 2–3 cleavages. However, tetraploid embryos produced by transfer of eight-cell and ICM cell nuclei into non-enucleated zygotes can give rise to blastocysts (Modliński 1978, 1981). In addition, haploid zygotes reconstituted with haploid eight-cell nuclei of the opposite parental origin can develop into live young (Surani *et al.* 1986). When diploid eight-cell nuclei were introduced into recipient zygotes that retained either the male or the female pronucleus, blastocysts were produced (Howlett *et al.* 1987). However, when the resident pronucleus was removed (even as late as 5 h after nuclear transfer) or silenced with either transcriptional inhibitor or DNA synthesis inhibitor, no development beyond two-cell stage followed (Howlett *et al.* 1987). These observations suggest ‘an active and continued helper role of the resident pronucleus for the participation of eight-cell nucleus in reconstituted eggs’ (Howlett *et al.* 1987). The question arises what the mechanism of such helper action is.

In *Xenopus*, the remodelling of introduced somatic nuclei is much more evident in an egg than in an oocyte cytoplasm. However, if the germinal vesicle (GV) is ruptured prior to nuclear transfer – which would allow the mix of GV material with the oocyte cytoplasm – the efficiency of remodelling increases significantly (Gurdon 1968, 1976, Gurdon *et al.* 1979). Also, recent studies concerning the nuclear transfer into germinal vesicle mouse oocytes indicate that GV material is essential for nuclear remodelling (Gao *et al.* 2002, Cheong *et al.* 2004). This suggests that both in amphibians and mammals, the nuclear components released from germinal vesicle may play an important role to facilitate remodelling and reprogramming of the introduced foreign nuclei. Moreover, it is suggested that some of the GV components released into the cytoplasm of the

oocyte after GVBD, appear to be incorporated – after oocyte activation – into growing pronuclei (Polanski *et al.* 2005). The nature of these components remains unknown, but the results obtained from the earlier amphibian studies suggest that the possible candidates, which could be involved in remodelling of foreign nuclei are such molecules as nucleoplasmin (Laskey *et al.* 1978) and N1/N2 nuclear proteins (Kleinschmidt *et al.* 1986).

The first overt event, interpreted as morphological evidence of remodelling of the introduced nucleus, is nuclear swelling. Furthermore, this nuclear enlargement is probably a *conditio sine qua non* for successful nuclear reprogramming (Gurdon 1976, Czołowska *et al.* 1984, Tani *et al.* 2003). The amphibian studies indicated that it could be the result of a considerable movement of cytoplasmic proteins into the nucleus (Merriam 1969, Barry & Merriam 1972). In the mouse, the specific examples of oocyte-made karyophilic materials that could flow into the nucleus are nuclear lamins (Kubiak *et al.* 1991) and snRNA and snRNPs (Dean *et al.* 1989), which are exhausted from the cytoplasm shortly after oocyte activation. If the cytoplasmic components essential for successful development are incorporated into growing pronuclei, then the removal of the whole pronuclei by the ‘classical’ method of enucleation (CE) may lead to depletion of those factors resulting in the low development of reconstructed embryos (Campbell & Albeiro 2004). One cannot exclude that some of those

Figure 4 A mouse cloned from a reconstructed zygote. DBA/2 pup (female) derived from F1 (C57BL/6 × CBA/H) zygote reconstructed with DBA/2 1/8 blastomere nucleus. The coat colour of the pup indicates its origin from DBA/2 nucleus. The cloned mouse is accompanied by a Swiss albino foster sibling, 3 days older.

Figure 5 Electrophoretic separation of GPI isozymes. (A) GPI-AA standard (blood of DBA/2 male); (B) GPI-BB standard (blood of F1 (C57BL/6×CBA/H) individual); (C) cloned newborn, brain sample; (D) the same cloned newborn, lung sample; (E) 2-month-old cloned male, blood sample.

factors released from pronuclei during the first mitosis remain in the cytoplasm of two-cell stage blastomeres which could explain – to a certain degree – the ability of reconstituted blastomeres for further development.

When the pronuclei are removed using the SE method, the nucleoli remain in the cytoplasm. The nucleolus is a specialized domain of the nucleus in which the production of rRNA and synthesis of ribosomes take place. However, the recent studies revealed that the activity of some cell cycle regulators depends on sequestration in the nucleolus (Olson *et al.* 2000, 2003, Leung & Lamond 2003) as well as that nucleoli contain proteins with no known or clear relationship to ribosome biogenesis (Scherl *et al.* 2002, Politz *et al.* 2005). One cannot exclude that some of these proteins may enhance, after being released to the cytoplasm, the developmental capabilities of the reconstituted zygotes.

Our results clearly show that usefulness of zygotes as recipients of embryonic nuclei strongly depends on the enucleation method used. In CE method, all nuclear structures and components are taken out from the zygote, while in SE, most likely, only the pronuclear envelope-attached structures are removed. Since there are several lines of evidence that a filamentous scaffold structure underneath the inner nuclear membrane is the anchorage site for chromatin to the nuclear lamina (Marshall *et al.* 1996, Foisner 2002, Gasser 2002), we believe that – when using SE – the chromatin is being removed along with the pronuclear envelope. The birth of mice entirely derived from the introduced DBA/2

nuclei supports that hypothesis. Perhaps, this explains partially the results of Illmensee & Hoppe (1981), whose enucleation technique employed narrow pipettes that may have resulted in the removal of the pronuclear envelope and a release of a part of the pronuclear contents back to the cytoplasm.

The efficiency of development to the blastocyst stage of zygotes (our results) and oocytes (Cheong *et al.* 1993) receiving eight-cell nuclei was the same (47 and 46.2% respectively) and higher than that obtained after the reconstruction of two-cell blastomeres with eight-cell nuclei (35%; Tsunoda *et al.* 1987). This efficiency was also higher than the efficiency of development to the blastocyst stage of oocytes microsurgeically injected with nuclei of adult female fibroblast (38.8%), thymus (3.1%), spleen (22.4%), brain (22.4%) and Sertoli cells (39.6%; Wakayama & Yanagimachi 2001) and also with ES cells nuclei (28.7%; Wakayama *et al.* 1999), but lower than in oocytes injected with nuclei of fetal ovarian (59.3%) or testicular (56.4%) cells and adult male fibroblasts (59.5%) or cumulus cells (53.3%; Wakayama & Yanagimachi 2001).

According to our best knowledge, the presented results and also those by Cheong *et al.* (1993) and Hiiragi & Solter (2005) are the only ones to show that in the mouse obtaining of full-term development is possible after direct transfer of embryonic nuclei from beyond the two-cell stage. In all other cases, live young were produced by serial nuclear transfer (re-cloning) of the nuclei from the NT-derived embryos (obtained after transfer of early stage, morula and ICM/trophectoderm cell nuclei) into enucleated zygotes or two-cell blastomeres (Kwon & Kono 1996, Tsunoda & Kato 1997, 1998).

The efficiency of blastocyst formation upon the use of re-cloning procedures is high – 83% (Kwon & Kono 1996). The reason why re-cloning is so effective in mice is not clear, but it is speculated that factors present in the cytoplasm of the zygote can enhance the developmental potential of reconstituted embryos (Tsunoda & Shioda 1988). Indeed, the development to the blastocyst stage was improved significantly (from 16 to 83%) when pseudopronuclei from the reconstituted oocytes were introduced into the enucleated zygotes rather than to the activated oocytes (Kwon & Kono 1996).

The use of zygotes as recipient cells would eliminate the need for activation of reconstituted cytoplasts and simplify the cloning procedures.

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