Animal Science Papers and Reports vol. 33 (2015) no. 4, 323-336
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Developmental potential of selectively enucleated mouse zygotes reconstituted with embryonic cell, embryonic stem cell and somatic cell nuclei*

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(Accepted September 3, 2015)

In an overwhelming majority of experiments, both mammalian embryonic and somatic cloning have relied on introducing exogenous nuclei into enucleated metaphase II (MII) oocytes. Since attempts at cloning using interphase zygotes as recipient cells have failed, these cells were – until quite recently – commonly regarded as poor recipients for nuclear transfer. However, we have recently shown that interphase zygotes can be successfully used as recipients of embryonic nuclei. In a previous study, we used our original method of selective enucleation (SE), in which the pronuclear envelope with attached chromatin is removed while the liquid pronuclear contents and nucleoli in the zygote's cytoplasm are left intact, to obtain fertile mice upon transfer of 8-cell (1/8) nuclei into SE zygotes. Here we report that 16-cell (1/16) nuclei can also support full-term development. Additionally, full pre-implantation development, albeit to a limited degree, was obtained after transfer of embryonic stem (ES) cell and foetal fibroblast (FF) nuclei (2.4% and 2.5%, respectively). Sporadically, SE zygotes reconstructed with FF nuclei were able to implant, but they never developed beyond mid-pregnancy. Our results clearly indicate that SE zygotes can be successfully used as competitive recipients of embryonic nuclei from, at least, the 16-cell (morula) stage. However, their use as recipients of ES cell and somatic cell nuclei seems to be questionable.

KEY WORDS: embryonic cells / embryonic stem cells / foetal fibroblasts / nuclear transfer / selective enucleation / zygotes

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*This study was financed by the State Committee for Scientific Research (grant N302 04132/3424 given to JAM)

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Animal cloning after the transfer of embryonic and somatic cell nuclei into enucleated MII oocytes has demonstrated the remarkable ability of the oocyte to reprogram foreign nuclei and to give rise to offspring. Cloning with embryonic nuclei was first demonstrated in amphibians by Briggs and King [1952] and later in mammals [sheep – Willadsen 1986]. As more differentiated cells were used as nuclear donors, obtaining full development proved to be difficult, suggesting a biological restriction for the reprogramming of fully differentiated somatic cells [Gurdon 1975]. However, improvements in somatic cell nuclear transfer (SCNT) techniques overcame this problem. The births of Dolly the sheep [Wilmut *et al*. 1997] and the mouse Cumulina [Wakayama *et al.* 1998], the first mammals born after the transfer of adult somatic cell nuclei into enucleated MII oocytes, have ultimately demonstrated that genetic information can be reprogrammed in the oocyte's cytoplasm. Thereafter, many mammals (including practically all livestock and domestic species) have been cloned successfully using SCNT. Furthermore, using interspecies somatic cloning (iSCNT), some wild species (e.g., gaur, banteng, mouflon, ibex, grey wolf and African wildcat) have also been cloned [for a review see Loi *et al*. 2013].

Although these achievements demonstrate that terminally differentiated cells can be used for cloning when improved SCNT procedures are employed, cloning efficiencies are still very low. The efficiency of cloning depends upon a variety of factors including methods of embryo reconstruction and, to a greater extent, on biological factors, such as sources of recipient and donor cells, activation protocols, cell cycle co-ordination between recipient and donor cells and possibly some other yet unknown factors. All of these factors influence nuclear reprogramming and remodelling. Successful nuclear reprogramming should therefore convert the transferred genome of a differentiated cell to totipotency, which is the *sine qua non* condition of normal embryonic development.

For over 20 years, zygotes have been considered poor recipients for nuclear transfer, and enucleated metaphase II (MII) oocytes have been the preferred recipient cytoplast in almost all cloning procedures. Although in the early 1980s, Illmensee and Hoppe [1981] reported the successful cloning of a mouse after transferring inner cell mass (ICM) nuclei into enucleated prophase zygotes, this experiment has been regarded as controversial, as all further attempts to clone mammals from zygotes reconstructed with embryonic/ES cell nuclei have failed [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 and Smorąg 1991; pig – Prather *et al*. 1989; cattle – Prather & First 1990; rhesus – Meng *et al.* 1997]. Therefore, until recently, it has been commonly believed that enucleated prophase zygotes are inappropriate recipients for nuclear transfer.

However, it should be mentioned that in all cloning procedures since 1983, zygotes have been enucleated using the complete enucleation (CE) method of McGrath and Solter [1983], in which karyoplasts containing intact pronuclei are removed. It is fairly certain that in the CE method, factors essential for the reprogramming of introduced

nuclei and the successful development of reconstructed zygotes are removed with the pronuclei. This hypothesis is supported by the results in mouse [McGrath and Solter 1984, Wakayama *et al*. 2000], pig [Prather *et al*. 1989] and cattle [Prather and First 1990], indicating that the proper development of embryos reconstructed using zygotes is, on the whole, restricted to the exchange of pronuclei. Thus, we have developed an alternative method of interphase zygote enucleation based on a technique described earlier [Modliński 1975] called selective enucleation (SE), which allows the removal of the pronuclear envelope with attached chromatin while leaving the pronuclear contents in the zygote's cytoplasm. With SE, it was possible – after the transfer of 8-cell nuclei – to obtain full-term development [Gręda *et al*. 2006]. This was followed by reports of: (1) calves born after the transfer of adult fibroblast nuclei into early zygotes from which both the decondensing sperm chromatin and the maternal telophase II spindle had been previously removed [Schurman *et al*. 2006]; and (2) mice born after the reconstruction of late zygotes enucleated at the metaphase stage of the first mitosis with chromosomes from mitotically arrested 2-cell, 8-cell blastomeres and ES cells [Egli *et al*. 2007].

Using zygotes as recipient cells in cloning procedures thus remains an intriguing possibility. From a biological point of view, zygotes have a great advantage over unfertilised oocytes: they are naturally activated by sperm. Thus, the question arises: why are they so "stubborn" as competent recipients?

In this study, we tested whether nuclei from embryos beyond the 8-cell stage, as well as nuclei from somatic cells (foetal fibroblasts) and ES cells, could support development after their transfer into selectively enucleated mouse zygotes.

Material and methods

All inorganic and organic compounds were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA) unless otherwise stated. All mice 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 06:00 to 18:00 h). Food (Labofeed H, Kcynia, Poland; metabolic energy of 13.0 MJ/kg) and water were available *ad libitum*. 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.

Collection of zygotes (recipient cells)

Mature (C57BL10 x CBA/H) F1 and DBA/2 3- to 6-month-old mice were used as zygote donors. Females were superovulated by injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; Folligon, Intervet, Holland) followed by 7.5 IU human chorionic gonadotropin (hCG; Chorulon, Intervet, Holland) 48-52 h later, and were then mated either with F1 (C57BL10 x CBA/H) or DBA/2 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 (Specialty Media, Phillipsburg, NJ, USA) at 37.5°C (5% CO_2 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 mg/ml) and nocodazole (0.25 mg/ml).

Donor cells

Embryonic cells. Spontaneously ovulated DBA/2 and CBA/H-T6T6 females were mated with DBA/2 and CBA/H-T6T6 males, respectively; the resulting 16-cell embryos were collected and used as donors of embryonic nuclei. Embryos were flushed from oviducts and tubo-uterine junctions at 18:00 on the third day after mating (vaginal plug, day 1). Zonae pellucidae were removed by treatment with 0.5% pronase in PBS for 3-5 min. After rinsing the embryos in M2 medium (3X), they were transferred to Dulbecco's salt solution (Ca^{+2}; Mg^{+2} free) for 15 min. After this treatment, the zona-free embryos were pipetted with a flame-polished narrow-bore pipette in M2 medium to disaggregate them into single blastomeres. Prior to micromanipulation, the isolated blastomeres were incubated in M2 medium supplemented with CB (5 mg/ml) for 20-30 min.

Embryonic stem cells (ES cells). Two different ES cell lines were used: the CGR8 line (European Collection of Animal Cell Culture, UK) and the line established in our laboratory from epiblasts isolated from the blastocysts of the CBA/H-T6T6 strain of mice. Both strains were of the XY karyotype. Culture conditions and preparation of ES suspension were performed as described by Robertson [1987] and Abbondanzo *et al*. [1993].

Somatic cells (Foetal fibroblasts – FFs). To establish primary cultures of foetal fibroblasts (FFs), females of the CBA/H-T6T6 and CBA/H strains were mated with males of the same or the other strain to obtain either CBA/H-T6T6 foetuses or F1 (C57BL10 x CBA/H) foetuses. Foetuses were collected from uterine horns at day 11-14 p.c. After the foetuses were dissected out of their foetal membranes, they were decapitated and eviscerated. The carcasses were rinsed in sterile PBS, cut into small pieces and placed in 0.25% trypsin/EDTA for 30 min at 37°C. Trypsin digestion was stopped by adding Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS, Gibco). After centrifugation for 5 min (200 g, 1000 rpm), the cells were suspended in the same medium and seeded onto tissue culture Petri dishes (cell suspension from single carcass per one 6 cm dish) to be cultured at 37.5°C, in an atmosphere of 5% CO_2 in air. Upon reaching confluence, the cells were passaged and then frozen at passages 1-4 to be used later. For the experiments, an ampoule of frozen cells was thawed and the seeded cells were cultured until reaching confluence. For manipulation, the confluent culture was trypsinised, centrifuged and suspended in a small amount (200 ml) of M2 medium to be taken to the manipulation chamber.

Micromanipulation

All manipulations were performed under inverted Leitz Fluovert FS (Leitz, Wetzlar, Germany) and Eclipse Ti-U (Nikon, Japan) microscopes, connected to Leitz

mechanical micromanipulators and to an Integra manipulators system (Research Instruments Ltd., Falmouth, UK), respectively. Both microscopes were equipped with Nomarski differential interference contrast (DIC). Enucleation and injection pipettes were connected with CellTram Vario micropumps (Eppendorf, Germany). Pipettes were prepared from thin-walled borosilicate glass capillaries of an external diameter of 1 mm (GC 100T-15, Harvard Apparatus Ltd, Kent, UK). The narrow conical micropipettes for selective enucleation were pulled out on an M-97 micropipette puller (Sutter Instrument Co., Novato, CA, USA).

Enucleation of zygotes. Genetic material from zygotes was removed using the method of selective enucleation (SE) of Modliński [1975] and Gręda *et al*. [2006]. Briefly, a conical pipette with a diameter of 1-2 μm at its tip is used for enucleation. This pipette is introduced in the vicinity of an early- to mid-pronucleus and when strong negative pressure is applied, the nuclear envelope adheres to the tip of the pipette. Upon withdrawing the pipette from the zygote, the pronucleus tears open due to an increase in its internal pressure and the nuclear envelope, with the attached chromatin network, is removed. The liquid pronuclear contents and nucleoli remain in the cytoplasm. The second pronucleus is removed in the same way. The steps of SE are shown in Figure 1.

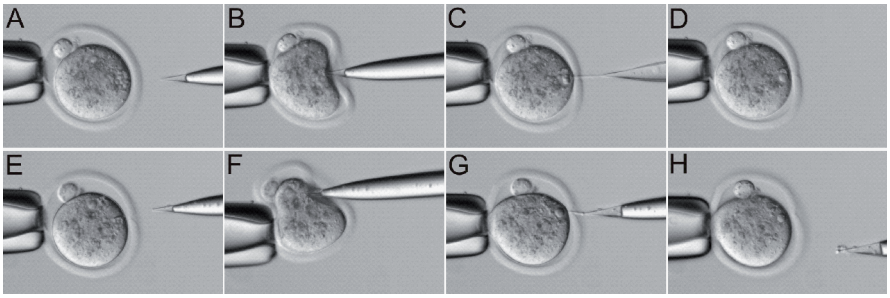

Fig. 1. Removal of pronuclear envelopes of both mouse zygote pronuclei by the selective enucleation method. (A)-(D) Removal of the envelope from the male pronucleus. During removal of the envelope, due to increased internal pressure, multiple pseudonucleoli of the male pronucleus fused and formed one pseudonucleolus (D). (E)-(H) Removal of the envelope from the female pronucleus. The female pronucleus usually contains one pesudonucleolus.

Nuclear transfer. Donor cell nuclei were introduced into SE zygotes by means of electrofusion [Kubiak and Tarkowski 1985, Ozil and Modliński 1986]. Enucleated zygotes were placed with isolated blastomeres/ES cells/FF in M2 medium+ CB (5 mg/ml) and a single cell 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. Electrofusion was performed in 0.3 M mannitol supplemented with 0.1 mM $MgS0_4$ and 0.05 mM $CaCl_2$ using an ECM 2001 Electro Cell Manipulator (BTX Genetronics, San Diego, CA, USA) or a Microporator (Eppendorf, Germany). Two direct current (DC) pulses (1.2 kV/cm) of 55 μs (1/16 blastomere), 60 μs (ES cell) and 65 μs (FF) each were applied. Nuclei from blastomeres isolated from 16-cell embryos were introduced: (1) DBA/2 blastomeres into F1 C57BL10 x CBA/H SE

zygotes; (2) CBA/H-T6T6 blastomeres into DBA/2 SE zygotes; (3) ES cell nuclei into DBA/2 SE zygotes; and (4) FF nuclei into DBA/2 and C57BL10 x CBA/H SE zygotes. Treated pairs were rinsed three times in M2 medium, incubated in M2 at 37.5°C and monitored for fusions.

Electrophoretic and karyological analysis

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

Chromosome preparations were made using an air-drying method [Tarkowski 1966] and stained with Giemsa stain. Whole-mount preparations were made according to the method of Tarkowski and Wroblewska [1976] and stained with Harris hematoxylin.

Results and discussion

SE zygotes reconstituted with 16-cell stage blastomere nuclei

Out of 183 DBA/2 and CBA/H-T6T6 zygotes, 148 (81%) survived double enucleation. After injection of 1/16 blastomeres under the zona pellucida of SE zygotes, 145 cytoplast-blastomere pairs were obtained and 136 (94%) of them fused. Reconstructed zygotes were cultured either for 2-4 h *in vitro* and transferred into the oviducts of pseudopregnant recipients or they were cultured overnight and were transferred into the oviducts the next morning as 2-cell embryos. Sixty-eight (68) DBA/2 and 52 CBA/H-T6T6 reconstructed zygotes/2-cell embryos were transferred into seven and five recipients, respectively, resulting in four (of DBA/2 origin) and two (of CBA/H-T6T6) young born (Tab. 1). Out of the four DBA/2 pups, one was stillborn and another was eaten by the foster mother. The remaining two developed normally and both, upon sexual maturation, proved to be fertile. Their coat colour indicated their DBA/2 origin (Fig. 2), which was also confirmed by the electrophoretic separation of glucose phosphate isomerase (GPI) isozymes. Analysis of skin and blood samples revealed the presence of GPI-1A isozyme, characteristic for DBA/Z strain. Of the two CBA/H-T6T6 pups, one was eaten after 5 days by the recipient and the second one (a male) reached sexual maturation and proved to be fertile. The dark pigmented eyes of both pups and the agouti coat colour of the 3-week-old male indicated their origin from the CBA/H-T6T6 strain (Fig. 3).

SE zygotes reconstructed with ES cell nuclei

Nine hundred and eighty-four (984) zygotes were selectively enucleated and 802 (81.5%) survived double enucleation. Single ES cells were introduced under the

Table 1. Post-implantation development of embryos obtained after reconstruction of SE zygotes with 1/16 blastomere nuclei

Strain of the donor nuclei	No. of reconstructed zygotes	No. of recipients	No. of transferred zygotes/2-cell embryos	No. of pregnant recipients	No. of young born (%)
DBA/2	74	7	68	3	4 (5.8)
CBA/H -T6T6	62	5	52	2	2 (3.8)

Fig. 2. Mice cloned from F1 (C57BL10 x CBA/H) SE zygotes reconstructed with DBA/2 1/16 blastomere nuclei. (A) DBA/2 pups (4 days old). (B) The same mice at the age of 3 weeks. Their characteristic silver-grey coat colour indicates their DBA/2 origin.

Fig. 3. Mice cloned from DBA/2 zygotes reconstructed with 1/16 CBA/H-T6T6 blastomere nuclei. (A) Two-day-old pups. Dark eye pigmentation indicates their CBA/H origin (DBA/2 mice have significantly lighter eye pigmentation). (B) One of those pups (male) at the age of 3.5 weeks. His agouti coat colour indicates his CBA/H-T6T6 origin.

zona pellucida of 789 of the SE zygotes. After electric field treatment, 458 (58%) reconstructed zygotes were obtained.

However, from the beginning, their development was severely disturbed. Although 374 (81.6%) of the reconstructed SE zygotes cleaved, the 84 zygotes blocked at the 1-cell stage revealed four serious types of abnormalities: (1) fragmentation; (2) lack of nuclear swelling, which is recognised as the first sign of nuclear remodelling; (3) formation of gigantic nuclei (Fig. 4) reaching half, or even three-quarters of the diameter of a zygote, indicating an improper pattern of remodelling; and (4) formation

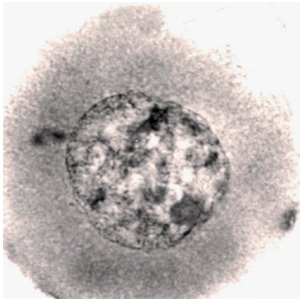

Fig. 4. SE zygote reconstructed with a CGR8 ES cell nucleus. The excessive growth of the introduced ES cell nucleus indicates its abnormal remodelling.

of an abnormal spindle of the first mitotic division. The dynamics of cleavage of SE zygotes reconstructed with ES cell nuclei are shown in Table 2. Out of the 142 zygotes reconstructed with CGR8 ES cell nuclei and the 232 reconstructed with CBA/H-T6T6 ES cell nuclei which underwent the first cleavage division, eight and 14 reached the compacted morula stage, while two and seven developed to the blastocyst stage, respectively. Five blastocysts had small cavities, relatively small cell numbers and no clearly visible inner cell mass (ICM). The remaining four blastocysts developed properly, with a large blastocoele and a well-formed ICM (Fig. 6). Karyological analysis of the two blastocysts developed from SE zygotes reconstructed with CBA/H-

Table 2. *In vitro* development of SE zygotes reconstructed with ES cell nuclei

ES cell line	Developmental potential of reconstructed SE zygotes								
	no. of reconstructed zygotes	FR No.	1-cell No. (no cleavage)	2-cell No. (%)	4-cell No. (%)	8- to 12-cell No. (%)	M No. (%)	BL No. (%)
CGR8	172	16	14	142 (82.6)*	32 (22.5)**	24 (16.9)**	8 (5.6)**	2 (1.4)** (25)***
CBA/H-T6T6	286	28	26	232 (81)*	72 (31)**	46 (19.8)**	14 (6)**	7 (3)** (50)***
Total	458	44	40	374 (81.6)*	104 (27.8)**	70 (18.7)**	22 (5.8)**	9 (2.4)** (40.9)***

FR – fragmentation; M – morulae; BL – blastocysts.
*As % of reconstructed zygotes.
**As % of embryos which passed the 1st cleavage.
***As % of morulae.

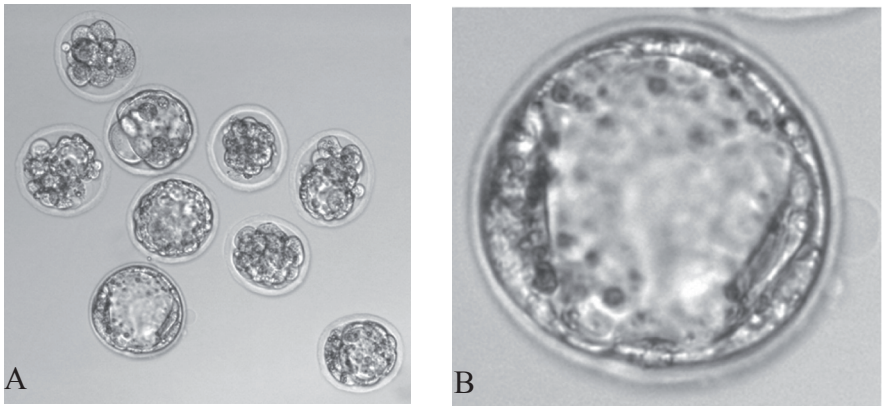

Fig. 5. (A) Group of embryos developed *in vitro* from SE zygotes reconstructed with ES cell nuclei. Fragmented zygotes/embryos, cleaving and arrested development embryos, the morula, a trophoblastic vesicle and one normal blastocyst (*) are visible. (B) The same blastocyst under higher magnification.

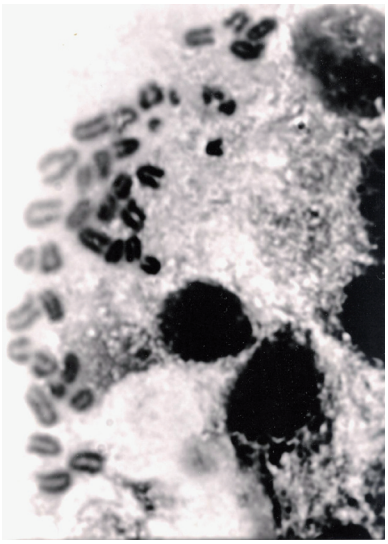

Fig. 6. Chromosome spread from a cloned blastocyst. F1 (C57/BL/10 x CBA/H0 SE) zygote was reconstructed with a CBA/H-T6T6 1/16 blastomere nucleus. The smallest 2 of the 40 chromosomes present are T6 marker chromosomes.

T6T6 nuclei revealed the presence of two T6 marker chromosomes, indicating their CBA/H-T6T6 origin (Fig. 5). For post-implantation development, 84 SE zygotes reconstructed either with DBA/2 nuclei or with CBA/H-T6T6 nuclei were transferred into 3 and 5 pseudopregnant recipients, respectively. On the 14th day, not a single implantation site was found.

SE zygotes reconstructed with FF nuclei

Foetal fibroblasts' nuclei were successfully introduced into 895 out of 1082 (82.7%) selectively enucleated zygotes. Around 50% of them passed the first cleavage division

and 2.6% (12/470) of the SE zygotes reconstructed with C57BL10 x CBA/H nuclei and 3.3% (14/425) of the dSE zygotes reconstructed with CBA/H-T6T6 nuclei developed to the morula stage. Out of a total of 26 morulae, 11 (42.7%) transformed into blastocysts (Tab. 3). Only five blastocysts had relatively normal morphology with medium-sized cavities and visible ICM. The remaining six cloned blastocysts were comprised of two trophoblastic vesicles without ICM and four blastocysts with small cavities and small, yet distinguishable, ICM. Karyological analysis of the four blastocysts developed from SE zygotes receiving CBA/H-T6T6 nuclei revealed the presence of two T6 marker chromosomes, confirming their CBA/H-T6T6 origin (Fig. 6). On the chromosome spreads from two of those blastocysts, numerous metaphase plates were found that indicated their high mitotic activity (Fig. 7).

Table 3. In vitro development of SE zygotes reconstructed with FF nuclei

Strain of recipient zygote	FF strain	no. of reconstructed zygotes	FR No.	Developmental potential of reconstructed SE zygotes					
				1-cell No. (no cleavage)	2-cell No. (%)	4-cell No. (%)	8- to 12-cell No. (%)	M No. (%)	BL No. (%)
DBA/2	C57BL10 x CBA/H	470	91	170	209 (44.5)*	48 (23)**	20 (9.6)**	12 (5.7)**	5 (2.4)** (41.7)***
C57BL10 x CBA/H	CBA/H-T6T6	425	67	128	230 (54)*	58 (25)**	26 (11.3)**	14 (6)**	6 (2.6)* (42.8)***
Total		895	158	298	439 (49)*	106 (24.1)**	46 (10.5)**	26 (5.9)**	11 (2.5)** (42.3)***

FR – fragmentation; M – morulae; BL – blastocysts.
*As % of reconstructed zygotes.
**As % of embryos which passed the 1st cleavage.
***As % of morulae.

After transferring 92 SE zygotes reconstituted with CBA/H-T6T6 nuclei into 11 pseudopregnant recipients, four females became pregnant. In two of them (killed at 14 dpc), two foetuses were found, one completely and one partially resorbed. The remaining two recipients – killed one day later (15 dpc) – carried two completely resorbed foetuses and one feotus that was partially resorbed.

Our previous [Gręda *et al.* 2006] and recent results clearly indicate that the usefulness of SE zygotes as recipients of exogenous nuclei depends on the type of nuclei used. Embryonic nuclei, up to at least the 16-cell stage, are able to support full-term development upon transfer to SE zygotes, with the recipients giving birth to normal, healthy and fertile progeny. On the other hand, SE zygotes reconstructed with

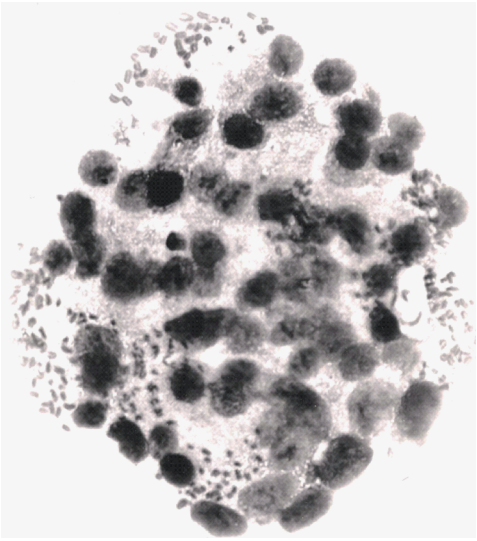

Fig. 7. Spread from the blastocyst developed from the SE zygotes reconstructed with CBA/H-T6T6 FF nuclei. The numerous metaphase plates visible indicate the high mitotic activity of that embryo. In some plates, T6 marker chromosomes are visible.

either ES cell or FF nuclei had very limited developmental potential, most likely due to improper remodeling/reprogramming of the introduced nuclei in their cytoplasmic environment.

It seems that the critical genomic factors required and sufficient for reprogramming embryonic nuclei are present inside the pronuclei but not in the zygotic cytoplasm, and that these are removed upon complete enucleation. However, thus far, no animals have been born after transfer of somatic cell nuclei into SE interphase zygotes, suggesting that indeed reprogramming/remodeling factors are present in pronuclei and maybe also in the zygote's cytoplasm, but that their activity is lower than those operating in MII oocytes and is not sufficient to reprogram somatic nuclei. The nature of these factors is unknown – one cannot exclude the possibility that some of them are of germinal vesicle origin. Studies by Polanski *et al*. [2005] on maturation of enucleated GV oocytes reconstructed with pronuclei, follicular cell nuclei and spermatocytes suggest that nuclei of GV oocytes and the pronuclei of fertilized oocytes contain factors crucial for controlling some events in the progression through meiosis and the first mitotic cycle. Earlier studies on *Xenopus* showed that remodeling of somatic nuclei occurred more often when the germinal vesicle was torn up before nuclear transfer and its contents were released into cytoplasm [Gurdon 1968, Gurdon *et al.* 1979]. After fertilization/activation, these crucial factors are probably gradually incorporated into pronuclei at the time of their formation and further growth. This idea is supported by the findings of Schurmann *et al*. [2006], who after transfer of bovine ear skin fibroblast nuclei into enucleated MII oocytes and early zygotes, reported: (1)

improved post-implantation development after sperm-mediated activation (IVF-NT: 61% *vs.* control MII-SCNT: 42%); and (2) decreased development to the blastocyst stage, depending on the time interval between insemination and nuclear transfer (50% 4.5 h after insemination *vs.* less than 10% 7 h after insemination). This is also confirmed by our recent studies in which piglets were born after transfer of fibroblast nuclei into SE zygotes enucleated at the very early stage of pronuclear formation [Modliński *et al.* submitted]. Additionally, Egli *et al*. [2007] reported that mouse zygotes arrested and enucleated in mitosis and reconstructed with ES cells chromosomes can support ES cell nuclei reprogramming and full-term development of cloned animals.

What could be the reason for such discrepancies in the potential of zygotic/ pronuclear factors to reprogram embryonic, ES cells and somatic cell nuclei? The full-term development of SE interphase zygotes reconstructed with 8- and 16-cell stage nuclei suggests that removal of the nuclear envelope releases factors required for their successful development. However, this release (sufficient for non-differentiated embryonic nuclei) is probably incomplete and thus insufficient to support the development of ES cell and somatic nuclei. Egli *et al*. [2010] suggested that enucleation of interphase zygotes resulted in depletion of Brg1 - the transcriptional regulator protein that is an important component of the Swi/SNF chromatin remodeling complex and is also required for proper zygotic genome activation – in early embryos. According to their interpretation, only a modest amount of Brg 1 is released into the cytoplasm as a result of SE since, *"activities required for transcriptional reprogramming are, in part, closely associated with chromatin in interphase and that they are removed with interphase chromosomes regardless of the enucleation method used."* They also showed that, in contrast to prophase, the nuclear reprogramming factors are not closely associated with mitotic chromosomes and are retained in the cytoplasm after removal of chromosomes during mitosis.

In conclusion, it is necessary to explore the nuclear reprogramming capacity of zygotes as recipients for nuclear transfer [for a comprehensive review see Lorthongpanich *et al*. 2010]. It is quite clear that SE enucleated zygotes can be used as recipient cell in embryonic cloning but their possible usefulness as recipients for SCNT is doubtful and solving this problem requires further study.

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