Articles

Fertilization of mouse oocytes using somatic cells as male germ cells

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Abstract

Female and male mouse somatic cells were injected into mouse F_1 oocytes. The cells used included cumulus cells (female) and muscle derived fibroblasts (male). The ability of the cells to fertilize oocytes and support embryonic development was examined. Following activation of the injected oocytes, two second polar bodies were extruded and two pronuclei were formed, one derived from the oocyte chromosomes and the other from the somatic cell chromosomes in a similar way to that observed following fertilization with secondary spermatocytes. Both second polar bodies contained DNA. The fertilization rates by cumulus cells were 10–29%. This was dependent on the artificial activation protocol and on the age of the oocytes. Older oocytes recovered 16–17 h after human chorionic gonadotrophin (HCG) injection were more likely to produce two second polar bodies and two pronuclei than young oocytes which were retrieved at 13–14 h after HCG injection ($P < 0.01$). The fertilization rates with fibroblasts were 29% using the most effective activation regime and aged oocytes. Most (80–90%) of the 'zygotes' produced by somatic cells cleaved to two cells in culture and ~50% reached the morula stage. However, the developmental competence of the embryos to reach blastocysts was limited. The present study demonstrates that mouse somatic cells undergo haploidization when injected into metaphase II oocytes, fertilize oocytes as diploid male germ cells and support preimplantation development to a degree.

Keywords: *artificial gametes, fertilization, nuclear transfer, somatic cells*

Introduction

Production of embryos and offspring from injections of nuclei isolated mechanically from haploid and diploid germ cells such as elongating and round spermatids, and secondary spermatocytes were reported in animals with varied success rates (Lacham-Kaplan and Trounson, 1997; Sofikitis *et al*., 1998). Injection of germ cells into mature oocytes has also been attempted in humans to overcome male infertility related to obstructive and non-obstructive azoospermia (Tesarik, 1996; Aslam *et al*., 1998; Vanderzwalmen *et al*., 1998). However, the small number of cells obtained and the failure to distinguish between the different stages of germ cell development and other cell types resulted in a limited number of oocytes injected, fertilization failure and reduced embryo development (Vanderzwalmen *et al*., 1998; Verheyen *et al*., 1998).

Maturation of human male germ cells *in vitro* for patients with germ cell arrest syndrome and xenogenic transplantation of spermatogonia for patients undergoing X-irradiation and chemotherapy treatments were suggested as possible treatments for male infertility. Attempts to mature human germ cells *in vitro* resulted in a time dependent loss of viability (Aslam and Fishel, 1998), with only a few spermatocytes and spermatids maturing into elongating spermatids or spermatozoa which were used to fertilize oocytes and result in pregnancies (Aslam and Fishel, 1998; Cremades *et al*., 1999; Tesarik *et al*., 1999). Intra-species and inter-species xenogenic transplantation of seminiferous tubules, male germ cells or stem cells has been successful in establishing spermatogenesis and the production of germ cells and fertile spermatozoa in animals (Brinster and Zimmermann, 1994; Clouthier *et al*., 1996; Schlatt *et al*., 1999). However, xenogenic transplantation of human spermatogonia and germ cells into sterile mice testes failed to

initiate spermatogenesis (Reis *et al*., 2000). Until a compatible recipient animal species for xenogenic transplantation of human testicular tissue is identified, treatment of human male infertility is restricted to intracytoplasmic injection of mature spermatozoa; if that is unsuccessful, patients are offered insemination with donor spermatozoa.

Cloning is proposed as the ultimate strategy for the treatment of male and female sterility. However, somatic cell nuclear transfer results in a high rate of embryo developmental abnormalities, birth defects and postnatal losses in animals due to epigenetic remnants that distort the regulation of gene expression of cloned embryos (Daniels *et al*., 2000, 2001; Humphreys *et al*., 2001; Trounson, 2001). An alternative based on the experiments of Kimura and Yanagimachi, 1995) is to induce haploidy of somatic diploid nuclei injected into oocytes to form artificial gametes (Trounson, 1998, 2001).

It has been demonstrated in mouse cloning studies that somatic cells undergo haploidization within metaphase II (MII) oocytes (Wakayama *et al*., 1998). The cell chromosomes divide into two poles in 64% of reconstituted oocytes and progress to the formation of two pronuclei following artificial activation in the presence of cytochalasin B (Wakayama *et al*., 1998). Recently, nuclear transfer of adult somatic cells into human oocytes has resulted in the formation of haploid pronuclei following artificial activation or insemination with spermatozoa (Takeuchi *et al*., 2001; Tesarik *et al*., 2001). Replacement of an oocyte's chromosomes with those of a somatic cell, creating a new 'healthy' oocyte, has also been suggested as a treatment for female patients with ovarian failure (Sauer *et al*., 1991) or defective oocytes (Rosenwaks, 1987).

The present study describes the use of somatic cells as artificial male germ cells. Mice (Kimura and Yanagimachi, 1995) and human (Sofikitis *et al*., 1997) secondary spermatocytes, which are diploid germ cells, are able to enter metaphase and undergo meiosis following insertion into MII oocytes and artificial activation. Two second polar bodies were extruded from the activated oocyte; one contained half of the oocyte chromosomes and the other contained half of the spermatocyte chromosomes. Following embryo transfer, live healthy offspring were born in mice (Kimura and Yanagimachi, 1995) and a birth has also been claimed in a human subject (Sofikitis *et al*., 1997). The present study examined the ability of somatic cells to undergo meiotic division within mature MII mouse oocytes, to extrude chromosomes in a second polar body, and to initiate fertilization in a similar way to male diploid spermatids. In addition, the effect of two different artificial activation methods and the oocyte age on fertilization and development rates of artificial gamete zygotes were investigated.

Materials and methods

For all experiments F_1 (C57BL females X CBA males) hybrid female mice were used as oocyte donors, somatic cell donors and as recipient foster mothers. F_1 males were used as sperm donors and somatic cell donors. Somatic cells used for the study include ovarian follicular cumulus cells from an adult female, and skin fibroblasts obtained from one male.

Preparation of oocytes and cumulus cells

Females underwent superovulation by the injection of 10 IU of pregnant mare's serum gonadotrophin (PMSG; Folligon; Intervet, Lane Cove, Australia) and 10 IU human chorionic gonadotrophin (HCG; Choralon; Intervet) 48 h later. Oocytes were recovered from the oviducts of superovulated females 13–14 h or 16–17 h after HCG injection. Oocyte–cumulus cell complexes were liberated into warm M2 handling medium (Quinn *et al*., 1982) containing 40 IU hyaluronidase (fraction IV-S; Sigma Chemicals Co., St Louis, MO, USA). The oocyte–cumulus cell complexes were incubated in the hyaluronidase solution for 5 min to separate cumulus cells from oocytes. Cumulus-free oocytes were collected and washed in fresh warm M2 medium before being cultured in M16 medium (Whittingham, 1971) previously equilibrated at 37°C in a 5% CO_2 in air atmosphere for 15 h. The remainder, hyaluronidase solution containing cumulus cells was collected into a 5 ml plastic tube (Falcon, Becton Dickinson, NSW, Australia). An additional 2 ml of fresh warm M16 was added to the tube before it was centrifuged at 300 *g* for 5 min. The supernatant was removed and the cells located at the bottom of the tube were collected and transferred into a drop of 5% CO_2 in air equilibrated M16 medium. Both oocytes and cumulus cells were cultured under mineral oil (Sigma) at 37°C in 5% CO_2 in air until used 1 h later for injection.

Preparation of adult fibroblasts

Adult male fibroblasts were prepared as previously described by Lacham *et al*. (2000). The muscle and skin tissues from adult male mice were removed and chopped finely using surgical blades. The tissue fragments were treated with 0.25% trypsin (Sigma) and 0.4% EDTA (Boehringer Mannheim, Mannheim, Germany) in Ca/Mg-free PBS (Oxoid, Hampshire, UK) for 30 min at 37°C. Disaggregated cells were washed and cultured in Dulbecco's Modified Eagles medium (DMEM, Trace Biociences, NSW, Australia) supplemented with 2 mmol/l L-glutamine (Gibco, Life Technology, NY, USA), 26 mmol/l sodium bicarbonate (BDH, Dorset, UK), 100 IU/ml penicillin (Life Technologies), 100 mg/ml streptomycin (Life Technologies) and 10% fetal calf serum (FCS) (Life Technologies) in 10 cm Petri dishes (Falcon) at 37°C under 5% CO_2 in air. The primary culture was allowed to reach 100% confluence before being passaged into 75 cm culture flasks (Falcon). Cells were stored at second passage and frozen in 90% FCS and 10% DMSO (Sigma) at –80°C.

For nuclear transfer, cells were thawed at 37°C in a water bath then washed and cultured in DMEM containing 10% FCS up to the fourth passage. The cell monolayer was trypsinized to detach cells from the tissue culture flasks and centrifuged at 1000 r.p.m. for 5 min. The cell pellet was re-suspended in M2 medium containing 4 mg/ml BSA (Fraction IV, Sigma).

Preparation of spermatozoa

Spermatozoa for intracytoplasmic sperm injection were prepared by removing one cauda epididymis from a 12- to 14-week-old F_1 male. A single slit was made in the tissue before it was placed in 1 ml warm and equilibrated with modified Tyrode's medium (MT6; Quinn *et al*., 1982) to allow spermatozoa to swim out into the medium. Spermatozoa were

capacitated for 2 h in 5% CO_2 in air at 37°C before they were used for injection.

Micromanipulation procedure

Oocytes were placed in 40 ml droplets of M2 medium in an-oil filled 10 cm tissue culture plastic dish (Falcon). Cumulus cells, fibroblasts or spermatozoa were dispersed into 10 ml 20% polyvinylpyrrolidone (PVP; Sigma) in a M2 drop also located on the cover. Randomly selected cells were aspirated into a glass microinjection pipette with an inner diameter of 5 mm (for cumulus cells and spermatozoa) or 7–8 mm (for fibroblasts) mounted on a Leica (Leica Microscopy Systems Ltd, Wetzlar, Germany) manually operated micromanipulator attached to a piezo system (Prime-Tech Ltd, Ibaraki, Japan). Micromanipulation was performed at room temperature. Cumulus cells or fibroblasts were aspirated into the injection pipette and their membrane broken. The separated nucleus was expelled once from the injection pipette into the PVP solution and re-aspirated into the pipette. For sperm injection, sperm heads were separated from tails using a high piezo pulse before being collected into the injection pipette. About five cumulus cells, fibroblasts or sperm heads were aspirated into the injection pipette. The injection pipette was moved into the drop containing the oocytes. A piezo pulse was applied and a hole was created in the zona pellucida. The injection pipette was inserted into the perivitelline space and a nucleus isolated from the somatic cells or a sperm head were brought as close as possible to the opening of the pipette. The oolema was ruptured by applying a gentle piezo pulse and the isolated nucleus or sperm head was injected slowly deep into the ooplasm. About 30–40 oocytes were injected with a single somatic cell nucleus or sperm head within 1 h in batches of 10 at a time.

Following injection, oocytes were transferred to 5% CO_2 in air equilibrated drops of M16 culture medium. At 2–3 h after injection nuclear transfer oocytes were artificially activated to initiate formation of pronuclei and embryonic development.

Artificial activation of nuclear transfer oocytes

For activation, the oocytes injected with somatic cells were exposed to either 8% ethanol in M2 medium for 5 min, or to 8% ethanol in M2 medium for 5 min, followed by 4 h incubation in 1 mg/ml of the protein inhibitor, cyclohexamide (Sigma) in M16 culture medium.

Reconstituted zygotes exhibiting two second polar bodies and two pronuclei were separated and cultured up to 5 days to examine their development to blastocysts. In a separate experiment, two-cell embryos resulted from zygotes exhibiting two second polar bodies and two pronuclei were transferred to pseudopregnant recipient females mated to vasectomized males.

Confocal analyses of zygotes exhibiting two second polar bodies

To determine if the two second polar bodies that were extruded following artificial activation contained DNA, reconstituted zygotes exhibiting two second polar bodies were fixed in 2.5% glutaraldehyde in cacodylate buffer and stained with the fluorescent DNA stain propidium iodide (PI) (Sigma; 20 mg/ml of M2 medium). Stained oocytes were examined by confocal microscopy using filters with excitation maxima 494 nm and an emission maximum at 617 nm. The confocal microscope (MRC-1000; Biorad Microscience, Hemel Hempstead, UK) uses the CoMos operating system (Version 7.0a: Biorad Microsciences)

Statistical analysis

Data were analysed by χ^2 tests for statistical significance.

Ethical approval

Monash University animal ethics committee approved all experiments.

Results

At ~1 h following exposure of nuclear transfer oocytes to 8% ethanol or following sperm injection, oocytes extruded one or two second polar bodies and within 2–3 h, pronuclei appeared within the manipulated oocytes. The second polar bodies were very large and easily distinguishable; the first polar bodies were fragmenting by this stage. There were no leakages of cytoplasm from the oocytes into the perivitelline space following injection using the Piezo system. Oocytes producing two second polar bodies and two pronuclei from injections of somatic cells

Table 1. Effect of different activation methods on the preimplantation development of zygotes produced from injection of cumulus cells into oocytes retrieved 13–14 h after human chorionic gonadotrophin.

No. oocytes injected	*No. oocytes survived*	*Method of activation*	*No. oocytes activated*	*No. oocytes producing two–2ndPB and 2PN*	*No. 2-cell embryos*	*No. morulae*	*No. blastocysts*
196	121 (62%)	8% ethanol for 5min	63	15[a] (24)	12 (80)	5 (33)	2 (13)
		8% ethanol for 5 min/4 h cyclohexamide	58	6[a] (10)	5 (83)	3 (50)	0
		Total	121	21 (17)	17 (81)	8 (38)	2 (10)

Values in parentheses are percentages.
PB = polar body(ies), PN = pronuclei
[a]Significantly different ($P < 0.05$).

Table 2. Effect of age of oocytes on the number of cumulus cell zygotes and their preimplantation development *in vitro*.

Oocyte age (h post-HCG)	*No. oocytes injected*	*No. oocytes survived*	*No. oocytes with two second PB and 2PN*	*No. 2-cell embryos*	*No. morulae*	*No. blastocysts*
13–14	396	242 (61)	48[a] (20)	45 (94)	21 (44)	4 (8)
16–17	329	187 (57)	54[a] (29)	50 (93)	26 (48)	9 (17)
Total	725	429 (59)	102 (24)	95 (93)	47 (46)	13 (13)

Values in parentheses are percentages.
PB = polar body(ies), PN = pronuclei, HCG = human chorionic gonadotrophin
[a]Significantly different ($P < 0.01$)

Table 3. In-vivo development of cumulus cell embryos.

Cell injected	*No. oocytes injected*	*No. oocytes survived*	*No. oocytes producing PB and 2PN*	*No. 2-cell embryos transferred*	*No. foster females*	*No. live pups*
Cumulus cells	180	108 (60)	23[a] (21)	20	2	0[b]
Epididymal spermatozoa	154	100 (65)	95[a] (95)	30	3	16[b] (53)

Values in parentheses are percentages.
PB = polar body(ies), PN = pronuclei
[a,b]Values with the same superscript are significantly different ($P < 0.001$)

(**Figure 1a,b**) and oocytes which produced one second polar body and two pronuclei following sperm injection were considered fertilized and were separated from the rest.

In-vitro development of zygotes produced by nuclear transfer of male and female somatic cells

A total of 196 oocytes were injected with cumulus cells, from which 121 (62%) survived. Two to 3 h later, 63 and 58 oocytes were exposed to 8% ethanol for 5 min or 8% ethanol for 5 min followed by 4 h incubation in cycloheximide respectively (**Table 1**). Higher proportions of oocytes produced two second polar bodies and two pronuclei when exposed to 8% ethanol, than when exposed to the combination of 8% ethanol and cycloheximide (24 and 10% respectively). These differences were significant ($P < 0.05$). In both activation groups, most of the fertilized oocytes cleaved to two cells (>80%) and 30–50% advanced to the morula stage in culture (**Table 1**). No blastocysts were obtained in the group of oocytes activated by the combination of 8% ethanol and cycloheximide and two (13%) embryos reached the blastocyst stage following activation by ethanol alone. The differences in development to the blastocyst stage were not statistically different.

Oocytes which were retrieved from superovulated females 16–17 h after HCG injection produced more zygotes with two second polar bodies and two pronuclei, and more blastocysts *in vitro* following nuclear transfer of cumulus cells and 8% ethanol activation than oocytes retrieved at 13–14 h after HCG injection (**Table 2**). From a total of 242 (61%) oocytes that survived the injection, 48 (20%) extruded two second polar bodies and formed two pronuclei, from which four (8%) developed to blastocysts in culture. From 187 (57%) older oocytes that survived the injection, 54 (29%) extruded two second polar bodies and formed two pronuclei, and nine (17%) developed to blastocysts in culture. The number of oocytes producing two second polar bodies and two pronuclei were significantly different between the two groups ($P < 0.01$).

In a separate experiment, 57 oocytes were injected with adult male fibroblast cells. A total of 31 (54%) of the oocytes survived the injection and nine (29%) extruded two second polar bodies and formed two pronuclei. The numbers of these embryos developing to two cells, morulae and blastocysts were nine (100%), six (67%) and two (22%) respectively.

In-vivo development of zygotes produced by cumulus cells

A total of 20 two-cell embryos produced from cumulus cell injection and 30 two-cell embryos produced by sperm injection were transferred to pseudopregnant female mice (**Table 3**). None of the embryos produced by cumulus cell injection developed to live pups, whereas 16 (53%) of the embryos produced by sperm injection developed to live offspring.

DNA analyses by confocal microscopy

A total of seven oocytes with two second polar bodies were fixed and five were recovered and analysed. Confocal analyses of these oocytes stained with propidium iodide (PI) showed that the somatic cell chromosomes were separated into two sets and one of these sets was extruded in a second polar body (**Figure 2**).

Discussion

The present study examined the possibility of producing embryos and offspring from somatic cells in mice. The study

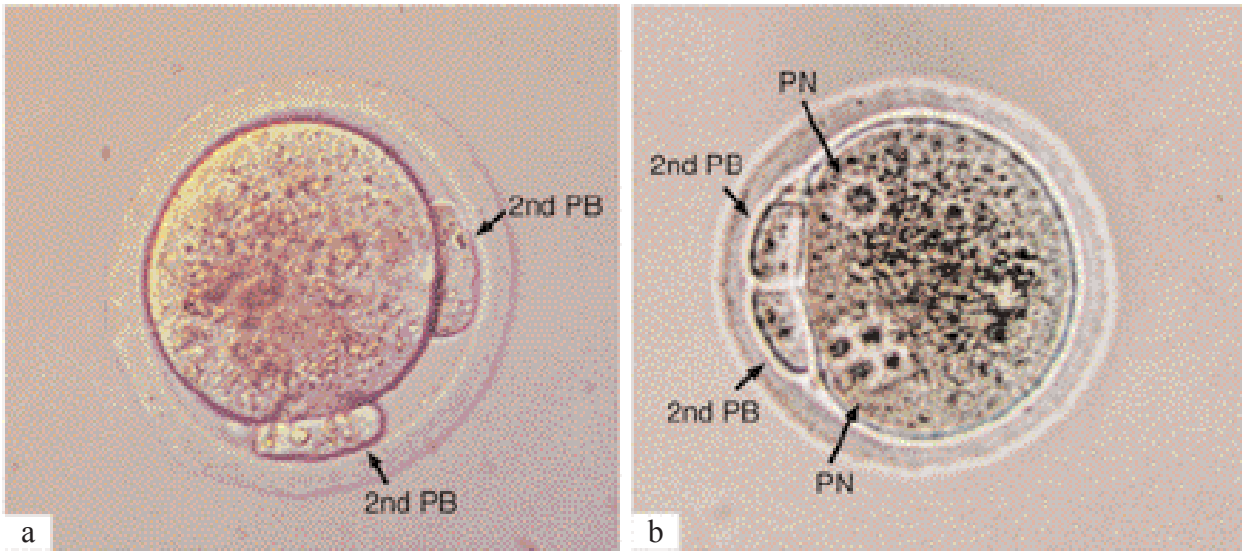

Figure 1. Mouse oocytes injected with cumulus cells. Injected oocytes were activated with 8% ethanol for 5 min. At 1 h after activation two second polar bodies (PB) are extruded from the oocyte (**a**), and about 6 h after the injection of the cell (**b**) two clear pronuclei (PN) originated from the oocyte, and cumulus cell chromosomes are observed (original magnification x200).

confirmed that mouse somatic cells are able to enter metaphase within the cytoplasm of metaphase II (MII) oocytes similarly to that demonstrated by Takeuchi (2001) and Tesarik *et al*. (2001) when human somatic cells were injected into human oocytes. In all studies, somatic cells were able to continue as metaphase cells, resulting in their chromosomes separating into two poles. Following artificial activation (Tsai *et al*., 2000; Takeuchi, 2001; present study) or insemination with spermatozoa (Tesarik *et al*., 2001), the oocytes extruded two second polar bodies and two pronuclei, or two pronuclei were produced within the oocyte with no polar body extrusion. While Tesarik (2001) and Takeuchi (2001) used somatic cells to replace the oocyte chromosomes and artificially activated or inseminated the reconstituted oocytes with spermatozoa to produce embryos, the present study used somatic cells to reconstitute diploidy in the oocyte. Mouse female or male somatic cells were injected into non-enucleated MII oocytes, which were activated 2–3 h later, resulting in the formation of two second polar bodies and two pronuclei (**Figure 1**).

In comparison with the high fertilization rate (75%) following nuclear transfer of diploid spermatocytes in mice, the efficiency in producing diploid zygotes following haploidization of somatic cells within MII oocytes is very low (10–29%). Moreover, although two second polar bodies were produced and two pronuclei were formed, the capacity of the reconstituted oocytes to develop in vitro was limited (0–17%) and no offspring were obtained following the transfer of somatic cell derived embryos to recipient females. Kimura and Yanagimachi (1995) reported 65% of oocytes injected with secondary spermatocytes develop to blastocysts and 24% developed to live offspring. Sofikitis *et al*. (1997) reported that from a total of 30 human oocytes injected with secondary spermatocytes isolated from testicular biopsies, 12 (40%) formed two pronuclei and developed normally in culture before being transferred to three patients, resulting in one (8%) pregnancy.

Haploidization of cumulus cells within enucleated human oocytes was confirmed by fluorescent in-situ hybridization

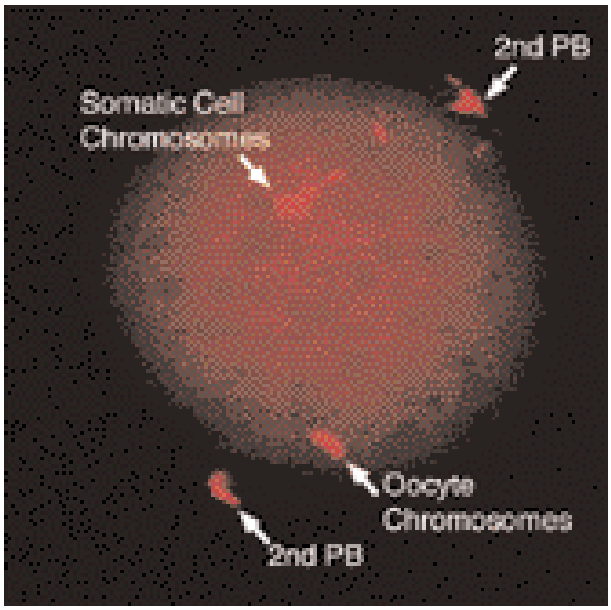

Figure 2. Confocal imaging of a mouse oocyte injected with a cumulus cell 1 h after activation with 8% ethanol. Activation took place 3 h after the cell was injected. Two polar bodies (PB) were extruded from the oocyte following activation. Both polar bodies contain DNA as identified by fluorescence following propidium iodide (PI) staining.

(FISH) analyses on the second polar bodies separated from the ooplasm of reconstituted oocytes after fertilization by spermatozoa (Tesarik *et al*., 2001). A single signal for each of the chromosomes 13, 18, 21 and X was identified in the polar bodies of two of the three oocytes analysed, indicating that they were haploid sets. In another study (Takeuchi *et al*., 2001), insertion of cumulus cells into enucleated human oocytes resulted in the formation of one or two pronuclei within the oocytes with no extrusion of a polar body. Nonetheless, when two pronuclei were formed, each contained a haploid set of chromosomes as identified by FISH using specific probes for

chromosomes 16, 18 and X. Unlike nuclear transfer into human oocytes, which results in either one or two pronuclei or one pronucleus and one polar body (Takeuchi *et al*., 1999, 2001; Tsai *et al*., 2000; Tesarik *et al*., 2001), somatic cells inserted into enucleated mouse oocytes were reported to produce two or more pronuclei (Wakayama *et al*., 1998). Although two pronuclei appeared in the majority of oocytes (64%), the rest (36%) had three or more pronuclei, suggesting that the separation of the chromosomes is random and that not all cells are capable of normal haploidization in MII oocytes in this species. In the present study, however, all oocytes that extruded two second polar bodies formed two pronuclei. One originated from the oocyte and the other from the cell inserted (**Figures 1 and 2**).

DNA was identified in both second polar bodies extruded from the oocytes and two distinct groups of DNA were identified within the cytoplasm (**Figure 2**). Although the somatic cell derived pronuclei are assumed to be haploid, and therefore the resultant embryos are diploid, their abnormal development may be related to structural aberrations of the chromosomes. This will need to be examined in further studies. Normal chromosome structure is important for gene expression during preimplantation development of the mammalian embryo (Thompson, 1996). In this study seven oocytes were fixed and five were examined: all these had DNA in the polar body and in the oocytes. However more eggs need to be examined in order to confirm these observations. Moreover, chromosome analyses, i.e. karyotyping and fluorescent in-situ hybridization, are essential and these studies have been initiated.

It has been suggested that the correct imprinting of genes is required for normal development, but that this may be overemphasized because of epigenetic modifications that occur following fertilization and embryo development are more likely to effect embryo development (Reik *et al*., 1993). Following fertilization, widespread demethylation of the zygotic DNA occurs, with the exception of imprinted genes, as the DNA is reprogrammed to enable the correct pattern of embryonic gene expression to be established (Monk *et al*., 1987). The high level of DNA methylation present in somatic cells is likely to affect this process. The maternally and paternally inherited alleles of imprinted genes are distinguished by epigenetic modifications, notably methylation, which allow the developing embryo to express either allele as and when required for normal development (Surani, 1998). In secondary spermatocytes both alleles are imprinted similarly to spermatozoa, hence inheritance of either allele would have no adverse effect on the expression of imprinted genes in the resulting embryo. In somatic cells, however, only one allele is imprinted, either maternally or paternally. In this case, inheritance of the maternal set of chromosomes or a random separation of chromosomes could result in the abnormal inheritance and ultimately expression of imprinted genes (Trounson, 2001). In a preliminary study (data not shown) involving three blastocysts developed from male fibroblast zygotes, the inheritance of the maternally imprinted *snrp* gene appeared to be random. Two of the embryos did not express the gene, which indicated that they inherited a maternally imprinted allele, and one expressed the gene, indicating that in this embryo the paternally imprinted allele was inherited. It is yet to be established if chromosomal separation during the expulsion of the second polar body of the somatic nucleus is random in all cases.

Regardless of whether the chromosomes are separated according to their origin or not, it is not known how this event occurs, as the information obtained from human and mouse studies is very limited. In mice, the nucleus of the somatic cell is transformed into disarrayed chromosomes 3 h after injection (Wakayama *et al*., 1998). Single condensed chromatids are each attached to a single spindle and are therefore not aligned on a metaphase plate. At ~1 h after activation the chromosomes are segregated into two groups and ~5 h later, two pronuclei appear. In the human, induction of premature chromosome condensation (PCC) in Hela cells, which are in a different stage of the cell cycle following fusion to metaphase cells, was not accompanied by the appearance of a mitotic spindle although the chromosomes migrated to two poles in the next cell division cycle (Johnson and Rao, 1970).

Only a limited number of embryos produced by cumulus cells in the present study were transferred to foster females, which may account for the inability to produce live offspring. It took >100 embryos to produce two pups in the initial cloning studies in mice (Wakayama *et al*., 1998; Wakayama and Yanagimachi, 1999). The proportion of live offspring from mouse cloning increased only slightly with time. The more recent mouse cloning studies reported 3–7% live offspring (Ogura *et al*., 2000; Wakayama *et al*., 2000). This minor improvement was related to the cell type used and the identification of the most receptive cytoplasm. Our attempts to increase the production of artificial gamete zygotes resulted in a slight increase when artificial activation was performed by exposure to ethanol alone and in a significant increase when older oocytes, which respond more readily to artificial activation, were used. For more efficient production of somatic cell zygotes with higher capability to develop to blastocysts and to term, other artificial activation procedures should be investigated (Sasagawa and Yanagimachi, 1996) using different cell types (Wakayama *et al*., 1998; Wakayama and Yanagimachi, 1999; Ogura *et al*., 2000; Ono *et al*., 2001).

The success of the present technique will be measured by the ability to produce progeny and if the progeny is genetically, physiologically and morphologically normal. If successful, the method will allow the production of embryos for couples where the male partner is sterile. Established cell lines from skin or muscle cells of men with spermatogenesis arrest or sterile men following chemotherapy or X-irradiation can be stored for many repeated treatment cycles. It would also be possible in the future to combine the artificial oocyte (Takeuchi *et al*., 2001; Tesarik *et al*., 2001) and artificial germ cell procedures, by injecting two somatic cells into enucleated oocytes (Trounson, 2001). Following artificial activation, the reconstituted oocytes would be expected to extrude a second polar body from both somatic cells and form two pronuclei, resulting in diploid zygotes. However, it is necessary to determine if epigenetic regulation of imprinted genes will allow normal development because the alleles derived from the somatic cells will probably retain the signature of their origins rather then the balanced set derived from male and female gametes. Unlike cloning where the complete set of alleles are derived from the somatic cell imprints, only half the genomic complement needs reprogramming because the oocyte chromosomes are retained for making artificial germ cell procedures. It would be interesting to see if this produces better outcome for the normality of development than does cloning (Trounson, 2001).

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