Zygote **15** (February), pp. 25–33. © 2007 Cambridge University Press
doi:10.1017/S0967199406003947 Printed in the United Kingdom

Somatic cell nuclear transfer using transported *in vitro*-matured oocytes in cynomolgus monkey

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Date submitted: 05.11.05. Date accepted: 09.03.06

Summary

Somatic cell nuclear transfer (SCNT) is not successful so far in non-human primates. The objective of this study was to investigate the effects of stimulation cycles (first and repeat) on oocyte retrieval and *in vitro* maturation (IVM) and to evaluate the effects of stimulation cycles and donor cell type (cumulus and fetal skin fibroblasts) on efficiency of SCNT with transported IVM oocytes. In this study, 369 immature oocytes were collected laparoscopically at 24 h following human chorionic gonadotrophin (hCG) treatment from 12 cynomolgus macaque (*Macaca fascicularis*) in 24 stimulation cycles, and shipped in pre-equilibrated IVM medium for a 5 h journey, placed in a dry portable incubator (37 °C) without CO_2 supplement. A total of 70.6% (247/350) of immature oocytes reached metaphase II (MII) stage at 36 h after hCG administration, MII spindle could be seen clearly in 80.6% (104/129) of matured IVM oocytes under polarized microscopy. A total of 50.0% (37/74) of reconstructive SCNT embryos cleaved after activation; after cleavage, 37.8% (14/37) developed to the 8-cell stage and 8.1% (3/37) developed to morula, but unfortunately none developed to the blastocyst stage. Many more oocytes could be retrieved per cycle from monkeys in the first cycle than in repeated cycles (19.1 vs. 11.7, $p < 0.05$). There were no significant differences in the maturation rate (70.0 vs. 71.4%, $p > 0.05$) and MII spindle rate under polarized microscopy (76.4 vs. 86.0%, $p > 0.05$) between the first and repeat cycles. There were also no significant differences in the cleavage rate, and the 4-cell, 8-cell and morula development rate of SCNT embryos between the first and repeat cycles. When fibroblast cells and cumulus cells were used as the donor cells for SCNT, first cleavage rate was not significantly different, but 4-cell (50.0 vs. 88.9%, $p < 0.05$) and 8-cell (0 vs. 51.9%, $p < 0.01$) development rate were significantly lower for the former. In conclusion, the number of stimulation cycles has a significant effect on oocyte retrieval, but has no effect on maturation and SCNT embryo development; however, different donor cell types (cumulus and fibroblast) resulted in different developmental potentials of SCNT embryos.

Keywords: Cloning, *In vitro* maturation, Monkey, Nuclear transfer, Oocytes, Transport

Introduction

Somatic cell nuclear transfer (SCNT) is a powerful technique for the multiplication of unique animal genotypes and the preservation of endangered animals (Wells *et al.*, 1998a). Since the first convincing demonstration of SCNT (Wilmut *et al.*, 1997) and production of the first transgenic cattle (Cibelli *et al.*, 1998), there has been a renewed interest in both understanding the concept of nuclear reprogramming and in developing more efficient ways of producing animals by nuclear transfer. Although successful production of animal clones from somatic cells has been achieved in many species, there has been no success in non-human primates. Blastocysts only have been developed from rhesus embryos after SCNT (Mitalipov *et al.*, 2002), and early pregnancies have been achieved by our group after transfer of SCNT embryos (Ng *et al.*, 2004).

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https://doi.org/10.1017/S0967199406003947 Published online by Cambridge University Press

The success of SCNT depends on several parameters that affect the cytoplast's reprogramming of the nucleus of the donor cell, or reversing the epigenetic changes that occur during development (Wilmut *et al*., 2002). Donor cell and recipient oocyte are the two key factors to successful SCNT. The potential of various cell types to serve as nuclear donors has been evaluated, for example cumulus (Ng *et al*., 2004), embryonic stem (ES) cells (Wakayama *et al*., 1999), fetal and adult fibroblasts (Mitalipov *et al*., 2002; Ng *et al*., 2004), granulosa (Arat *et al*., 2001), myoblasts (Gao *et al*., 2003a), neurons (Zawada *et al*., 1998), Sertoli cells (Inoue *et al*., 2003), tumour cells (Li *et al*., 2003), and B and T donor cells (Hochedlinger & Jaenisch, 2002). To date, no particular cell type has been shown to have an overwhelming advantage over another (Oback & Wells, 2002; Powell *et al*., 2004). Different cultured cell donors, however, have had great influence on the efficiency of SCNT (Wakayama & Yanagimachi, 2001a; Gao *et al*., 2003b; Wells *et al*., 2003; Powell *et al*., 2004). As well as the cell type, there has been some dispute in the literature regarding which donor cell's cell cycle stages are optimum (Cibelli *et al*., 1998). However, various combinations of donor cell stage and oocyte age have resulted in live healthy offspring (Kurosaka *et al*., 2002; Miyoshi *et al*., 2003; Wells *et al*., 2003).

As recipient cytoplast, MII-stage oocytes are the most often used; however, reports have shown that pig MI oocytes were able to support development to the blastocyst stage (Miyoshi *et al*., 2001). The use of aged cytoplasts resulted in the birth of calves (Vignon *et al*., 1998), and the use of oocytes enucleated in the telophase II stage resulted in embryonic development in cattle (Bordignon & Smith, 1998) and has resulted in the birth of cloned goats (Baguisi *et al*., 1999) and mice (Baguisi & Overstrom, 2000). Activated mouse zygotes, however, were not suitable for nuclear transfer (Wakayama & Yanagimachi, 2001b). In farm animals, large-scale application of SCNT methods are mainly based on IVM oocytes, especially cattle nuclear transfer (Dinnyes *et al*., 2002). Ovaries and immature/mature oocytes are now available from commercial suppliers or slaughterhouses (Hyun *et al*., 2003; Powell *et al*., 2004). Although IVM is still not fully optimized in all species, the birth of progeny following IVM, and then nuclear transfer, in sheep (Wells *et al*., 1997, 1998b), pig (Betthauser *et al*., 2000), and goat (Keefer *et al*., 2001) establishes the feasibility of using IVM oocytes in these species. However, in primates, oocytes or ovaries are not available commercially; moreover, the developmental competence of primate oocytes that mature *in vitro* is markedly inferior to that of their *in vivo*-matured counterparts (Schramm & Bavister, 1999). To obtain multiple oocytes in macaques, female monkeys are treated with human gonadotropic hormones to induce development of multiple follicles (VandeVoort *et al*., 1989). However, treatments of monkeys with exogenous gonadotropins especially HCG, can initiate an immune response, and eventually the animals no longer, respond to the human hormones (Ottobre & Stouffer, 1985; Bavister *et al*., 1986; Iliff *et al*., 1995; VandeVoort *et al*., 2003). It had been reported that initial cycles were more productive than repeated stimulation cycles (Wolf *et al*., 1999).

Oocytes and embryos need to be transported between laboratories, and it has been reported in the monkey and in mouse models that this transport does not affect maturation and further development (Chen *et al*., 2005, 2006). The objectives of this study were: (1) to investigate the effects of monkey stimulation cycle on the oocytes retrieval and *IVM*; and (2) to evaluate the effects of different monkey stimulation cycles and donor cell types on the efficiency of SCNT with transported IVM oocytes.

Material and methods

Animals

Twelve mature female long-tailed macaques (LoTM, *Macaca fascicularis*) weighing between 2.0 and 3.0 kg were used in this experiment; each monkey had had two oocyte retrievals (first and repeated cycles). All procedures were approved by the Animal Ethics and Welfare Committees, Animal Holding Unit, National University of Singapore and Department of Laboratory Animal Science, University of Malaya.

Macaque ovarian stimulation and oocytes recovery

Procedures for superovulation of the long-tailed macaque and collection of their oocytes have been described previously (Ng *et al*., 2002). Briefly, female monkeys were hyperstimulated with a fixed regimen comprised initially of downregulation with GnRH agonist, triptorelin (Decapeptyl, Ferring). Two weeks later, recombinant human follicle stimulating hormone (rhFSH; Gonal-F, 75 IU, Serono) was administered daily for 12 days. On the last day of FSH treatment, human chorionic gonadotrophin (hCG; Profasi, Serono) was administered intramuscularly. Laparoscopic oocyte recovery (OR) was performed at 24 h after hCG from sedated monkeys with a double-lumen needle (FAS Set C2, Gynetics Medical Products N.V.) attached to the Cook aspiration and flushing systems (V-MAR 5100; V-MAR 4000, respectively, Cook). The monkeys were rested for 3 months before the second stimulation cycle.

Oocytes maturation culture *in vitro* and transport

The cumulus oocyte complexes (COCs) were collected in HEPES-buffered Ferticult Flushing Medium

https://doi.org/10.1017/S0967199406003947 Published online by Cambridge University Press

(FFM, FertiPro N.V.). These were then washed and transferred into 5 ml tubes containing 2 ml of equilibrated IVM medium (TCM199-based medium: 10% FCS, 0.23 mM pyruvate, 1 IU/ml hCG, 1 IU/ml rhFSH, 1 μg/ml E2, 1 μg/ml P4), and cultured in an incubator (37 °C, saturation humidity, 5% CO_2 in air). After OR, tubes containing oocytes were capped tightly and transported in a dry portable CO_2-deficient incubator at 37 °C for a 5 h journey. Upon arrival at the laboratory, the COCs were transferred from tubes to IVM culture droplets, and cultured in a 5% CO_2 incubator at 37 °C until 36 h after hCG. Oocytes were stripped of cumulus cells by expose to hyaluronidase (80 IU/ml; Type IV-S bovine testes, Sigma H3884, Sigma–Aldrich), checked for their maturity and then placed in IVF-20 (Vitrolife) at 37 °C in 5% CO_2 until further use.

Macaque somatic cell nuclear transfer

Procedures for macaque SCNT have been described previously (Ng *et al.*, 2004). Donor cell preparation: cumulus cells were obtained from the 12 h *in vitro*-cultured IVM COCs of the macaques that had oocyte recoveries (ORs), and kept in HEPES-buffered IVF medium until further use. Frozen-thawed skin fibroblast cells derived from an adult male lion-tailed macaque (*M. silenus*) were plated in cell culture flasks (75 cm^3) at 1–3 × 10^6 cells/flask. When the cells were 75–85% confluent after 5–7 days of culture, they were cultured in DMEM with 2% FBS for 2 days and then 0.5% FBS for another 3 days with serum starvation. On the day of nuclear transfer, cell monolayers were disaggregated using PBS containing 0.15% (w/v) trypsin and 1.8 mM EDTA, pelleted by centrifugation and resuspended in DMEM until further use. Enucleation: the recipient MII oocytes were loaded individually into 5 μl microdroplets; a small opening on the zona pellucida was made by acidic Tyrode solution (pH 1.8), followed by aspiration of the second meiotic spindle under polarized microscopy (PolScope, SpindleView). After enucleation, the karyoplast was stained with 1 μg/ml DNA dye Hoechst 33342 to confirm complete removal of the maternal chromosomes under UV light. Oocytes were identified as present (positive) or absent (negative) of meiotic spindle under polarized microscopy. Some of the oocytes were used for other experiments. Nuclear transfer: LoTM fresh cumulus and starved fetal skin fibroblast cells were used as donor cells for nuclear transfer. A single donor cell collected from 10% polyvinylpyrrolidone (PVP; Sigma P0930) droplet was ruptured by gentle aspiration in and out of the injection needle, then directly microinjected into the enucleated oocyte 2 h after enucleation. Activation: cells were induced 2 h after cell microinjection by two consecutive direct current pulses (1.5 kV/cm, 50 μsec) from a BTX Cell Manipulator 2001 (Genentronics Inc.), and then after another 2 h combined with 7% ethanol for 5 min. After activation, SCNT embryos were cultured in IVF-20 containing 5 μM cytochalasin B and 10 μg/ml cycloheximide for 5 h and then transferred to IVF-20 for further culture.

SCNT embryo culture

The SCNT embryos were cultured in IVF-20 (Vitrolife) at 37 °C with 5% CO_2, 5% O_2 and 90% N_2. Nuclear formation was checked on day 1 before transfer to G1.2 (Vitrolife). Another 24 h later at D2, all SCNT embryos were transferred to G2.2 (Vitrolife) and thereafter to a new fresh G2.2 dish every 48 h.

Statistical analysis

Results were analysed using the Pearson's chi-squared test and the *t*-test. A probability value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Oocytes recovery and *IVM*

A total of 369 immature oocytes (15.4 per cycle) were retrieved from 12 long-tailed macaques at 24 h after hCG administration in 24 stimulation cycles (two cycles for every monkey) by laparoscopy. A total of 70.6% (247/350) of oocytes were matured after 12 h culture *in vitro* including 4–5 h shipment in portable dry incubator. MII spindles can be seen clearly (SPV positive) in 80.6% (104/129) of morphologically mature oocytes under polarized microscopy (Table 1). Comparing the OR efficiency in the first and second cycles, only one monkey produced more oocytes in the second OR cycle than in first OR. There were more oocytes collected in the first cycle (19.1 vs. 11.7 oocytes/cycles, $p = 0.01$), but there was no significant difference at 36 h after hCG in maturation rate (70.0% vs. 71.4%, $p = 0.77$) and SPV positive rate (76.4% vs 86%, $p = 0.17$).

Efficiency of somatic cell nuclear transfer using IVM oocytes

A total of 84 *in vitro*-matured oocytes from 17 monkey cycles were used for SCNT, 88.1% (74/84) of matured oocytes were successfully enucleated under polarized microscopy and microinjected with somatic cell types (LoTM cumulus cell and LoTM fetal skin fibroblast cell). After activation, 50.0% of SCNT embryos cleaved to 2-cell, 32.4% arrested at one cell and 17.6% were fragmented; 4-cell and 8-cell development rate of 2-cell

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Table 1 Effect of monkey stimulation cycle on oocyte retrieval and IVM[a]

Stimulation cycle	Oocytes retrieved (*n*)	Oocytes per cycle	Oocytes for IVM (*n*)	Maturation rate (%)	Oocytes for SPV[b]	SPV positive rate %
First	229	19.1 ± 8.1[c]	210	70.0 ± 23.1	72	76.4 ± 12.9
Second	140	11.7 ± 5.8[d]	140	71.4 ± 26.8	57	86.0 ± 15.5
Total	369	15.4 ± 7.8	350	70.6 ± 25.0	129	80.6 ± 15.6

[a] The value indicated is the mean ± SD, each monkey had two cycles, with a total of 12 monkeys.
[b] SPV: checking the spindle under polarized microscopy (SpindleView CRI, Woburn MA); SPV positive = clear spindle viewed under SpindleView system.
[c,d] Letters indicate that figures within the column show a significant difference, i.e. *c* is significantly different from *d* ($p < 0.05$).

Table 2 Effect of stimulation cycle number on the efficiency of SCNT in macaques[a]

Stimulation cycle	Number of monkey cycles	Oocytes before enucleation (*n*)	Oocytes after cell injection (*n*)	Frag. (%)[b]	1-cell (%)[b]	2-cell (%)[b]	4-cell (%)	8-cell (%)	Morula (%)
First	8	41	36	19.4	36.1	44.4	75.0	31.3	6.3
Second	9	43	38	15.8	28.9	55.3	81.0	42.9	9.5
Total	17	84	74	17.6	32.4	50.0	78.4	37.8	8.1

[a] Division rate in first cell cycle were based on number of embryos after cell injection; 4-cell, 8-cell and morula rates were based on the cleaved 2-cell number.
[b] First cell cycle.

Table 3 Efficiency of SCNT in macaques with different donor cells

Donor cell	Monkey cycles (*n*)[a]	Oocytes before enucleation (*n*)	Oocytes after cell injection (*n*)	Frag. (%)[b]	1-cell (%)[b]	2-cell (%)[b]	4-cell (%)	8-cell (%)	Morula (%)
Fibroblast	5	28	24	33.3	25.0	41.7	50.0[a]	0[a]	0
Cumulus	15	56	50	10.0	36.0	54.0	88.9[b]	51.9[b]	11.1

[a] Oocytes from same monkey may be injected into different type of donor cell after enucleation.
[b] First cell cycle.

SCNT embryos were 78.4% and 37.8%, respectively; only 8.2% of SCNT embryo developed into morula; unfortunately, none developed into blastocyst.

There was no significant differences in 2-cell, 4-cell, 8-cell and morula development rates between the two cycles (Table 2). The efficiency of SCNT was affected by donor cell type (Table 3). Between the donor cells, there was no significant difference in 2-cell rate; but 4-cell development rate was significantly lower (18.5% vs. 51.1%, $p = 0.0058$), and none developed into 8-cell when fibroblast cells were used as the donor cells. Eight-cell and morula development rates of SCNT embryos using cumulus cells as donor cells were 29.8% and 6.4%, respectively.

Discussion

Overall OR efficiency and IVM

In this study, a total of 369 immature oocytes were obtained at 24 h after hCG treatment for an average of 15.4 per surgical intervention (range 4–32). In total, 70.6% of immature oocytes reached MII stage after a 12 h culture *in vitro*, including a 5 h journey. This OR efficiency is higher than previously reported for 9.8 oocytes collected per cycle (Ng *et al.*, 2002), but similar to our later study of 16.5 oocytes per cycle (Ng *et al.*, 2004) and 16.7 per surgery by Wolf *et al.* (1999), but is less than the 23–34 oocytes collected per animal (VandeVoort *et al.*, 2003). However, this difference may due to use of different methods for oocytes collection and stimulation protocol. Maturation rate (70.6%) was almost same as reported, 64.7% (Ng *et al.*, 2002) and 61–91% (VandeVoort *et al.*, 2003). Although there was a report that the optimal time from hCG injection to oocyte collection was a 27 h interval (Wolf *et al.*, 1996), a subsequent report by same group showed that lengthening this interval to 30–34 h during nuclear transfer studies produced a higher yield of mature MII oocytes (Wolf *et al.*, 1999). Regarding the number of oocytes collected in different times after hCG, the results in this study (15.4 oocytes/retrieval at 24 h after hCG) was no different to our previous report of 16.5 oocytes retrieved at 34–36 h after hCG (Ng *et al.*, 2004). The maturation rate in this study (70.6%) after IVM culture was even slightly higher than the maturation rate (61.3%) *in vivo* of oocytes collected at 34–36 h after hCG as previous reported (Ng *et al.*,

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2004). Of course, this maturation rate refers only to morphological maturation, and not necessarily to cytoplasmic maturation. In this study, the MII spindle only can be seen clearly in 80.6% of mature oocytes under polarized microscopy. This may due to a small spindle, which may not be visible under polarized microscopy, or due to abnormal and/or dysfunctional spindle formation (Chen *et al*., 2006).

Effect of stimulation cycle on OR and IVM

Collection of oocytes is carried out routinely after ovarian stimulation, usually with human recombinant gonadotropins. Availability of these hormones has revolutionized non-human primate assisted reproduction technology (ART) because each female can usually be stimulated four or five times before becoming refractory. Previously, when using natural hormones (e.g., human urinary FSH, hCG, equine chorionic gonadotropin) animals became refractory after only one cycle of stimulation (Bavister *et al*., 1986). In this study, the initial cycle was more productive (19.1 oocytes/retrieval) than the repeat stimulation cycle (11.7 oocytes/retrieval, Table 1); however, there was no difference in maturation rate after IVM between the first and second cycles. This result was similarly to that reported by Wolf in 1999 (19.3/retrieval in initial cycle and 12.3/retrieval in repeat stimulation cycle). It is likely that the monkeys produce antibodies and become less responsive to the exogenous gonadotropins in the repeat stimulation cycles (Ottobre and Stouffer, 1985; Bavister *et al*., 1986; Iliff *et al*., 1995; VandeVoort *et al*., 2003). However, the generation of neutralizing antibodies did not have any deleterious effects on future reproductive capability, because of the failure of antibodies to cross-react appreciably with monkey LH or FSH and monkey chorionic gonadotropins (Iliff *et al*., 1995). To obtain more oocytes in macaques, female monkeys were treated with human gonadotropic hormones to induce development of multiple follicles (VandeVoort *et al*., 1989). The longer half-life and higher doses of hCG that are commonly used to induce maturation and ovulation of oocytes may be more antigenic than that of hFSH. It has been suggested that until the level of gonadotropic hormones may be decreased, by increasing the purity of the preparations and decreasing the amount of hormones given (Zelinski-Wooten *et al*., 1996), and if hCG were not used to induce oocytes maturation, it might be possible to stimulate each monkey more times, using only FSH to induce follicular development (Wolf *et al*., 1989). However, embryos resulting from oocytes recovered from monkeys stimulated only by FSH and matured *in vitro* exhibit impaired development compared with those embryos resulting from oocytes that undergo maturation *in vivo* (Lanzendorf *et al*., 1990; Schramm & Bavister, 1999). In this study, hCG was used to help the oocytes mature, and there was no difference in maturation rate between the initial and second stimulation cycles (Table 1). Given the costs of animals, their maintenance and hormones, macaque oocytes are extremely valuable, and it is worthwhile to collect oocytes a few times, if the oocytes collection still continue to yield good numbers of oocytes. Actually in another experiment, some monkeys were used for up to 7–10 stimulation cycles, and still produced six oocytes per cycle (Liow *et al*., 2002).

SCNT with IVM oocytes

The developmental competence of primate oocytes matured *in vitro* is markedly inferior to that of their *in vitro*-matured oocytes. This is primarily due to our poor understanding of the molecular processes involved in cytoplasmic maturation of primate oocytes and the lack of tools to study this process (Schramm & Bavister, 1999). In our previous study, cytoplasm maturation of the oocytes collected at 24 h after hCG and in 12 h culture *in vitro* is better than oocytes collected at 12 h after hCG and cultured 24 h *in vitro* for SCNT (Chen *et al*., 2006). In this paper, 84 transported *in vitro*-matured oocytes, collected at 24 h after hCG treatment, were used for SCNT. A total of 88.1% of oocytes were successfully enucleated and injected with donor cells. During the first cell cycle, 50% of SCNT embryos cleaved, 17.6% fragmented and 32.4% arrested at the 1-cell stage. This is similar to our previous study with *in vivo*-matured oocytes, i.e.: 48.5% cleaved (normal and abnormal), 14.7% fragmented and 36.8% arrested at the 1-cell stage (Ng *et al*., 2004). After cleavage, the 4-cell, 8-cell and morula stage developmental rates were 78.4%, 37.8% and 8.1%, respectively (Table 2); and none developed into blastocyst in this study using transported IVM oocytes. This developmental result was lower than that found in other reports using *in vivo*-matured oocytes: Mitalipov *et al.* (2002) reported that 27% of oocytes developed into morula and 1% developed into blastocysts. Our SCNT results show that there was no difference in the first cleavage rate between *in vivo*-matured oocytes and transported IVM oocytes for SCNT, but there were developmental consequences. We have previously reported in the mouse model that a few hours of transportation has no effect on IVM and embryos development (Chen *et al*., 2005). In our previous report with macaque oocytes collected at MII, pregnancies were achieved with transfer with 8-cell stages (Ng *et al*., 2004), but in this study, no SCNT embryos developed to blastocysts with transported IVM oocytes; we believe that this is due to the suboptimal IVM oocyte maturation, especially cytoplasmic maturation (Chen *et al*., 2006).

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This result shows that IVM oocytes are not as suitable as *in vivo*-matured oocytes for SCNT. It was also reported in porcine SCNT that no blastocysts were produced in three trials involving reconstruction using fibroblast karyoplasts with 88 IVM cytoplasts, whereas 2–5% developed into blastocysts with ovulated cytoplasts (De Sousa *et al*., 2002).

Effect of stimulation cycle on SCNT

In this study, there were no significant differences in the 2-cell cleavage, 4-cell, 8-cell and morula development rates between oocytes from first and second stimulated cycles. This shows that there is no difference in the quality of the matured oocytes retrieved from monkeys in first and repeat cycles. The same results were found in humans, that the oocyte quality, assessed as fertilization, implantation and pregnancy rates, was similar through three to five repeated controlled ovarian stimulation cycles (Caligara *et al*., 2001; Jain *et al*., 2005). And in mouse, the *in vitro* developmental competence of *in vivo* oocytes retrieved after repeated stimulation was not significantly different, and *in vivo*, similar implantation and absorption rates were observed following mating of animals subjected to repeated stimulation (Combelles & Albertini, 2003).

Effect of donor cell on SCNT

During initial SCNT experiments with sheep, donor cells were subjected to serum starvation in an effort to force them out of an active cell cycle and into a quiescent G_0 stage (Campbell *et al*., 1996; Wilmut *et al*., 1997). The chromatin in G_0 was thought to be more amenable to the structural changes required for reprogramming due to dramatically reduced transcriptional activity (Campbell, 1999). Since, many other offspring have been produced using cells that were expected to be quiescent. However, somatic cell cloning has also been achieved with donor cells in G_1 (Wilmut *et al*., 1997; Kasinathan *et al*., 2001), G_2 (Wakayama *et al*., 1999) and M (Ono *et al*., 2001) phases. But the studies showed a 3–7-fold increase in the production of viable offspring with embryos derived from adult quiescent G_0 cells (Wells *et al*., 2001, 2003). Donor cells at metaphase may be considered optimally compatible with MII oocytes and have resulted in improved rates of blastocyst development using murine embryonic stem (ES) cells, as opposed to nuclei at interphase; however, the postimplantation development was then characterized by a high mortality (Zhou *et al*., 2001). In this study, we used two cell types (cumulus and fibroblasts) that were expected to be quiescent at the $G_{0/1}$ stage, and are most commonly used as donor cells.

In the last few years, various differentiated cell types has been used as sources of nuclei for cloning domestic and laboratory animals. Almost all cell types tested so far have resulted in live offspring, although with great differences in their cloning efficiency (Renard *et al*., 2002). But to date, no particular somatic cell type has shown an overwhelming advantage over another (Oback & Wells, 2002; Powell *et al*., 2004). Conversely, it has been reported by many groups that the use of different donor cultured cells had great influence on the efficiency of SCNT (Wakayama & Yanagimachi, 2001a; Gao *et al*., 2003b; Wells *et al*., 2003; Powell *et al*., 2004). Differences in cloning efficiency are frequently observed between subcultures (batches) derived from the same biopsy (thus from the same genotype) in bovine species (Renard *et al*., 2002); this has also been reported between mouse embryonic cell lines (Humpherys *et al*., 2001) and between clonal fibroblasts cell lines derived from the same pig fetus (Kühholzer *et al*., 2001). In this study, we compared the most commonly used cells: fresh cumulus and cultured fibroblast cells. The results show that there was no significant difference in 2-cell cleavage rate during the first cell cycle, as with *in vivo*-matured oocytes (Ng *et al*., 2004). But there was significant difference in fragmentation rate and further development (4-cell and 8-cell); 51.9% and 11.1% of 2-cell SCNT embryos using fresh cumulus as the donor cell developed to 8-cell and morula, but none from fibroblasts as donor cells developed to the 8-cell and morula stages. The reason for this difference between donor cells is unclear, but could be because more than 90% of fresh cumulus are in the $G_{0/1}$ phase of the cell cycle (Schuetz *et al*., 1996). The situation of the cumulus cells was not studied 12 h after culture *in vitro* with COC. This result supports the hypothesis that the source of donor cells may be the most important factor in determining the success of a SCNT project (Miyoshi *et al*., 2003; Powell *et al*., 2004).

In conclusion, this study shows that the number of stimulation cycles has an effect on efficiency of oocyte retrieval and that the use of IVM oocytes may not be the ideal source for SCNT in non-human primates. There is a need for more research on IVM in non-human primates.

Acknowledgements

This study was supported mainly by A*STAR and partly by the National University of Singapore (R174–000–065–112/303) and the University of Malaya (FP013/2004A). The authors are grateful to Razali Jonit, Natasha Raina, Parani Baya, Nooraain Hashim, Anna Aryani Aryan, Alice Phua and Yuhaniza Shafinie Kamsani of University of Malaya, Kuala Lumpur, Malaysia, for their assistance.

https://doi.org/10.1017/S0967199406003947 Published online by Cambridge University Press

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