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Assessment of the developmental competence of human somatic cell nuclear transfer embryos by oocyte morphology classification

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BACKGROUND: The oocyte plays a key role in reprogramming the epigenetic status of donor cell nuclei, and the absence of reprogramming elements in the cytoplasm or aberrant accumulation of proteins can trigger the abnormal development of nuclear transfer (NT) embryos. Previous studies have demonstrated the relationship between oocyte morphology and both embryo development and pregnancy outcome. In the present study, we compared the morphology of oocytes with subsequent development of human somatic cell NT (SCNT) embryos.

METHODS: Piezo-assisted SCNT technology was used to produce reconstructed embryos, with almost 92% of oocytes reconstructed successfully. Depending on their morphologies, we separated metaphase II oocytes into four grades according to criteria which assess oocyte morphology, first polar body and perivitelline space, and especially, cytoplasm granula distribution.

RESULTS: Embryos from oocytes of Grades A and B could develop to the blastocyst stage with similar development efficiency for every developmental stage. However, embryos from Grade C oocytes arrested at or before the 8-cell stage then degraded, and the donor cell genome could not be activated and reprogrammed in such oocytes. For Grade D oocytes, cleavage was not observed in the reconstructed embryos, suggesting that the oocytes themselves have no developmental potential.

CONCLUSIONS: Our study revealed that different levels of developmental competence of SCNT embryos resulting from different oocyte reprogramming potentials associated with different morphologies. The results suggest that effective methods for improving oocyte quality should be studied, and that human SCNT efficiency would be increased following simple assessment of established oocyte morphology criterion.

Key words: human somatic cell nuclear transfer (SCNT) / oocyte morphology / oocyte donation / blastocyst development

Introduction

Stem cell therapy has tremendous treatment potential for replacing certain damaged cells, with proven feasibility in the mouse (Rideout *et al*., 2002; Barberi *et al*., 2003). However, the problem of immune incompatibility remains unresolved. Establishment of a homologous embryonic stem cell line derived from patient autologous cells using somatic cell nuclear transfer (SCNT) technology is widely regarded as a feasible solution, with success in the mouse and a non-human primate (Wakayama *et al*., 2001; Byrne *et al*., 2007). However, there are obstacles in the production of human blastocysts using SCNT technology, although one successful human SCNT study was recently reported (Stojkovic *et al*., 2005; French *et al*., 2008).

Interspecies studies have indicated that bovine and rabbit oocytes are capable of reprogramming human somatic cells, and interspecies blastocysts were produced (Chen *et al*., 2003; Chang *et al*., 2004). However, owing to ethical issues, such interspecies embryos can

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only be used in scientific research and are a long way from clinical application (Tecirlioglu *et al*., 2006; Minger, 2007). Recently, scientists in different laboratories have performed human SCNT experiments and tried to derive human SCNT blastocysts; these developed to the morula stage, but failed to develop to the blastocyst stage (Hall *et al*., 2007; Heindryckx *et al*., 2007). Successful derivation of human SCNT blastocysts was reported in 2005 (Stojkovic *et al*., 2005), and recently French *et al*. (2008) reported that human SCNT blsastocysts were successfully derived with higher efficiency from adult fibroblasts.

With the development of medical technology to assess oocytes prior to retrieval, follicular volume can be observed using B-type ultrasonic inspection and the oocytes in the follicles with determinate diameters can be regarded as matured *in vivo*. However, metaphase II (MII) oocytes collected from follicles are not uniform because of individual differences resulting from ovarian stimulation and the hormonal environment (Racowsky *et al*., 2005). In clinical treatment, some studies have reported successful pregnancy after embryo transfer using low-quality MII oocytes (Serhal *et al*., 1997; Balaban *et al*., 1998), but with lower efficiency compared with normal MII oocytes. For SCNT, the cytoplasm of the oocyte is regarded as the key environment for reprogramming the epigenetic status of donor cell nuclei. The molecular mechanisms of oocyte reprogramming remain unclear; however, poor-quality oocytes are probably deficient in reprogramming elements in the cytoplasm or have aberrant accumulation of proteins, such as major vault protein, which triggers the abnormal development of nuclear transfer (NT) embryos, even in fertilized embryos (Gioia *et al*., 2005; Sutovsky *et al*., 2005). It is impossible to perform molecular and biochemical testing of oocyte quality before SCNT. Therefore, it is necessary to establish a simple criterion to predict the oocyte's ability to reprogram the donor nucleus and to allow the subsequent development of an SCNT embryo.

To investigate this issue, we separated donated oocytes into four categories based on clinical standards in our assisted reproductive technology (ART) center, and compared their development efficiency after SCNT. Our aim was to establish an evaluation standard to predict the development potential of human SCNT embryos and to aid in understanding the normal mechanism of cytoplasmic reprogramming of human nuclei.

Materials and Methods

Donors

All donated oocytes came from the Reproductive Medical Center of the First Affiliated Hospital of SUN YAT-SEN University, which is certified by the Ministry of Health of the People's Republic of China. All oocyte donors were on an ART cycle. No financial benefit was involved in the donation process. Oocyte donors were clearly informed of all the study details, including the oocyte's use and research destination, and they voluntarily signed detailed informed consent documents. We guaranteed that all oocytes would be used in basic scientific research and not for reproductive purposes.

While conducting ART, we collected oocyte donations under the therapeutic cloning guidelines passed by the Ministry of Health of the People's Republic of China. We considered donation as an option when >30 oocytes were collected from a patient. Normally, only one to four oocytes were obtained from a donor and they were selected stochastically, so as not to have any impact on the patient's clinical treatment.

Ovulation induction and oocyte retrieval

A total of 21 couples were involved in this study (17 donors for SCNT and four donors for parthenogenetic activation). Each patient underwent a basic physical examination before ovulation induction, including tests for human immunodeficiency virus, hepatitis B virus, hepatitis C virus and contagious venereal disease. Patients had regular menstrual cycles every 29–32 days. Starting in the luteal phase of the previous cycle, on cycle day 21, eligible patients received 1.3 mg of a gonadotrophin-releasing hormone agonist (Dapherin) and then recombinant follicle-stimulating hormone (Gonal-f, Serono, Sweden) at a dose of 150–300 IU/day from Day 3 of the menstrual cycle to promote the growth of multiple follicles. When the diameter of dominant follicles reached 18 mm, a single dose of 10 000 IU of human chorionic gonadotrophin (Profasi, Serono, Sweden) was administered and transvaginal follicular aspiration was performed 36 h later. The cumulus–oocyte complex was cultured in culture medium (Quinn's Advantage^{TM} Fertilization Medium with 12% *v/v* Quinn's Advantage^{TM} SPS Serum Protein Substitute, SAGE IVF, Inc.) for 3–4 h in at 37°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 before SCNT.

Oocyte classification by morphologic criterion

Maturation and morphological features of the oocytes were investigated immediately before SCNT manipulation. Analyzed anomalies included dark central granulation of the cytoplasm, refractile bodies, vacuoles, aggregation of smooth endoplasmic reticulum (sER) an abnormal zona pellucida and an irregular first polar body (De Sutter *et al*., 1996; Ebner *et al*., 2003).

A normal matured MII oocyte should have a clear, moderately granulate cytoplasm, a small perivitelline space and an intact first polar body. Oocyte morphology criterion should be divided into two parts: one assesses extracytoplasmic abnormalities, including the shape of the oocyte itself, enlargement of the perivitelline space, presence of debris in the space and fragmentation of the first polar body, while the other determines if the cytoplasm is abnormal, with granulation and aggregation of the sER. Oocytes in the first grade or group are round with a smooth first polar body, dispersed cytoplasmic granula and normal perivitelline space (the widest space is similar to the diameter of the first polar body). The oocytes in the second group have similar morphology except slightly centralized granula. The oocytes in the third group are totally different from the first two groups. Although the oocyte morphology is round, the first polar body is inconspicuous, the morphology is fragmented or degenerated, the granula status is extensive centralization, and importantly the widest part of the perivitelline space is much smaller than the diameter of a normal first polar body. Sometimes there is no space which resembles an MI oocyte. The oocytes in the forth group have abnormal morphology, dispersed granules, and the widest part of the perivitelline space is much bigger than the diameter of a normal smooth first polar body with the oocytes able to wander in the perivitelline space; however, the morphology of the first polar body is similar to that in the third group (Fig. 1).

Preparation of donor cells

Donor cells were obtained by foreskin excision during the surgical procedure on a 3-year-old boy, and were named HFSF-1. The cells were cultured in medium containing 10% FBS and 90% low-glucose DMEM (Gibco) and were cryopreserved after two passages. At 48 h before NT, the fibroblasts were thawed and cultured for 2 days. Single cells were retrieved by

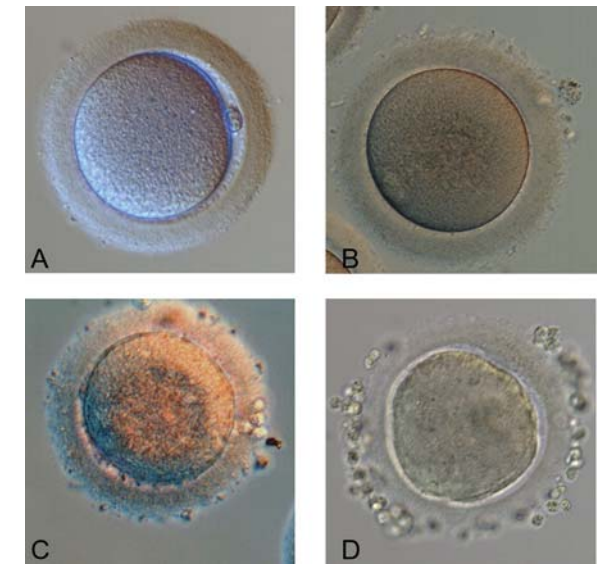

Figure 1 Four types of oocytes used in human SCNT experiments. (**A**) Grade A oocytes could support donor nuclei to develop into a blastocyst with high efficiency. (**B**) Grade B oocytes could support donor nuclei to develop into a blastocyst, but with low efficiency. (**C**) Grade C oocytes could not support donor nuclei to develop beyond the morula stage, but could be fertilized by IVF and could then develop further. (**D**) Grade D oocytes could support neither donor nuclei nor sperm.

trypsinization, and only cells in Passages 4–6 were used as donor cells (Fig. 2).

Karyotype and cell cycle of donor cells

Foreskin fibroblasts were incubated for 3 days and then collected and subjected to trypsinization. After washing with phosphate-buffered saline (PBS) and incubation in 0.075 mol/l potassium chloride for 10 min at 37°C, cells were fixed three times with 1:3 methanol/glacial acetic acid and dropped onto glass slides. Chromosome spreads were Giemsa-banded and photographed (Fig. 3). The karyotype of donor cells was determined in Passage 4–6, the same time as the cells were used in SCNT experiments.

HFSF-1 cells were cultured to 80% confluence and then washed in PBS without Ca^{2+} or Mg^{2+}. After treatment with 0.5% trypsin, the cells were collected and centrifuged at 350 g for 5 min at 4°C. The cell pellet was collected and the centrifugation was repeated. The cell pellet ($\sim 1.5 \times 10^6$ cells) was then resuspended in 70% ice-cold alcohol and stored at 4°C overnight. The next day the cells were centrifuged at 350 g for 10 min at 4°C and then resuspended in ice-cold PBS. After washing twice in ice-cold PBS, the cells were centrifuged again at 350 g for 10 min at 4°C. Then, 500 μl of propidium iodide/Triton X-100 staining solution was used to resuspend the cells, and 100 μl/ml DNAse-free RNAse A was added. After incubation for 20 min at 37°C, the sample was stored at 4°C and protected from light. The cells were analyzed by flow cytometry within 48 h, and the data were assessed using MulticycleAV (IBM-PC) (Fig. 4).

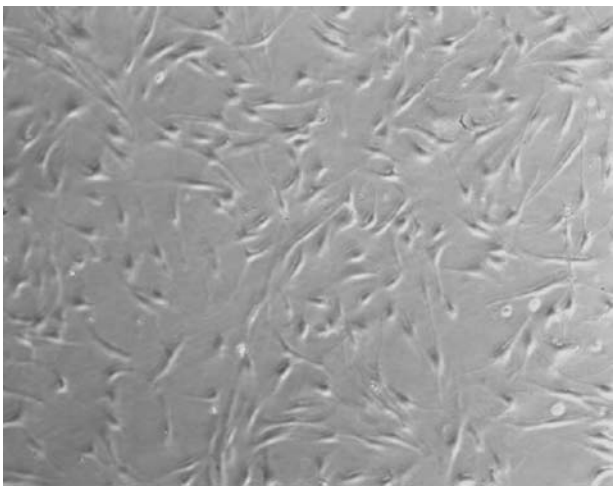

Figure 2 Morphology of HFSF-1 donor cells (×200 under an inverted microscope).

Somatic cell nuclear transfer

Cumulus–oocyte complexes were cultured *in vitro* in culture medium (Quinn's Advantage™ Fertilization Medium with 12% *v/v* Quinn's Advantage™ SPS Serum Protein Substitute), and then treated with 80 IU/ml hyaluronidase 30 min before SCNT micromanipulation. Mechanical handling was performed after brief hyaluronidase treatment. The oocytes were then cultured in an incubator for 30 min to balance the effects of *in vitro* manipulation. After this, three to four oocytes were transferred into a droplet of HEPES medium (Quinn's Advantage™ Medium with HEPEs with 12% *v/v* Quinn's Advantage™ SPS Serum Protein Substitute) containing 5 μg/ml cytochalasin B, and placed in an operation chamber on the microscope stage. The spindle, observed using Spindle View (Cri Inc.), was fixed at the 3 o'clock position using a hold needle. The one-step method reported by Zhou *et al*. (2003, 2006) was used; an injection needle was used to break down the zona pellucida using a piezo current, a donor cell was selected to inject into the cytoplasm along the cut, and then the spindle oocyte was removed.

After manipulation, reconstructed embryos were immediately transferred back into culture medium (Quinn's Advantage™ Fertilization Medium with 12% *v/v* Quinn's Advantage™ SPS Serum Protein Substitute) at 37.5°C and incubated for 2 h before activation. The reconstructed embryos were to be divided, according to oocyte morphology criterion, into different drops for culture.

Embryo activation and culture

According to our previous report (Mai *et al*., 2007), electric-activation was combined with chemical treatment to activate the reconstructed embryos. Electric-activation was performed in 0.3 M mannitol medium without calcium, and involved stimulation with two 20-μs pulses of 1.6 kV/cm using an electro cell manipulator (BTX 2001, San Diego, CA, USA). Reconstructed embryos were rinsed and exposed to 5 μM ionomycin (Sigma–Aldrich) for 5 min. After extensive washing in HEPES-buffered medium, the embryos were incubated at 37°C under 5% CO_2 in humidified air at 1.9 or 2 mM 6-DMAP (Sigma–Aldrich) for an additional 5 h.

After 6-DMAP treatment, groups of two to three reconstructed embryos in the same category were cultured in one droplet of cleavage culture medium (Quinn's Advantage™ Cleavage Medium with 12% *v/v* Quinn's Advantage™ SPS Serum Protein Substitute) under mineral oil (Sigma–Aldrich) at 37°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After 68–72 h of embryo activation, the embryos that developed to the 8-cell stage were transferred to blastocyst culture

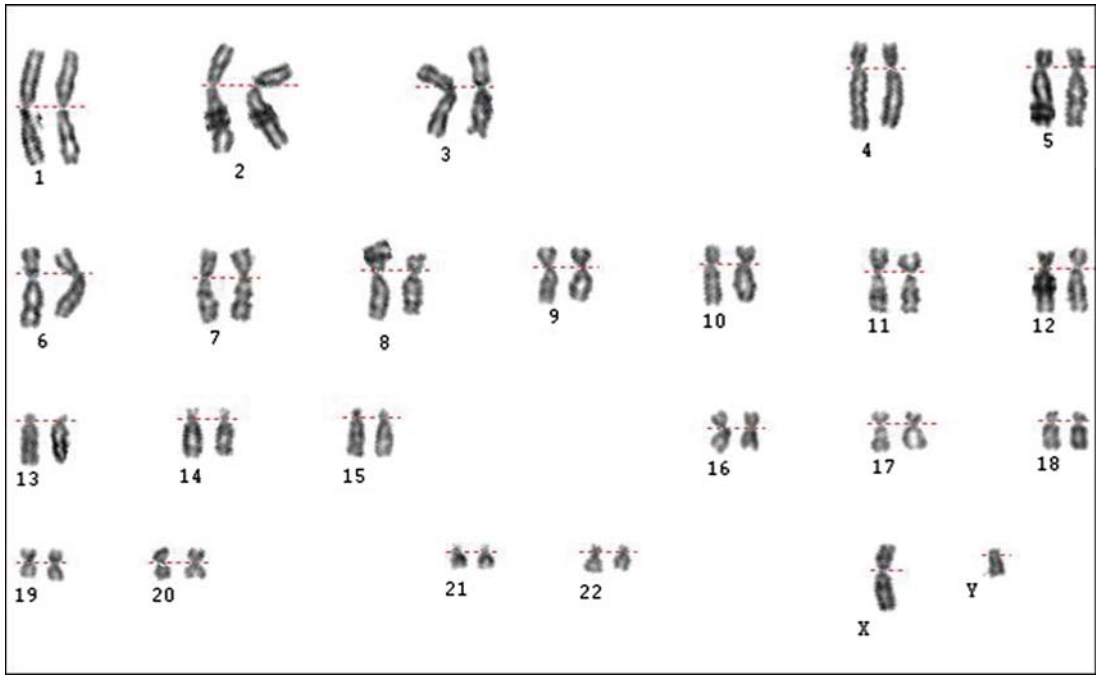

Figure 3 Karyotype of HFSF-1 donor cells at Passage 8.

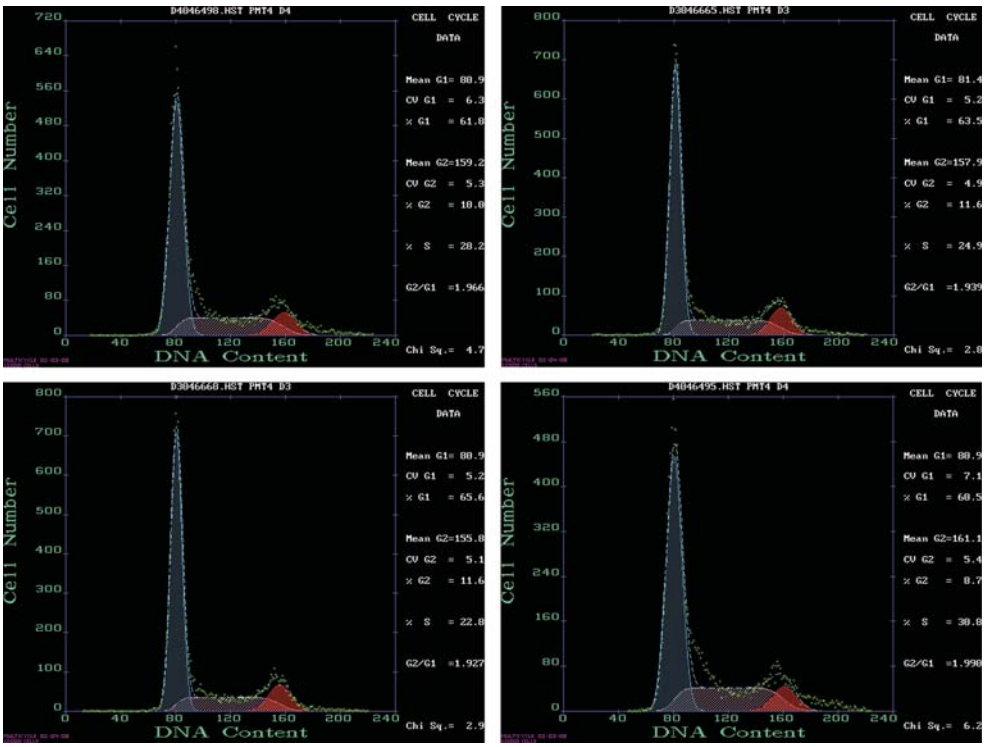

Figure 4 Cell cycle distribution of HFSF-1 donor cells.

medium (Quinn's Advantage™ Blastocyst Medium with 12% *v/v* Quinn's Advantage™ SPS Serum Protein Substitute) for sequential culture to the blastocyst stage.

Parthenogenetic activation

While performing the SCNT procedure, some fresh MII oocytes were retained for parthenogentic activation. Immature oocytes identified during the treatment of cumulus–oocyte complexes were incubated for a further 24 h (Quinn's Advantage™ Cleavage Medium with 12% *v/v* Quinn's Advantage™ SPS Serum Protein Substitute). Oocytes that exhibited a first polar body were considered mature and were also subjected to parthenogenetic activation. Activation and culture conditions were the same as for SCNT embryos.

Statistical analysis

All experiments, including SCNT experiment, karyotyping indentification and flow cytometry, were repeated at least three times. Embryo developmental data were analyzed by *t*-test using SPSS 13.0 software. Statistical significance was accepted at $P < 0.05$.

Results

Karyotype and cell cycle of HFSF-1 donor cells

HFSF-1 cells retained the normal morphology of fibroblasts during propagation (Fig. 2) and exhibited the 100% normal diploid 22-XY karyotype ($n = 50$) in Passage 4–6, which is when they were used in SCNT experiments (Fig. 3).

Different phases of the cell cycle in HFSF-1 cells were detected by flow cytometry (Fig. 4). The intense DNA peaks (represented by the blue grids) show that the majority of HFSH-1 cells at 80% confluence were in G0/G1-phase (60.5–65.6%). The distribution of S-phase (represented by light red diagonals) was 20.8–30.8%, and that of G2/M-phase was 8.7–11.6%.

Assessment of developmental efficiency

To investigate the development potential of human oocytes after artificial activation, some oocytes were subjected to parthenogenetic activation without an SCNT attempt, and the developmental efficiency was observed and recorded every 24 h. From a total of eight oocytes, seven fresh oocytes were activated after culture for 2 h in an incubator; of these, all embryos developed to the 2-cell stage, and two successfully progressed to blastocysts (Table I). Such blastocysts are capable of expanding and hatching, and these indeed hatched as normally fertilized blastocysts (Fig. 5I). In addition, a total of 28 GV or GVB oocytes were identified during cumulus–oocyte complex treatment, and 18 of these spontaneously expelled the first polar body after a further 24 h in *in vitro* culture. After artificial activation treatment, there was no difference from the cleavage rate between fresh and IVM oocytes; however, the activation rate and the blastocyst-development efficiency of fresh oocytes were significantly higher than for IVM oocytes (Table I).

Oocyte morphology score for prediction of embryo development

Poor-quality and immature oocytes have been regarded as a viable resource for human SCNT from an ethical point of view, but actual manipulation usually results in failure. To investigate this issue, we classified all donated oocytes into four categories using morphology criteria in our ART center (Table II).

Overall, regardless of oocyte group, 91.8% of the 61 oocytes survived after piezo-activation; of these surviving oocytes, 69.6% were activated with 39.3% cleaving to at least the 2-cell stage (Table III). Embryos from oocytes of Grades A and B developed with similar efficiency (33.3 versus 25%, respectively). However, embryos from Grade C oocytes developed no further then the 8-cell stage before arresting. Embryos from Grade D oocytes were activated with low efficiency and none could develop to the 2-cell stage (Table III). Good morphology and a distinct inner cell mass could be observed in the cloned blastocysts which had the ability to hatch (Fig. 5A–H).

Discussion

Human blastocysts can be obtained using SCNT technology

In the present study, human cloned blastocysts were obtained using a piezo-assisted injection method, which is different from the traditional electrofusion method previously described (Stojkovic *et al*., 2005). Oocytes are regarded as a valuable resource because of their limited numbers and the ethical issues involved, and the reconstruction efficiency using electrofusion is very low compared with the piezo-assisted injection method (Yu *et al*., 2007a, b). Comparison of these NT methods has been reported in some species, including the pig (Kurome *et al*., 2003), mouse (Yu *et al*., 2007a, b) and rhesus monkey (Zhou *et al*., 2006). Our previous mouse SCNT experiments suggested that there is no difference in blastocyst formation between the two SCNT methods, but the reconstruction efficiency is different. It is apparent that more oocytes are used for the electrofusion method, although a recent study reported increased fusion efficiency (Li *et al*., 2005). Compared with a recent human SCNT study (Stojkovic *et al*., 2005), our higher efficiency of activation and cleavage

Table I Development efficiency of parthenogenetic embryos

Oocytes	No. oocytes	No. oocytes matured *in vitro*	No. surviving oocytes that were activated	Development of activated oocytes (%)		
				2-cell	Morula	Blastocyst
Fresh	8	8	7 (87.5)[a]	7 (100)[a]	6 (85.7)[a]	2 (28.6)[a]
IVM	28	18	11 (61.1)[b]	11 (100)[a]	3 (27.3)[b]	1 (9.1)[b]

Different superscripts in the same line indicate a significant difference ($P < 0.05$).

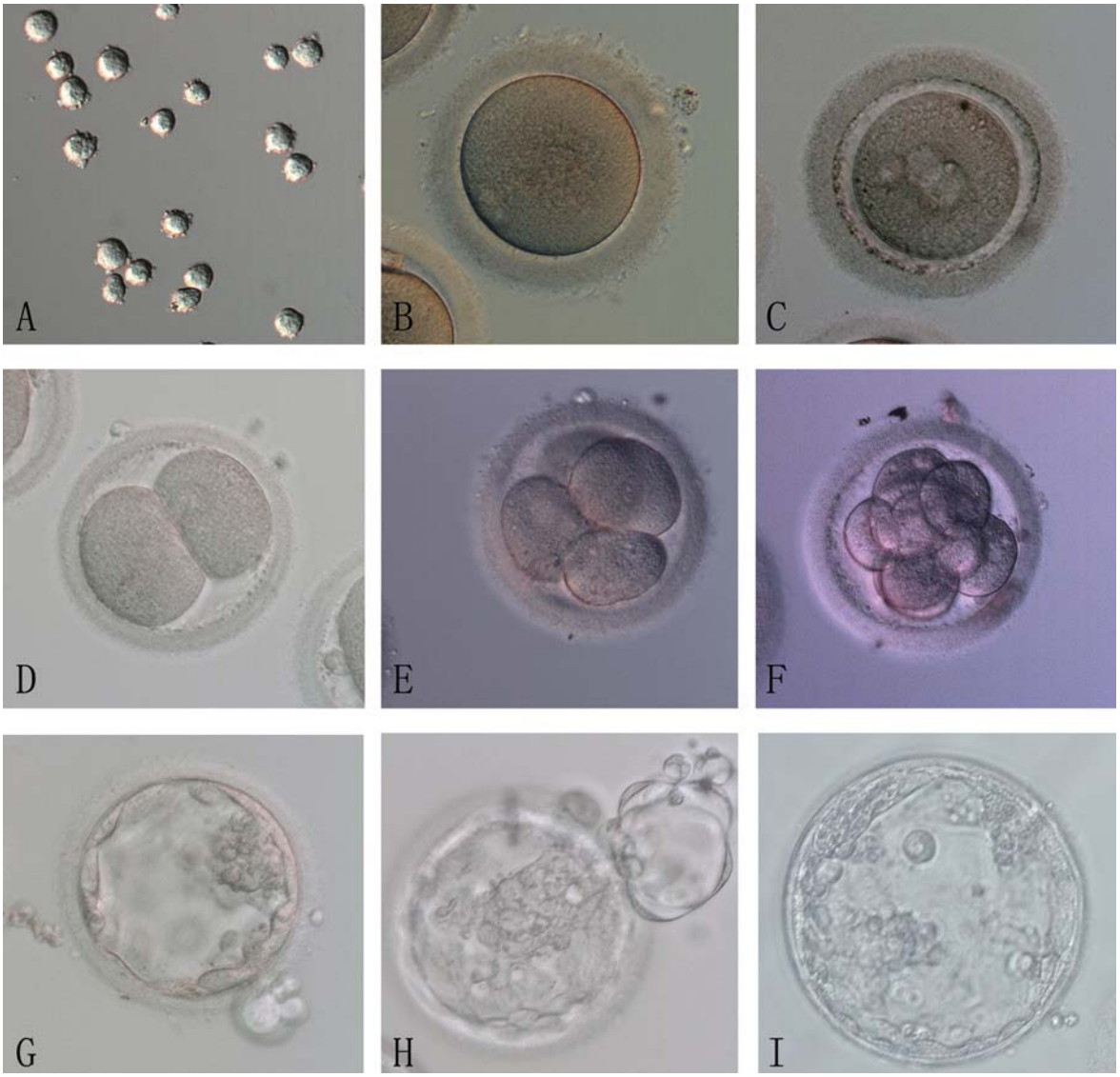

Figure 5 SCNT embryo development process *in vitro*.(**A**) Donor cells from foreskin fibroblast, (**B**) fresh MII oocyte, (**C**) pseudo-pronuclear embryo, (**D**) 2-cell stage, (**E**) 4-cell stage, (**F**) 8-cell stage, (**G**) expanded blastocyst and (**H**) hatching blastocyst. (**I**) Expanded blastocyst after parthenogenetic activation.

and similar efficiency of blastocyst formation for oocytes of Grade A or B was mainly attributed to the NT technology itself. Our previous study indicated that micronuclei are induced by piezo-assisted NT, and this is similar to apoptosis post-implantation (Yu *et al*., 2007b), but it is not harmful to blastocyst development. Thus, piezo-assisted methods are better than electrofusion techniques from the point of view of oocyte usage.

G0/G1 or pre-S-phase donor nucleus exhibita capacity for reprogramming when placed in a MII oocyte with a high level of maturation promoting factor, is shown in SCNT studies of many species. The usual methods of cell cycle synchronization of cultured cells are serum starvation and contact inhibition. Serum starvation is widely used for synchronizing somatic cells by arresting them in the G0/G1 phase of the cell cycle, but aberrant gene transcription has been observed in human primary fibroblasts treated using serum starvation method (Iyer *et al*., 1999). Also serum starvation method often reduces cell survival and increases DNA fragmentation (Kues *et al*., 2000), which causes high-embryonic losses after NT (Lawrence *et al*., 2005). Growing cells to confluence is another strategy used for synchronizing cells at G0/G1 stage of the cell cycle, and in the present study, ~65% of the cells were at G0/G1 after culturing to confluence. Although our efficiency is lower compared with the previous report in porcine (Boquest *et al*., 1999), which is possibly attributed to less confluence of somatic cells because the cells were only thawed and cultured for no more than 48 h, our approach coupled with assessment of cell diameter and other criteria, was sufficient to select a suitable donor cells.

Table II Morphological classification of oocytes

Grade	Oocyte morphology	PB I	Distribution of cytoplasm granula	Perivitelline space
A	Round	Obvious, smooth intact PB I retained—normal morphology	Dispersal	Normal, the widest space is similar to the diameter of the first polar body
B	Round	Obvious, smooth intact PB I retained—normal morphology	Slight centralization	Normal, the widest space is similar to the diameter of the first polar body
C	Round	Inconspicuous, fragmented or degenerated morphology	Excessive centralization	Smaller or none, the widest space is much smaller than the diameter of a normal smooth first polar body. Sometimes there is no space which looks like MI oocytes
D	Abnormal	Inconspicuous, fragmented or degenerated morphology	Dispersal	Larger, the widest space is much bigger than the diameter of a normal smooth first polar body, and the oocytes can wander in the perivitelline space

Table III Effect of oocyte morphology on the development efficiency of human SCNT embryos

Oocyte grade	No. oocytes	No. oocytes that survived piezo-activation	No. surviving oocytes that activated	Development of activated oocytes (%)			
				2-cell	8-cell	Morula	Blastocyst
A	10	9 (90)	9 (100)	8 (88.9)	6 (66.7)	3 (33.3)	3 (33.3)
B	4	4 (100)	4 (100)	4 (100)	2 (50)	1 (25)	1 (25)
C	41	38 (92.7)	24 (63.2)	10 (41.7)	2 (8.3)	0 (0)	0 (0)
D	6	5 (83.3)	2 (40)	0 (0)	0 (0)	0 (0)	0 (0)
Total	61	56 (91.8)	39 (69.6)	22 (39.3)	10 (17.9)	4 (7.1)	4 (7.1)

Oocytes score aids in evaluating oocyte developmental potential

Oocyte quality is regarded as a key factor in SCNT because the oocyte provides the space and materials for reprogramming of donor genetic materials, as well as the nutrition and substances required during further embryo development. The hope that IVM oocytes could be used for reprogramming using somatic cell nuclei (Takeuchi *et al*., 1999; Hall *et al*., 2007) arose from successful pregnancies established using IVM oocytes (Buckett *et al*., 2007). However, some researchers have reported low efficiency for IVM compared with fresh oocytes, with IVM oocytes even exhibiting abnormal expression of Oct-4, an important factor in embryo differentiation (Chen *et al*., 2004; Chang *et al*., 2005). Our parthenogenetic activation results suggest that the developmental potential of such oocytes is limited during the IVM process. IVM oocytes represent a tremendous cloning resource, but their low development potential remains a problem. Serial SCNT seems to represent a solution for cloning in mice (Heindryckx *et al*., 2002), but in humans it is impossible to obtain a normal zygote after a second SCNT.

Zygote scores have been predominantly used in ART, and some studies have indicated that such a score could predict the development and pregnancy efficiency of embryos in patients treated by IVF or ICSI (Edirisinghe *et al*., 2005; Payne *et al*., 2005). Although there are conflicting results from different ART centers (James *et al*., 2006; Nicoli *et al*., 2007), we still consider that a scoring system would be helpful in embryo classification and research. Oocyte classification is still scarcely reported, although zygote scores have been widely investigated. Serhal *et al*. analyzed ICSI results based on oocyte morphology and reported that while early and preimplantation development efficiency was not affected by oocytes of abnormal morphology or cytoplasm-containing oocytes, implantation failed for such abnormal oocytes (Ebner *et al*., 2003). In our SCNT study, we established oocyte grades based on maturation signs, including oocyte morphology, first polar body, cytoplasmic granula distribution and perivitelline space. Owing to different physiological reactions in patients to extrinsic hormones, different types of oocytes can be obtained from the ovulation induction procedure (Racowsky *et al*., 2005). Although strict criteria for oocyte collection have been established, including B-type ultrasonic inspection, follicular volume and clinical examination, so that few immature oocytes are obtained, there are still differences among mature MII oocytes.

A mature nucleus and cytoplasm are regarded as equally important for embryo development, and each is closely associated with oocyte morphology. The occurrence of the first polar body is proof of nuclear maturation, but another issue is to identify an evaluation standard for cytoplasm maturation. Moreover, cytoplasm contains accumulating materials, and provides proteins, RNA, morphorgenetic factors and protective chemicals. Thus, a mature cytoplasm seems to play a key role in reprogramming of the donor cell genome. Our results indicate that oocytes of grade A or B had the ability to support SCNT embryo development to the blastocyst stage, however when using oocytes of grade C, the reconstructed embryos arrested at or before the 8-cell stage, which indicated that

the zygotic genome was incompletely activated for such a reprogramming environment.

Although different studies have shown varying ICSI results (De Sutter *et al.*, 1996), oocyte quality is very important in SCNT. Successful SCNT is also highly dependent upon oocyte quality, as demonstrated in ferret cloning (Li *et al.*, 2006). In a bovine study, oocytes from different backgrounds had different effects on cloned embryo development *in vitro*, and this differed from the effect on IVF embryos (Yang *et al.*, 2008). According to our own experiments in mice, oocytes from mice of different backgrounds and ages exhibit different blastocyst development efficiency, even for embryonic stem cell establishment (data not shown).

Future perspectives

Although the present results are encouraging, the numbers of oocytes of Grades A, B and D were small, and the findings require verification with large sample sizes. SCNT still has low efficiency and is affected by many factors, including donor cells, recipient oocytes, NT technology and method, activation treatments, etc. Therefore, a predictable standard should be established for human SCNT because human oocytes are a rare resource. Recently, the potential of induced pluripotent stem (iPS) cell technology has been identified for therapeutic cloning (Takahashi *et al.*, 2007; Yu *et al.*, 2007a) and the technique was successfully applied in a mouse model (Hanna *et al.*, 2007). However, some problems need to be resolved before clinical application, including consideration of virus utility, epigenetic analysis, etc. (Stojkovic and Phinney 2008). Therefore, it cannot be assumed that iPS will replace ES cells in clinic therapy because of their lack of moral and ethical issues. For clinical therapeutic cloning, the realistic approach is still derivation of stem cell lines from cloned human blastocysts, because more studies have been attempted in ES cells, including differentiation, self-renewal and therapeutic security (tumor formation and epigenetic stability). Here, we set up an evaluation standard that predicts the development potential of human SCNT embryos and may help in the production of human SCNT blastocysts for therapeutic cloning.

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