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RESEARCH ARTICLE

Successful reprogramming of differentiated cells by somatic cell nuclear transfer, using *in vitro*-matured oocytes with a modified activation method

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Abstract

Therapeutic cloning has tremendous potential for cell therapy and tissue repair in some diseases. However, the efficiency of development of cloned human embryos by somatic cell nuclear transfer is still low. In the present study, the activation of cloned human embryos was investigated while using *in vitro*-matured oocytes. Pseudo-pronuclear formation and the subsequent development was compared with different activation parameters, including different durations of ionomycin and 6-dimethylaminopurine treatment. The results showed that somatic cells were successfully reprogrammed by modification of activation treatments while using *in vitro*-matured oocytes. The activation efficiency of cloned human embryos was significantly increased at durations of ionomycin at both 5 and 7 min, despite different durations of 6-DMAP treatment. The results of blastocyst development showed that 20% of activated embryos developed to the blastocyst stage when the embryos were activated with 5 μM ionomycin for 5 min and 2 mM 6-DMAP for 5 h, which was significantly higher than those activated with other parameters. Moreover, we found that an increasing duration of 6-DMAP induced the formation of a single, large, pseudo-pronucleus in cloned human embryos and impaired subsequent development competence. In conclusion, successful reprogramming of human somatic cells was achieved using *in vitro*-matured oocytes by somatic cell nuclear transfer and improved with a modified activation method.

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Supporting information may be found in the online version of this article.
Supplementary Movie 1. Spindle and nucleus in human IVM MII oocyte

Keywords somatic cell nuclear transfer; reprogramming; therapeutic cloning; activation; ionomycin; 6-dimethylaminopurine; pronuclear morphology

1. Introduction

Therapeutic cloning has tremendous potential for cell therapy and tissue repair (Pomerantz and Blau, 2004) in a variety of medical conditions, such as neurodegenerative disorders, diabetes and cardiac myocyte disorders (Hochedlinger and Jaenisch, 2003). However, some factors have limited the development of therapeutic cloning, including immune rejection and graft origin. Homologous embryonic stem cells appear to be a solution to this dilemma (Hall *et al*., 2006), with cloned blastocysts being derived from homologous somatic cells by nuclear transfer technology.

Cloned human blastocysts were first derived by nuclear transfer technology using embryonic stem cells (Stojkovic *et al*., 2005), but recent successes have been obtained for somatic cell nuclear transfer (SCNT). Hall *et al*. (2007) performed human SCNT using aged oocytes that failed to fertilize oocytes as recipients, but the cloned embryos did not develop beyond the eight-cell stage. In the same year, Heindryckx *et al*. (2007) used geminal vesicle (GV)

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oocytes as recipients and the embryos developed to the morula stage but no further. French *et al*. (2008) obtained five blastocysts from 21 fresh mature oocytes by SCNT technology, representing a blastocyst efficiency of 23%, which is comparable to the results obtained with rodents and livestock cloning; however, the cleavage efficiency was ≤50%, which is much lower than rodents and livestock. Our previous study (Yu *et al*., 2009) suggested that oocyte morphology is key to the development of cloned human embryos, and blastocyst efficiency was higher only in those oocytes with the best grading. Recently, Fan *et al*. (2011) suggested that Trichostatin A, a histone deacetylase inhibitor, could facilitate derivation of cloned human blastocysts using patient fibroblasts. The oocytes used in these studies were all donated fresh oocytes; however, it is key to finding a replaced resource for hSCNT because of the well-known ethical issues associated with donated fresh oocytes; *in vitro*-matured (IVM) oocytes appear to be a better substitution for SCNT owing to potentially avoiding or minimizing public concerns over ethical issues, but these are no reports about somatic cell reprogramming by IVM oocytes to date.

Embryo activation is a key step in the development of fertilized and cloned embryos (Alberio *et al*., 2001). In a study of SCNT, adequate activation could be helpful to keep correct ploidy of somatic nuclei after transfer into enucleated oocytes. The combination of ionomycin and 6-dimethylaminopurine (6-DMAP) is a routine tool in the activation treatment of cloned embryos of ruminants and non-human primates. Ionomycin was first identified as a polyether antibiotic with properties characteristic of a divalent cation ionophore in 1978 (Liu and Hermann, 1978), and was found to elevate the level of intracellular calcium in cells, and the study indicated that ionomycin enhances Ca^{2+} influx via activation of endogenous entry pathways and not by plasmalemmal translocation stimulation (Morgan and Jacob, 1994). 6-DMAP is a protein serine/threonine kinase inhibitor (Neant and Guerrier, 1988), and Ledda *et al*. (1996) suggested the close relation of 6-DMAP on DNA synthesis in activated mammalian oocytes. The condition of ionomycin and 6-DMAP in some specials has been indicated in a previous study (Choi *et al*., 2004); most of the protocols used for activation of reconstructed embryos involved exposure of oocytes to 5 μM ionomycin for 4–5 min, followed by treatment with 2 mM 6-DMAP for 2–6 h. The study also suggested that inadequate or erroneous duration of treatment with 6-DMAP will impair chromosome remodelling and sequential development (Choi *et al*., 2004). Moreover, in some studies 6-DMAP was involved in the control of post-fertilization, such as pronuclear (PN) formation (Szollosi *et al*., 1993) and chromatin remodelling by inhibiting DNA synthesis (Ledda *et al*., 1996).

In the present study, we investigated the effects of different activation parameters of ionomycin and 6-DMAP on pre-implantation of human SCNT embryos, with the aim of increasing the reprogramming efficiency of somatic cells in IVM oocytes. The number and morphology of pseudo-pronuclei under experimental conditions were analysed to study the relationship to the development of cloned human embryos by SCNT technology.

2. Materials and methods

2.1. Informed consent

All of the ART and therapeutic cloning procedures closely followed the guidelines legislated and posted by the Ministry of Health of the People's Republic of China, including *Technical Regulation for Human Assisted Reproductive Technology* and *Ethical Guiding Principles for the Research of Human Embryonic Stem Cell*. The present study was approved by the Institutional Review Board of Peking University Third Hospital.

All patients undergoing intracytoplasmic sperm injection (ICSI) cycles were clearly informed that it is possible for immature oocytes to be collected during the process of oocyte collection after super-ovulation, and only mature MII oocytes will be used in routine fertilization assisted with ICSI, so the immature oocytes will be discarded. Moreover, two choices for patients in the informed consent were unambiguously given regarding how the discarded immature oocytes would be handled: one, that the oocytes could be used for basic scientific research; or the other, that the oocytes would be discarded. The couples in ICSI cycles signed detailed informed consent documents voluntarily. Only the couples that declared that the immature oocytes would be given up and used for basic scientific research were involved in the present study. We guaranteed that the embryos would only be used for basic scientific research and not for reproductive purposes.

2.2. Experimental design

In the first experiment, direct and indirect proof were given to indicate the cloning origin of the embryos. In the second experiment, embryos were activated under different durations of ionomycin and 6-DMAP treatment, including ionomycin for 0, 3, 5, 7 and 10 min, and 6-DMAP for 2, 5 and 8 h. In the third experiment, the development of cloned human embryos was observed and compared among different experimental groups, according to the results in experiment 2. Finally, the PN morphology of cloned embryos activated under different durations of 6-DMAP treatment was determined and the development of cloned human embryos with different PN morphology was compared in three groups treated for 2, 5 and 8 h.

2.3. Oocyte collection

All chemicals were purchased from Sigma Aldrich Co. (Shanghai, China), unless otherwise indicated. In this study, 221 couples undergoing ICSI treatment cycles were involved. The cumulus–oocyte complex was treated using

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0.3 mg/ml hyaluronidase and oocytes with typical polar body I (PB I) were selected for clinical ICSI micromanipulation, whereas the immature oocytes at the GV and metaphase I stages were collected for IVM culture. In the process of immature oocyte selection, only oocytes with normal morphological characteristics (including regular oocyte morphology, appropriate thickness of the zona pellucida and dispersed cytoplasmic granules) were incubated in IVM medium in a humidified atmosphere at 5% CO_2 in air at 37 °C.

Commercial IVM basal medium (SAGE In Vitro Fertilization Inc.) was used in this study and preheated for at least 6 h before using. An additional 75 IU FSH and LH was added and mixed into the medium before IVM culture. Normally three to five oocytes were incubated in one 25 μl drop of IVM medium, overlaid with embryo-tested light mineral oil in a humidified atmosphere at 5% CO_2 in air at 37°C.

The mature oocytes were selected from the dish after 24 h for MI oocytes and 48 h for GV oocytes. The existence of PB I is the best evidence for defining oocyte maturation, as observed by an inverted microscope (DMI 3000B; LEICA, Germany) with Hoffman modulation contrast optics and a magnification of 200X.

2.4. Preparation of donor cells

The donor cells (hFF), which originated from a piece of discarded foreskin tissue from a 4 year-old child after circumcision and had a normal 46 XY karyotype, were used in our previous study (Lu *et al*. 2010). The cells were cultured in medium containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 90% Dulbecco's modified Eagle's medium [DMEM (no pyruvate, high-glucose formulation); Gibco), and cryopreserved after two passages. At 48 h before nuclear transfer, the fibroblasts were thawed and cultured for 1 day. Single cells were retrieved by trypsinization and only cells in passages 4–6 were used as donor cells.

2.5. Somatic cell nuclear transfer

In primary culture, residual cumulus cells around the oocytes were dispersed with 0.3 mg/ml hyaluronidase 30 min before SCNT micromanipulation. In the process of enzyme digestion, the oocytes were put into one drop of hyaluronidase, and meanwhile treated with blowing–suction using a mouth-controlled hand-made pipette with an inner diameter of 150–180 μm. The oocytes were cultured in an incubator for 30 min to eliminate the effects of *in vitro* manipulation. Then a group of five oocytes were transferred to a droplet of G-MOPES medium (Vitrolife, Gothenburg, Sweden) containing 5 μg/ml cytochalasin B, placed in an operation chamber on the microscope stage. The spindle was observed and fixed at the 3 o'clock position, using a hold needle, after observation using an spindle imaging apparatus (Oosight, Cri Inc., USA). The one-step method (OSM) reported previously by Zhou *et al.* (2006) was used to reconstruct an embryo clone in 1–2 min.

After manipulation, reconstructed embryos were immediately transferred back into G_1 culture medium (Vitrolife) supplemented with 5% v/v 100 mg/ml human serum albumin (HSA; Vitrolife) at 37.5°C and incubated 1 or 2 h before activation.

2.6. Embryo activation and culture

According to our previous study, electrical stimulation combined with chemical treatment was used to activate the cloned human embryos (Yu *et al*., 2009). Electrical activation was performed in 0.3 M mannitol medium, using stimulation with two 20 μs pulses (1.6 kV/cm) using an electro-cell manipulator (BTX 2001; Genetronics Inc., USA). Reconstructed embryos were rinsed in G-MOPES medium at least three times to remove the electrical activation buffer. The embryos were then immediately exposed to 5 μM ionomycin for different durations, then the embryos were incubated at 37.0°C under 5% CO_2 in humidified air with 2 mM 6-DMAP for an additional duration of time, according to the experimental design.

After activation treatment, groups of five cloned embryos were cultured in 1 droplet of G_1 culture medium under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 and 5% O_2. Embryos that developed to the eight-cell stage by day 3 were transferred to G_2 culture medium (Vitrolife) for sequential culture to the blastocyst stage. PN data were recorded approximately 6 h after activation and development data were recorded every 24 h.

2.7. Embryo biopsy

The cloned human embryos at the eight-cell stage were selected stochastically on day 3 and one blastomere in an eight-cell embryo was removed randomly with an enucleation pipette, as described for human blastomere biopsy (Sermon *et al*., 2004). After manipulating the embryos, the biopsied 'seven-cell' embryos and the single blastomere were transferred back into G_2 culture medium at 37.0°C under 5% CO_2 in humidified air. The seven-cell embryos were cultured to day 5 for morula/blastocyst development, whereas the single blastomere underwent Y chromosome identification by fluorescence *in situ* hybridization (FISH).

2.8. Fluorescence *in situ* hybridization

The single blastomere was treated following our previous study (Jin *et al*. 2010). FISH for chromosomes 18, X and Y was performed in 20 samples. The FISH test used a standard panel of commercially available probes for chromosomes 18, X and Y (FISH Kit for the Detection of Chromosome Numerical Anomalies in Prenatal Diagnosis®, Jinpujia Medical Technology Co. Ltd, Beijing,

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China). The X probe was labelled with a green fluorochrome (CSP spectrum green), the Y probe with a red fluorochrome (CSP spectrum orange) and the 18 probe with blue fluorochrome (CSP spectrum blue). Biopsied blastomeres were spread on slides by the HCl–TWEEN 20 method (Coonen *et al*., 1994). (a) Pre-preparation: the slides were incubated in 2× SSC, pH 7.0, prewarmed to 37°C for 10–20 min and then dehydrated sequentially in a 70%, 85% and 100% ethanol series (2–3 min each) and finally air-dried at room temperature. (b) Probe preparation, slide denatured and hybridization: the probes were prepared following the manufacturer's instructions from the kit, and the probes or slides were denatured for 5 min in denaturant solution (70% formamide/2× SSC) in a 75°C water bath inside a Coplin jar, and then the hybridization was applied in a hybridization oven for 16 h at 42°C after adding denatured probes to the slides. (c) Elution, DAPI staining and examining slides: 50 ml 2× SSC and 2× SSC/0.1% NP-40 solution was placed in Coplin jars, and the slides were placed into 2× SSC solution for 10 min. Then the slides were put into 2× SSC/0.1% NP-40 solution for 5 min. The slides were soaked in 70% ethanol for 3–5 min and air-dried at room temperature. DAPI solution (20 μl) was applied to the target area and a cover glass was applied. The slides were examined using a fluorescence microscope. The results were available after 48 h.

2.9. Statistical analysis

The experiments for examination of cloned embryo activation and subsequent development were repeated at least three times. Development data were analysed by *t*-test or one-way ANOVA, using SPSS 13.0 software. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Confirmation of successful enucleation and reconstruction of human oocytes

Some direct and indirect proof has confirmed the origin of cloned human embryos. The spindle of IVM oocytes can only be observed clearly using the Oosight spindle-imaging system that uses polarized light to visualize the oocyte meiotic spindle (see Supporting information, Movie 1). Next, the karyoplasts removed from oocytes were analysed using Hoechst 33342, and the presence of the meiotic spindles consistently confirmed a 100% efficiency of spindle removal using this approach (Figure 1A–C). Moreover, the FISH results indicated that the Y chromosome could be detected in blastomeres biopsied from cloned embryos at the eight-cell stage (Figure 2B), which contributed to the male 46 XY karyotype (Figure 2A).

3.2. Effects of duration of ionomycin and 6-DMAP treatment on activation of cloned human embryos

The duration of ionomycin exposure to cloned human embryos was varied in the range 0–10 min in this study at a concentration of 5 μM, and every experimental group was sequentially treated by 2 mM 6-DMAP for 5 h after ionomycin treatment. The rate of activated cloned human embryos exposed to ionomycin for 5 or 7 min was significantly higher than those treated for 0, 3 and 10 min. Moreover, in the 0 min group the activation rate was only 16.6%, which is significantly lower than other groups (Table 1). This indicated that ionomycin treatment is a necessary step for the activation of cloned human embryos.

Following testing the effect of different durations of ionomycin treatment on the activation of cloned human embryos, the effect of different durations of 6-DMAP was also tested. Following treatment with ionomycin for 5 or 7 min, cloned human embryos were cultured in 2 mM 6-DMAP for 2, 5 and 8 h, respectively. The results showed that the activation rates of embryos treated in ionomycin for 5 or 7 min did not have any difference under the definite duration of 2 mM 6-DMAP. The activation rates of embryos incubated in 6-DMAP for 2 and 5 h did not differ significantly, but were significantly higher than embryos incubated for 8 h under conditions of 5 μM ionomycin for 7 min; the data obtained under the condition of 5 μM ionomycin for 5 min showed similar results (Table 2).

3.3. Effects of different duration of ionomycin and 6-DMAP on the developmental competence of cloned human embryos

To ascertain which conditions were suitable for the reprogramming of human somatic cells, the subsequent development was observed every day. While the duration of ionomycin was 5 min, the optimal development results were found in the 5 h group of 6-DMAP treatment, in which the efficiency at cleavage (two cells), zygotic activation (eight cells) and morula/blastocyst stages were all significantly higher than in the other two groups treated with 2 mM 6-DMAP for 2 and 8 h. In contrast, the development at every stage in the 8 h group of 6-DMAP treatment was significantly lower than the other two groups treated by 2 mM 6-DMAP for 2 and 5 h. While the duration of ionomycin was 7 min, there were no significant differences in development in the 2 and 5 h groups, except the result of cleavage. Similar to the above data obtained with 5 μM ionomycin treatment for 5 min, the development at every stage in the 8 h group of 6-DMAP treatment was still significantly lower than the other two groups treated by 2 mM 6-DMAP for 2 and 5 h. Overall, the activation conditions of 5 μM ionomycin for 5 min and 2 mM 6-DMAP for 5 h are more suitable for the development

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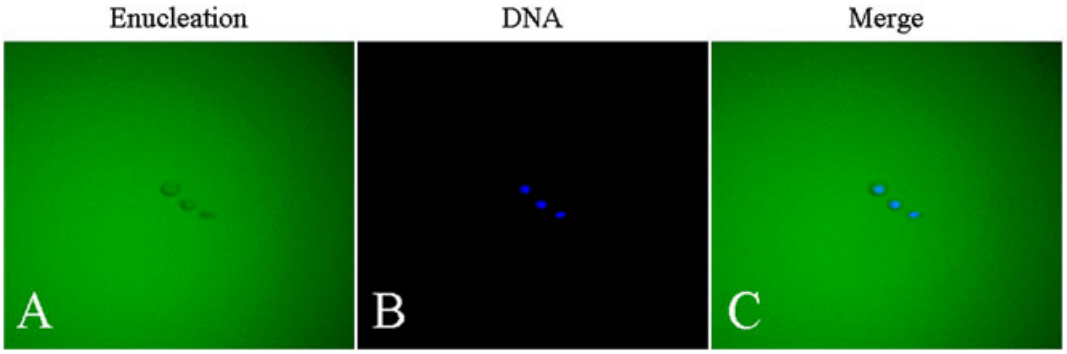

Figure 1. DNA staining proof for successful enucleation of *in vitro*-matured oocytes. (A) Cytoplasm mass removed from *in vitro*-matured oocytes; (B) DNA staining by Hoechst 33342; (C) merged images

of cloned human embryos, even though the morula/blastocyst could be derived from four groups (Table 3).

3.4. The effects of different duration of 2 mM 6-DMAP on the morphology of pseudo-pronuclei of cloned human embryos

The PN morphology and quantity were observed after activation under the conditions of 5 μM ionomycin for 5 min and 2 mM 6-DMAP for 2, 5 and 8 h. Three types of PN morphology were observed: a single large PN; a single normal PN; and a double PN (Figure 3). The number of single large PN increased significantly with the duration of 6-DMAP treatment, but the number of double PNs was not significantly different among the three groups (Table 4).

To study the effect of PN morphology on the development of cloned human embryos, we separated the cloned embryos into classes according to the PN morphology after 2, 5 and 8 h of 2 mM 6-DMAP treatment. Embryos with a single large PN could not develop beyond the eight-cell stage, irrespective of the duration of 2 mM 6-DMAP treatment (2, 5 or 8 h). The morula/blastocyst rates did not differ between normal single and double PN groups after 2 and 5 h of treatment by 2 mM 6-DMAP, even though the developmental efficiencies at the two- and eight-cell stages were significantly different between normal single and double PN groups in the two groups. However, the rate of morula/blastocyst development of embryos with a double PN was significantly higher than those with normal single PN (Figure 4).

4. Discussion

Efficient oocyte activation is a key step in successful nuclear transfer for cloning when genomic reprogramming and maintenance of correct ploidy for introduced nuclei occur in activated enucleated oocytes (Alberio *et al.*, 2001). In the present study, the development of cloned human embryos using IVM oocytes was compared under different activation conditions, and electric pulse stimulation followed by sequential exposure to 5 μM ionomycin for 5 min and 2 mM 6-DMAP for 5 h is more suitable for the development of cloned human embryos.

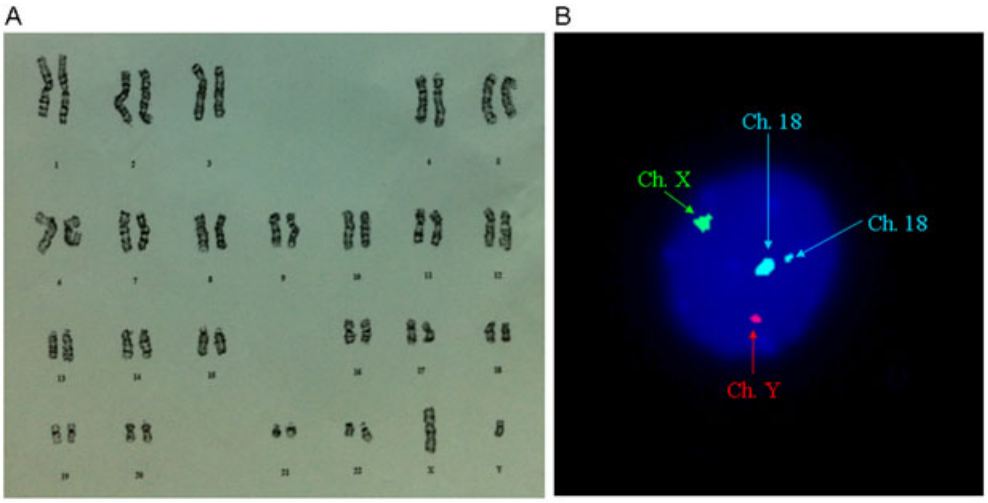

Figure 2. Proof of cloned embryos originated from somatic cells of male gender. (A) Karyotyping of somatic cells, 46 XY; (B) Y chromosome detected in one blastomere biopsied from cloned human eight-cell embryos by the FISH method (arrows: green points to chromosome X; red points to chromosome Y; and blue points to chromosome 18)

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Table 1. Effects of different durations of 5 μM ionomycin on the activation of cloned human embryos before 5 h exposure to 2 mM 6-DMAP

Duration of ionomycin treatment (min)	No. of cloned human embryos	No. of activated cloned human embryos (%)
0	18	3 (16.6)[a]
3	16	12 (75.0)[b]
5	22	20 (90.9)[c]
7	18	18 (100.0)[c]
10	23	15 (65.2)[b]

[a–c]Values in the same column differ significantly ($p < 0.05$).

Table 2. Effects of different durations of 2 mM 6-DMAP on the activation of cloned human embryos after exposure to 5 μM ionomycin

Duration of 6-DMAP treatment (h)	Duration of ionomycin (min)	No. of cloned human embryos	No. of activated cloned human embryos (%)
2	5	14	12 (85.7)[a,b]
5		22	20 (90.9)[a]
8		23	18 (78.3)[b,c]
2	7	19	16 (84.2)[a,b]
5		25	22 (88.0)[a]
8		18	13 (72.2)[c]

[a–c]Values in the same column differ significantly ($p < 0.05$).

In the current study, enucleated IVM oocytes were utilized to reprogramme the somatic nuclei by somatic cell nuclear transfer technology. Successful pregnancies have been reported since 1989 using IVM oocytes in assisted reproduction centres (Cha *et al*., 1991), but previous attempts with nuclear transfer were unsuccessful (Hall *et al*., 2007). Here we first indicated that enucleated IVM oocytes could reprogramme somatic nuclei to the blastocyst stage, which would facilitate the study of therapeutic cloning and potentially avoid or minimize public concerns over ethical issues.

Failed enucleation in human SCNT could produce parthenogenetic embryos, as reported (Hwang *et al*., 2004; Kim *et al*., 2007). To avoid pathenogenetic embryos, donor cells from a 4 year-old boy were used and these cells were also used as feeder cells to support the derivation of human parthenogenetic embryonic stem cells in our previous study (Lu *et al*. 2010). Furthermore, to prove the enucleation of human oocytes, the spindle was observed carefully using an Oosight apparatus (which has been used in rhesus monkeys and humans; Byrne *et al*., 2007; Yu *et al*., 2009) and the aspirated cytoplasm containing spindles was stained using Hoechst 33342 as a routine dye for DNA staining. The current results showed that the nuclei of human oocytes could be removed with the assistance of the Oosight apparatus and Hoechst 33342 staining. Moreover, FISH was performed to identify whether or not there was a Y chromosome in the cloned human embryos, and positive results showed that the eight-cell embryos originated from SCNT. Subsequent data were derived from cloned embryos.

The combination of ionomycin and 6-DMAP was a routine procedure for cloned embryos, especially for ruminants and monkeys (Im *et al*., 2007; Keefer *et al*., 2001; Wells *et al*., 1999; Zhou *et al*., 2006). Ionomycin was first identified as a polyether antibiotic with properties characteristic of a divalent cation ionophore and was found to elevate the level of intracellular calcium in cells (Liu and Hermann, 1978). Before the application of ionomycin A23187, another calcium ionophore (Reed and Lardy, 1972), was also widely used in activating parthenogenetic or cloned embryos combined with 6-DMAP. These researchers compared the characteristics of divalent metal ion transport in both ionomycin and A23187 in rat liver cells, and they indicated that the former is more specific for divalent metal ions than the latter. Another study (Kauffman *et al*., 1980) indicated that ionomycin is theoretically capable of transporting twice as much divalent cation as A23187, so the ionomycin may play a specific role in the process of inducing cellular responses and release of intracellular Ca^{2+} stores. In a cloning study, Shen *et al*. (2008) compared the activation and development potential by A23187 or ionomycin combined with 6-DMAP in cloned cattle embryos, and demonstrated that there was no significant difference in either group, but they did not suggest the development potential after implantation and development to term. In a study involving non-human primate, Okahara-Narita *et al*. (2007) achieved cloned blastocysts in *Cynomolgus* monkeys under the activation conditions of ionomycin combined with 6-DMAP, using amniotic epithelial cells. While using cumulus cells as donor cells, the results of comparison between ionomycin and

Table 3. Development competence of cloned human embryos activated with different duration of 5 μM ionomycin and 2 mM 6-DMAP

Duration of ionomycin (min)	Duration of 6-DMAP (h)	No of cloned human embryos	No. of activated embryos (%)	Development stage (%)		
				Two-cell	Eight-cell	Blastocyst
5	2	14	12 (85.7)[a, b]	6 (50.0)[a]	3 (25.0)[a]	1 (8.3)[a]
	5	22	20 (90.9)[a]	13 (65.0)[b]	9 (45.0)[b]	4 (20.0)[b]
	8	23	18 (78.3)[b]	7 (38.9)[c]	2 (11.1)[c]	0[c]
7	2	19	16 (84.2)[a]	10 (62.5)[b]	4 (25.0)[a]	1 (6.3)[a]
	5	25	22 (88.0)[a]	11 (50.0)[a]	7 (31.8)[a]	2 (9.1)[a]
	8	18	13 (72.2)[b]	5 (38.5)[c]	1 (7.7)[c]	0[c]

[a–c]Values in the same column differ significantly ($p < 0.05$).

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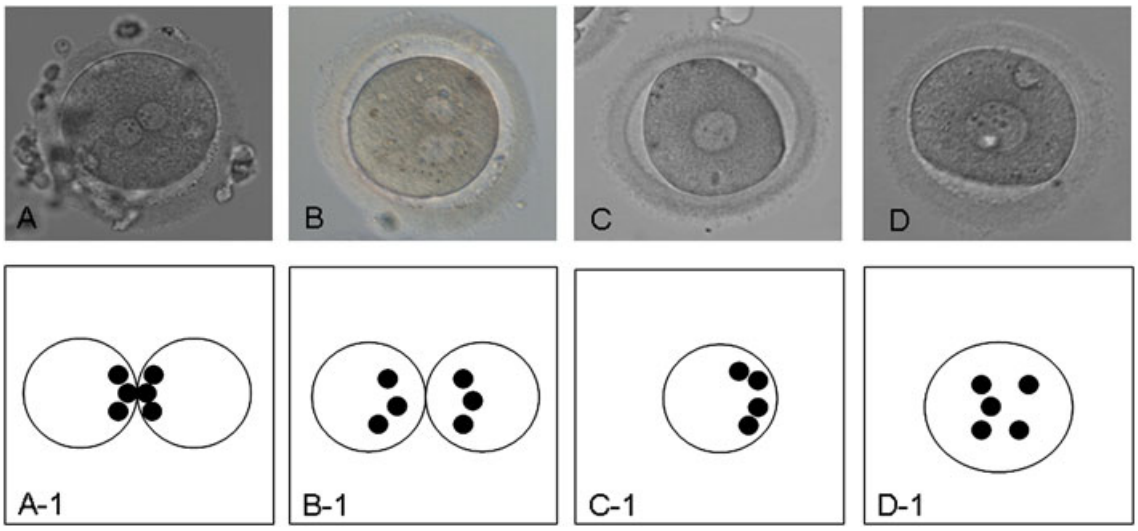

Figure 3. Configurations of pseudo-pronuclear morphology, nucleolar morphology and pronucleus diameter of cloned human embryos after activation. (A, A1) Fertilized zygote with double pronucleus; (B, B1) cloned zygote with double pronucleus; (C, C1) cloned zygote with normal single pronucleus; (D, D1) cloned embryo with abnormal single large pronucleus

A23187 suggested that the developmental efficiency of four and eight cells in the ionomycin group was higher than the A23187 group, although no morula or blastocyst could be derived from either group. In the present study, the activation rate would be very low compared with other treatment group if the embryos were not treated by ionomycin and induced activation by 6-DMAP only, which indicated the active function of ionomycin in the activation of cloned human embryos.

6-DMAP is a protein serine/threonine kinase inhibitor (Neant and Guerrier, 1988), widely used to activate cloned embryos in SCNT in bovines (De Sousa *et al*., 1999), goats (Lan *et al*., 2005), horses (Choi *et al*., 2004), dogs (Jang *et al*., 2007), ferrets (Li *et al*., 2006), monkeys (Zhou *et al*., 2006) and humans (French *et al*., 2008; Yu *et al*., 2009). Studies in mice have indicated that 6-DMAP can accelerate PN formation and promote the development of parthenogenetic embryos (Szollosi *et al*., 1993). 6-DMAP was also helpful in inactivating maturation promoting factor (MPF) and mitogen-activated protein kinase (Liu and Yang, 1999). Therefore, it is important to clarify whether the development of human clone embryos can be improved by modified 6-DMAP treatment. Lan *et al*. (2005) reported that the timing of 6-DMAP treatment plays a key role in the development of goat cloned embryos; inefficient 6-DMAP treatment could impair embryo development and PN formation. Lan *et al*. (2004) found that long incubation with 6-DMAP impaired the development of mouse parthenogenetic embryos. It is possible that 6-DMAP enhances the efficiency of activation and the speed of PN formation but has no effect on DNA synthesis and further development (Ledda *et al*., 1996); however, the present study showed that 8 h of treatment with 6-DMAP impaired cleavage, zygotic genome activation (ZGA) and morula/blastocyst formation, whereas approximately 20% morula/blastocyst efficiency was accepted when the activation conditions were 5 μM ionomycin for 5 min and 2 mM 6-DMAP for 5 h.

During PN formation, nuclear precursor bodies migrate and merge to form nucleoli. Nucleoli are closely related to the synthesis of pre-rRNA (Tesarik and Kopecny, 1990); thus, PN formation is a crucial stage for maternal–paternal genome integration and a key criterion to evaluate embryo development potential. In our human SCNT experiments, three main PN morphologies were observed. A large PN was regarded as an abnormality, and the number of cells with a large PN increased with the duration of 6-DMAP treatment. In human assisted reproductive technology, PN morphology is a key evaluation criterion for fertilized embryo development and screening of chromosome abnormalities (Borges *et al*., 2005; Scott *et al*., 2000), and is used to select embryos of good quality and development potential. In the present study, abnormal PNs had a larger diameter than normal PNs and the nucleoli were dispersed within them. Only 20–30% of zygotes with an abnormal PN developed to the two-cell stage, but few exceeded the eight-cell stage. In contrast, embryos with a normal single or double PN developed to blastocysts with different efficiency. In both the 2 and 8 h groups, blastocyst formation efficiency was equivalent, and only in the 5 h group was the blastocyst efficiency of embryos with double PN significantly higher than those with normal single PN. The single large PN ratio was 66.7%, 27.9% and 16.7% for 6-DMAP treatment of 8, 5 and 2 h, respectively. Embryos with an abnormal PN completed the first division but did not exceed the eight-cell stage, resulting in decreased blastocyst formation. Chromosome remodelling of donor cells also reflects the PN morphology (Collas and Robl, 1991), therefore extended 6-DMAP duration possibly impaired chromosome remodelling of donor cells, so that embryos could not complete the transformation from the maternal to the zygotic genome.

In conclusion, one activation parameter proved to be suitable to PN formation and subsequent development of cloned human embryos by SCNT while

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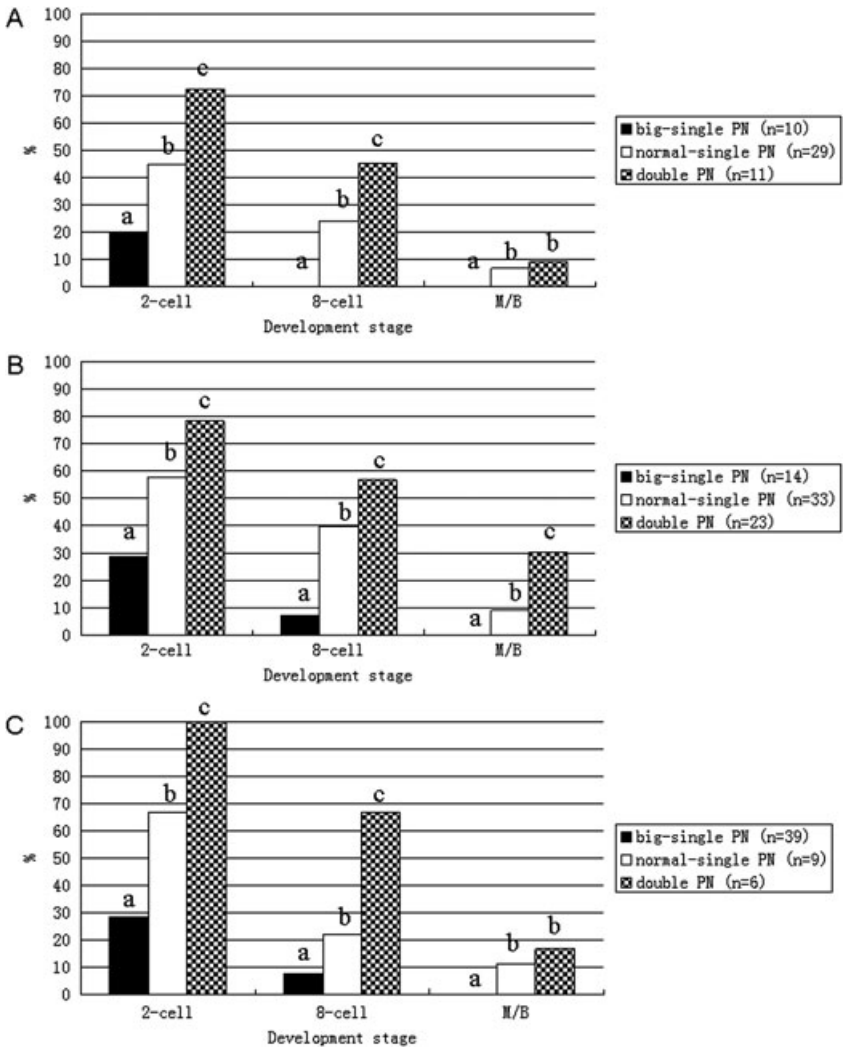

Figure 4. The development efficiency of cloned human embryos with different pseudo-pronuclear morphology activated by different durations of 2 mM 6-DMAP: (A) 2 h; (B) 5 h; (C) 8 h. [a–c]Significant ($p < 0.05$) differences from other groups

using IVM cytoplasm. An increase in the reprogramming efficiency of human donor cells would improve the possibility of deriving human homologous embryonic stem cells and facilitate basic research and clinical applications in regenerative medicine and cell therapy.

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