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Developmental potential of human oocytes reconstructed by transferring somatic cell nuclei into polyspermic zygote cytoplasm

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ABSTRACT

The generation of patient-specific nuclear transfer embryonic stem cells holds huge promise in modern regenerative medicine and cell-based drug discovery. Since human in vivo matured oocytes are not readily available, human therapeutic cloning is developing slowly. Here, we investigated for the first time whether human polyspermic zygotes could support preimplantation development of cloned embryos. Our results showed that polyspermic zygotes could be used as recipients for human somatic cell nuclear transfer (SCNT). The preimplantation developmental potential of SCNT embryos from polyspermic zygotes was limited to the 8-cell stage. Since ES cell lines can be derived from single blastomeres, these results may have important significance for human ES cells derived by SCNT. In addition, confocal images demonstrated that all of the SCNT embryos that failed to cleave showed abnormal microtubule organization. The results of the present study suggest that polyspermic human zygotes could be used as a potential source of recipient cytoplasm for SCNT.

Introduction

Therapeutic cloning, whereby embryonic stem cells (ESCs) are derived from patient-specific, cloned blastocysts via somatic cell nuclear transfer (SCNT), holds great promise for treating many human diseases in regenerative medicine. Indeed, experiments in mice have shown that nuclear transplantation, combined with gene and cell therapy, represents a valid strategy for treating genetic disorders [1]. However, there are several obstacles to successful human therapeutic cloning. One major hindrance is the availability of human oocytes. To our knowledge, only three studies have described successful generation of human SCNT blastocysts [2–4]. In these studies, however, the cloned blastocysts are derived from fresh oocytes recovered from artificially stimulated volunteers.

Human oocytes are difficult to obtain and their collection raises ethical issue concerning the potential risk to female donors. This issue could be circumvented if the discarded oocytes or embryos from in vitro fertilization (IVF) procedures could be used. Several preliminary studies have generated early cleavage stage, nuclear transfer (NT) embryos using cytoplasts obtained from both in vitro and in vivo, matured oocytes that failed to fertilize in clinical IVF procedures [5–7]. Recently, Egli et al. reported that mouse zygotes that are temporarily arrested in mitosis can support somatic cell reprogramming, the production of embryonic stem cell lines and the full-term development of cloned animals [8]. Therefore, human polyspermic zygotes, which have no clinical use and are routinely discarded, may be a potential source of human oocytes for the creation of patient customized embryonic stem cells.

In the present study, we have investigated the use of mitotic human polyspermic zygotes as recipients for injection of heterologous donor somatic cells and observed the preimplantation development of these human NT embryos.

Material and methods

All chemicals used for SCNT were purchased from Sigma–Aldrich unless otherwise stated.

Source of polyspermic zygotes. All of the polyspermic zygotes were donated for this research from stimulated patients undergoing IVF treatment in the Reproductive Medical Center, The Third Affiliated Hospital of Guangzhou Medical College between October 2007 and December 2008. The patients were clearly informed of all the research details and were approved by the ethical committee of the hospital.

Preparation of donor cells. Donor cells were obtained from a foreskin excision of a 5-year-old boy. The cells were cultured in DMEM supplemented with 10% FBS and 1% non-essential amino acids. Cells were arrested in mitosis by culturing with 0.1 μg/mL nocodazole (Sigma M1404) for 6 h before nuclear transfer. Cells were

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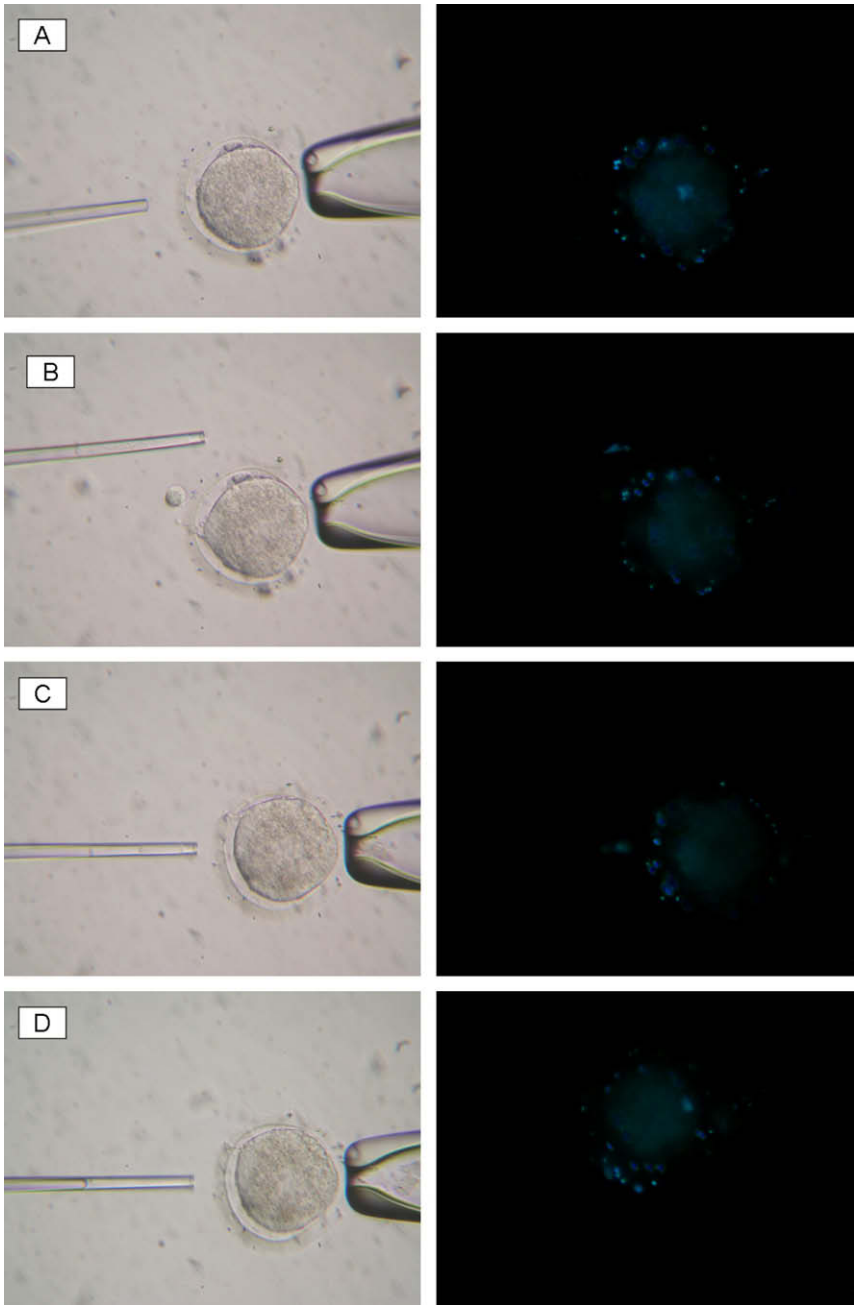

Fig. 1. The method of nuclear transfer into polyspermic zygotes arrested in mitosis. (A) Arrested polyspermic zygote before spindle and chromosome removal, 5 min after the shift from nocodazole to HTF with CB, Hoffman modulation contrast. (B) Removal the polyspermic zygote spindle and chromosomes by micromanipulation. (C) Piezo-actuated injection of a nocodazole-arrested somatic cell into a mitotic polyspermic cytoplasm. (D) Somatic cell chromosomes in the polyspermic zygote immediately after transfer (×200 under an inverted microscope).

Table 1
Preimplantation development of polyspermic zygotes after cell cycle arrest by nocodazole.

Drug treatment (total egg number)	2-cell (%)	8-cell (%)	Blastocyst (%)
No drug treatment (11)	11 (100)[a]	7 (63.6)[a]	1 (9.1)[a]
0.1 μg/mL nocodazole, arrested 5 h (8)	8 (100)[a]	6 (75)[a]	1 (12.5)[a]

Numbers with the same superscript denote that values that do not differ significantly within that column ($P > 0.05$).

obtained from culture dishes using the mitotic shake-off technique and were then mixed with HTF medium (Quinn's AdvantageTM Medium with HEPES) containing 0.1 μg/mL nocodazole.

Nuclear transfer into enucleated polyspermic zygotes arrested in mitosis. Polyspermic zygotes were obtained 18–20 h after insemination by conventional IVF. Zygotes were transferred into G1.5 media (Vitrolife Sweden AB) containing 0.1 μg/mL nocodazole for 5 h. Zygotes arrested in mitosis were washed with three drops of G1.5 to remove residual nocodazole and then transferred into oil-covered droplets of HTF supplemented with 7.5 μg/mL Cytochalasin B (CB, Sigma, C6762).

All manipulations were done on a heated stage of a Nikon microscope equipped with Hoffman modulation contrast optics. A 12 μm inner diameter (ID) blunt-tip pipette was passed through the zona using a piezo device. The pipette was then slowly withdrawn into the proximity of the metaphase chromosomes as visu-

alized by Hoffman microscopy. The chromosomes, along with a small amount of ooplasm, were aspirated into the pipette, removed from the zygote and stained with Hoechst 33342 in a separate drop to confirm spindle removal. After culturing for 1 h in G1.5, the enucleated zygotes were transferred into HTF without CB. A mitotic fibroblast was aspirated into a 12 μm ID blunt-tip pipette, breaking the cell membrane. The cell was then injected into an enucleated zygote (Fig. 1). After manipulations were completed, nuclear transfer embryos were cultured in G1.5 and covered with mineral oil. Embryo development was observed daily.

Immunofluorescent staining of microtubules and nuclei. After removing the zona pellucida in acidic Tyrode's medium (pH 2.5), arrested embryos were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. Embryos were then permeabilized with 0.5% Triton X-100 for 30 min at room temperature, blocked in 2% BSA-supplemented PBS (blocking solution) for 1 h, and incubated overnight at 4 °C with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against α-tubulin (Sigma F2168) diluted 1:100. The embryos were washed three times and stained with 10 μg/mL propidium iodide for 30 min. The embryos were mounted on slides and observed under a Confocal Laser Scanning Microscope (Nikon, C1si) within a week of preparation.

Statistical analysis. Embryo developmental data were analyzed by *t*-test using SPSS 13.0 software. The level of significance was set at $P < 0.05$.

Results

In vitro developmental potential of nuclear transfer embryos

To investigate the developmental potential of polyspermic zygotes after cell cycle arrest by nocodazole, eight zygotes were cultured and observed every 24 h. All eight polyspermic zygotes that were arrested by nocodazole for 5 h developed to the 2-cell stage. Six of the zygotes developed to the 8-cell stage and one progressed to the blastocyst stage. Untreated polyspermic zygotes were used as controls. Our results indicated that there was no significant difference in preimplantation development between the nocodazole treated and untreated polyspermic zygotes (Table 1). In 92 zygotes arrested in mitosis, 80.4% survived after enucleation. Of these surviving zygotes, 55.4% survived after nuclear transfer and 46.3% cleaved to at least the 2-cell stage (Fig. 2A). The developmental potential was limited to the 8-cell stage (Fig. 2B). However, when interphase polyspermic zygotes were used as recipients for nuclear transfer, development of the embryos arrested at the first cleavage (Table 2).

Distribution of microtubules in arrested cloned embryos

To investigate the relationship between developmental arrest (cleavage failure) and the distribution of microtubules, arrested cloned embryos were examined using immunofluorescent staining and confocal microscopy. All of the arrested embryos showed abnormal microtubules at the first cell division stage. While 31.2% (5/16) of the arrested embryos showed defective mitotic spindles with misaligned chromosomes (Fig. 3A), 68.8% (11/16) did not show any microtubules in the cytoplasm (Fig. 3B).

Discussion

Human therapeutic cloning has been difficult, particularly due to the limitations in obtaining donated oocytes. A recent study by Egli et al. demonstrated that cell reprogramming could occur by replacing the chromatin from a zygote that is arrested in metaphase just prior to the first cell division with chromatin from a metaphase-arrested somatic cell. This technique had success rates that were comparable to NT into mature oocytes [8]. Interestingly, Egli and co-workers further demonstrated that polyspermic zygotes could be used for successful nuclear transfer [8]. Polyspermic fertilization occurs in ~7% of cases in human IVF programs, and these embryos are routinely discarded [9]. These polyspermic IVF embryos may present a valuable source of oocytes that could be used in human NT.

In the present study, we have demonstrated that mitotic polyspermic zygotes can be used as recipients for SCNT, whereas interphase polyspermic zygotes can not. Unlike the traditional electrofusion method previously described, we used a piezo-assisted method to produce viable SCNT embryos [3,10]. Embryo survival rates after NT using polyspermic zygotes as recipients (55.4%) were comparable to those when using failed-to-fertilize oocytes [5], but significantly lower than when using in vivo oocytes

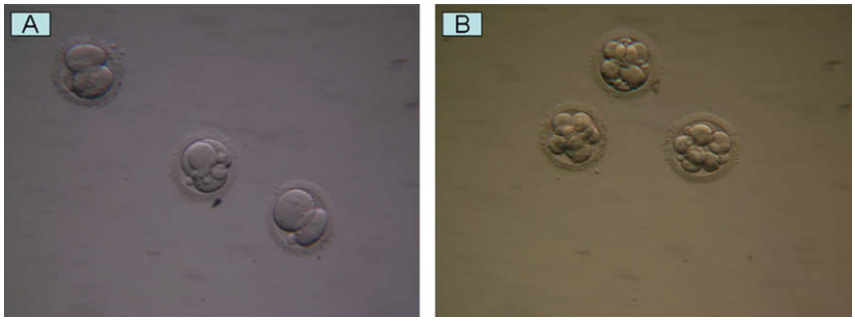

Fig. 2. Development of preimplantation embryos after polyspermic zygote nuclear transfer. (A) 2-cell stage. (B) 8-cell stage (×100 under an inverted microscope).

Table 2
In vitro developmental potential of nuclear transfer embryos.

Recipient (cell cycle stage)	Donor (cell cycle stage)	No. of manipulated	No. (%) of survival after enucleation	No. (%) of survival after NT	No. (%) of cleaved	No. (%) of 8-cell embryo
Polyspermic zygotes (mitosis)	Somatic cell (mitosis)	92	74 (80.4)[a]	41 (55.4)[a]	19 (46.3)[a]	5 (12.2)[a]
Polyspermic zygotes (interphase)	Somatic cell (interphase)	13	10 (76.9)[a]	6 (60.0%)[a]	0 (0)[b]	0 (0)[b]

Numbers with different superscripts denote values that differ significantly with a column ($P < 0.05$).

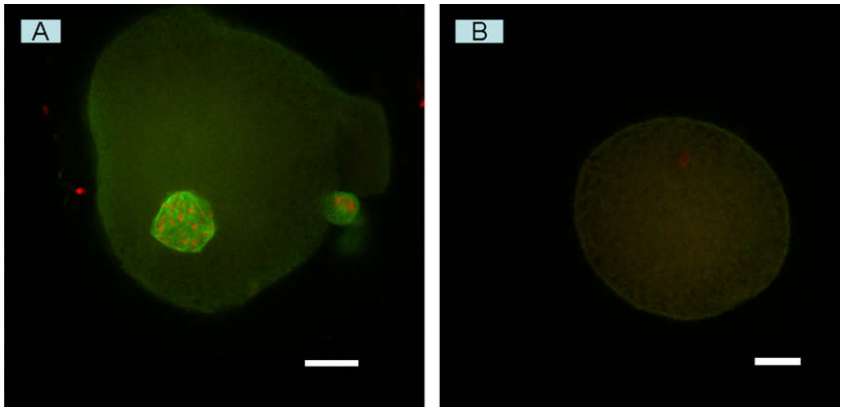

Fig. 3. Confocal images of microtubule distributions in cloned embryos that failed to cleave after nuclear transfer. Green indicates microtubules and red indicates DNA. Scale bar = 20 μm. (A) Defective mitotic spindle with misaligned chromosomes. (B) Absent microtubules distribution in the cytoplasm.

(78%). This is likely due to the fragility of the oolemma and the invasive method of directly injecting donor cells using piezo-assisted technology [7]. Lavoir et al. demonstrated that the artificial activation rates and the developmental potential of human NT embryos were poor using failed-to-fertilized oocytes, with few embryos progressing beyond the pronuclear stage [5]. Nuclear transfer using mitotic zygotes bypasses the need for artificial activations because the sperm has already initiated development. In our study, 46.3% of cloned embryos developed to the 2-cell stage, and 12.2% of cloned embryos developed to the 8-cell stage. DNA fingerprinting analysis revealed that both donor cells and 8-cell stage NT embryos had the same genetic origin (data not shown). Developmental results showed that polyspermic zygotes were more efficient recipients for human SCNT than failed-to-fertilize oocytes. However, 50% of cloned embryos did not undergo cleavage or were fragmented. It has been reported that primates were different from others animals, as disarrayed abnormal mitotic spindles with misaligned chromosomes were formed in most SCNT embryos [11]. Confocal images showed that microtubule organization was abnormal or absent in the cloned embryos arrested at the one-cell stage and in fragmented embryos. These results suggest that abnormal organization of microtubules is closely associated with the developmental arrest of the reconstructed embryos.

The developmental ability of cloned embryos is related to nuclear transfer techniques. It has been suggested that the developmental potential of monkey SCNT embryos is limited because of the deletion of microtubule motor and centrosomal proteins during meiotic spindle removal. These embryos showed formations of defective mitotic spindles originating from the transferred nuclei [11]. To some degree, this limitation was overcome with modified techniques [12]. Zhou et al. also reported that a one-step micromanipulation technique was superior in the routine production of SCNT embryos in monkeys [13]. Unlike human MII oocytes, we found that the pronuclei of polyspermic zygotes broke down upon entering into mitosis, and the supernumerary chromosome sets congregated on a single spindle in the center of the polyspermic zygote. The spindle and the chromosomes lying on the metaphase plate were visible with currently used microscopic technology. Enucleation was performed by aspirating the chromosome-spindle complex without Hoechst staining and UV irradiation. This may increase the developmental potential of cloned embryos. Previously, it was recognized that fresh oocytes were essential for mouse NT experiments. However, after improvements were made to the mouse NT protocol [14], it was clearly demonstrated that failed-to-fertilize oocytes could be used to generate mouse NT-ES cells [15]. In this report, the survival rates and developmental potential were low, suggesting that further optimization of the human NT technique is required.

In this study, the preimplantation developmental potential of SCNT embryos from polyspermic zygotes was limited to the 8-cell stage, when embryonic genome activation takes place. In mice, blockage of developmental progression through the 2-cell stage, when embryonic genome activation occurs, could be overcome with the appropriate culture media [16,17]. It is necessary that appropriate culture media is developed for supporting the development of human cloned embryos. Several reports demonstrated that cloned mouse embryos prefer somatic cell-like culture conditions, which differs from those required by normally fertilized control embryos [18,19]. The effect of improved culture conditions on successful human SCNT needs to be investigated.

During the IVF process, only a small number of all zygotes will develop successfully into blastocysts [20,21], and most of these embryos display abnormal or delayed cell division, frequently accompanied by cellular fragmentation and developmental arrest before the blastocyst stage [21,22]. However, not all blastomeres within the arrested embryo are abnormal nor are they responsible for developmental arrest [22–25]. A similar situation may also exist in arrested cloned embryos. Arrested embryos derived from NT could be a crucial source for successful development of patient-specific stem cells as derivation of human NT blastocysts is an inefficient process [26]. Currently, we are pursuing the generation of embryonic stem cell lines from SCNT embryos using polyspermic zygotes. Recent achievements in the development of ESC lines from human [27] and cloned mouse individual blastomeres [28], human arrested embryos [29,30] and primate somatic cell blastocysts [31] may hasten the attainment of this goal.

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