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RESEARCH ARTICLE

Experimental cloning of embryos through human-rabbit inter-species nuclear transfer

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Abstract Therapeutic cloning, which is based on human somatic cell nuclear transfer, is one of our major research objectives. Though inter-species nuclear transfer has been introduced to construct human somatic cell cloned embryos, the effects of type, passage, and preparation method of donor cells on embryo development remain unclear. In our experiment, cloned embryos were reconstructed with different passage and preparation methods of ossocartilaginous cell, skin fibroblast, and cumulus cells. The cumulus cell embryos showed significantly higher development rates than the other two ($P < 0.05$). The development rate of embryos reconstructed with skin fibroblasts of different passage number and somatic cells of different chilling durations showed no significant difference. Also, fluorescence *in situ* hybridization (FISH) was conducted to detect nuclear derivation of the embryos. The result showed that the nuclei of the inter-species cloned embryo cells came from human. We conclude that (1) cloned embryos can be constructed through human-rabbit inter-species nuclear transfer; (2) different kinds of somatic cells result in different efficiency of nuclear transfer, while in vitro passage of the donor does not influence embryo development; (3) refrigeration is a convenient and efficient donor cell preparation method. Finally, it is feasible to detect DNA genotype through FISH.

Keywords therapeutic cloning, nuclear transfer, somatic cell nuclear transfer, inter-species cloned embryo

1 Introduction

Stem cell is a kind of low differentiated cell with the potential of differentiating to many kinds of cells. This stem cell characteristic may be used to repair injured tissues or organs, thus to cure diseases. The successful establishment of human embryonic stem cell from early human embryos by Thomson et al (1998) made its clinical use possible. However, immune rejection must be overcome. Through nuclear transfer, cloned embryos can be constructed with the patient's somatic cell as donor and then embryonic stem cells can be obtained (Smith, 1998; Solter and Gearhart, 1999). Immune rejection will not occur if this kind of embryonic stem cell is transferred back into the very patient's body. This process, which is named "therapeutic cloning", is based on the construction of somatic cell cloned embryos.

It is very difficult to achieve "therapeutic cloning" because of the difficulties in obtaining the human oocyte. Therefore, the inter-species nuclear transfer technique, often used in rescuing animals on the verge of extinction, was tried to solve the problem (Wells et al., 1998). Our experiment, based on demonstrating whether human cloned embryos could be constructed through inter-species nuclear transfer, discussed the effect of donor cell type and passage number on embryo development to look for the most suitable donor cell. Furthermore, preparing donor cells using the refrigeration method and detecting DNA genotype of embryos by fluorescence in situ hybridization (FISH) were tried in this study.

2 Materials and methods

2.1 Preparation of donor cells and recipient oocytes

Adult female neck skin was obtained from discarded tissue after surgery. Fibroblasts were isolated, *in vitro* cultured and then packed in 1.5 mL centrifugal tube at a concentration of 2×10^5 mL^{-1} and finally stored at 4°C. Granulose cells were stripped from oocytes during IVF procedure with the consent of patients. Ossocartilaginous cells were given by Dr Zhang Mei from Anhui Provincial Hospital, China. Japanese rabbits were superovulated with PMSG-HCG and killed 15–18 h after HCG injection. Oocyte-corona-cumulus complex) was

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collected from oviducts using TCM 199. Cumulus cells were dissociated from oocytes.

2.2 Micromanipulation

Oocytes were put into M2 medium with 7.5 μg/mL cytochalasin B together with donor cells. Enucleation was performed by aspirating the first polar body and 1/4–1/3 cytoplasm beneath it (nuclei included). One single donor cell was inserted into the perivitelline space of enucleated oocyte through the same break.

2.3 Electrofusion and stimulation

The reconstructed eggs were placed between two electrodes filled with electrofusion solution in a cell fusion chamber of an electrofusion apparatus. The donor/recipient touch dimension was paralleled the electrodes. Electrofusion reference was 140 V/mm, 80 μs, 2 direct current pulses, with 1 second interval. After electrofusion, reconstructed eggs were cultured at 38°C, 5% CO_2, 95% air 20 minutes later, fusion was observed. This process was repeated among those eggs which failed to be fused previously. Electrical pulses (140 V/mm, 20 μs, two direct pulses, with one second interval) were applied to those which had been fused to stimulate the embryos.

2.4 *In vitro* culture

Embryos were washed in MS (M2 supplemented with 15% fetal bovine serum), and then cultured in 5 μs minidrop of the same medium at 38°C, 5% CO_2 / 95% air and observed daily.

2.5 Cell fixation

Four to eight cell stage embryos were selected. Donor cell sex of every embryo was recorded. Embryo cells were isolated after dissolution of the zona pellucida with streptoproteinase and placed in Tween-HCl drops [0.1% Tween 20–0.01 mol/L HCl] which were already on slides. After that, observations and documentations were conducted through microscope. Having been dehydrated in ethanol, the slides were placed at room temperature (25°C) overnight. Adult male peripheral lymphocyte chromosome slides were used as control.

2.6 FlSH

Denatured in (73 ± 1)°C, 70% deionized formanide for 5 minutes, the slides were dehydrated in ethanol. (37 ± 1)°C denatured probe mixture was added to the target area. Hybridization was carried out in the target area in a hybridization oven overnight at 42°C after the slides were covered with another slide and sealed with rubber mud. The next morning, after the cover was removed, slides were washed in succession in 0.4*SSC/0.1NP-40 at (73 ± 1)°C, 2*SSC/0.1NP-40 at room temperature (25°C). After the slide was cooled down in the dark, 10 μL DAPI was added to every target area.

2.7 Outcome determination

Observation was made through fluorescent microscope. When one orange signal and one green signal were seen in the cell, the sex chromosome karyotype was determined as XY. If only two green signals were seen, the sex chromosome karyotype was determined as XX.

2.8 Statistical methods

χ^2 test and exact probabilities were used, and difference was considered significant when $P < 0.05$.

3 Results

3.1 Construction of cloned embryos with three different kinds of somatic cell as donor

Three hundred and sixty-one eggs were reconstructed with three different kinds of donor in our experiment. Oocyte's number, fusion rate, and embryo development in each group are shown in Table l.

The table shows that there was no significant difference in fusion rate among the three groups. The early development of the granulose cell group was obviously better than that of the other two ($P < 0.05$) and the blastocyst rate of the granulose cell group was significantly higher than that of the fibroblast group ($P < 0.05$). At the same time, no blastocyst was obtained in the osseocaritlaginous cell group while early development rate showed no significant difference between the ossocartilaginous cell group and the fibroblast group (Fig. 1).

Table 1 The development of cloned embryos reconstructed with human osseocartilaginous cells, granulose cells and fibroblasts

Group of donor cell	No. oocytes manipulated [1]	Fusion rate / %[2]	Cleavage rate / %[3]	8 cell embryo rate / %[3]	Morula rate / %[3]	Blastocyst rate[3]
osseocartilaginous cell	67	61.2 (41/67)[a]	39.0 (16/41)[a]	14.5 (8/41)[a]	7.3 (3/41)[a]	
granulose cell	116	56.9 (66/116)[b]	65.2 (43/66)[b]	53.0 (35/66)[b]	31.8 (21/66)[b]	18.0 (12/66)[b]
fibroblast	178	55.6 (99/178)[a]	42.4 (42/99)[a]	25.3 (25/99)[a]	12.1 (12/99)[a]	6.1 (6/99)[a]

a and b means that the values in the same column differed significantly (χ^2 test $P < 0.05$)

1: Number of oocyte fused / Number. of oocvte manipulated; 2: Number of two-cell embryos / Number of reconstructed embryos;

3: Number of embryos developed to different stage / Number of reconstructed embryos cultured

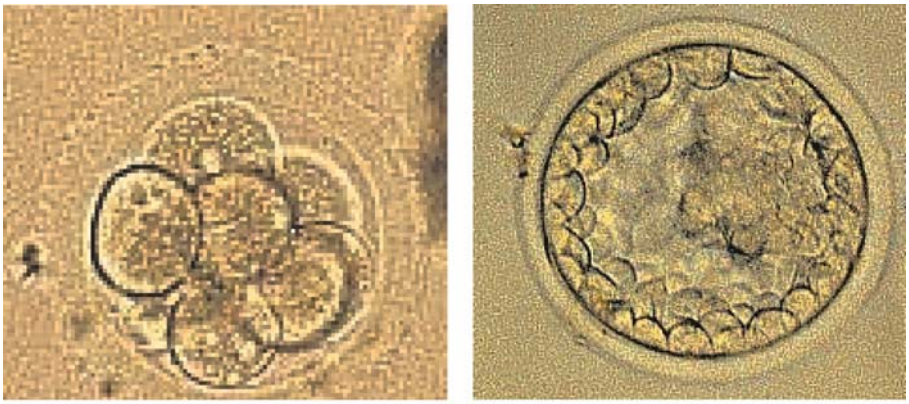


Fig. 1 Development of reconstructed embryos

3.2 The effect of passage number on the development potential of embryos

The passages of fibroblast in our experiment were 1–6, and the development potential of embryos from three of the six passages is shown in Table 2.

Table 2 Development of cloned embryos obtained from different passage number of fibroblast passage

Passage	Number of oocytes manipulated	Fusion rate / %	2–4 embryo rate / %	Blastocyst rate / %
Second passage	24	58.3 (14/24)[a]	35.7 (5/14)[a]	7.1 (1/14)[a]
Third passage	29	51.7 (15/27)[a]	66.7 (10/15)[b]	
Forth passage	66	35.1 (13/37)[a]	35.1 (13/37)[a]	10.8 (4/37)[a]

a and b meant that the values in the same column differed significantly (χ^2 test, $P<0.05$)

Table 3 Development of cloned embryos reconstructed from fibroblast chilling for different days

Chilling duration / day	Number of oocytes manipulated	Fusion rate / %	2–4 embryo rate / %	Blastocyst rate / %
0	24	41.7 (10/24)	50.0 (5/10)	
2	32	56.3 (18/32)	38.9 (7/32)	7.1 (1/14)
5	17	58.8 (10/17)	60.0 (6/10)	10.8 (4/37)
9	22	63.6 (14/22)	35.7 (5/14)	

Table 3 indicates that the electrofusion rate was most significantly different among the three groups. The 2–4 cell embryo rate of the third passage group was significantly higher than that of the other two. Except the second passage group, there were embryos from the other two passage groups that grew up to blastocyst, but their blastocyst rates were low and showed no difference.

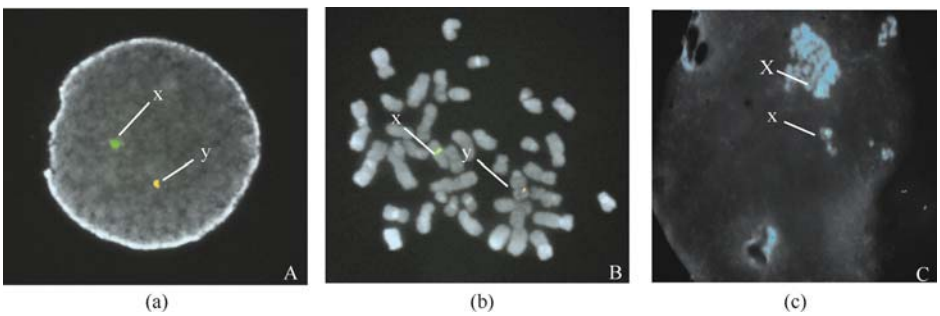
3.3 Effect of refrigeration on embryo development

One hundred and fifty-four eggs were reconstructed with chilled cells as donor. Embryo development of reconstructed embryos from fresh cell and cells chilled for 2, 5, and 9 days were compared, respectively.

The table indicates that compared with fresh cell, the fusion rate and early development rate of each refrigeration group showed no significant difference. No blastocyst was obtained in the fresh cell group and refrigeration for the 9-day group.

3.4 FISH outcome

Twenty-four blastomeres were conducted in our FISH experiment (Fig. 2). Clear, bright signals were detected in 22 of them, suggesting that genetic materials of reconstructed embryos from human were consistent with the sex chromosome karyotype of the donor cell.



(a): Male peripheral interphase lymphocyte cell; (b): Male peripheral middlephase lymphocyte chromosome; (c): Cleavage cell in reconstructed embryos

Fig. 2 FISH detection of reconstructed embryos

4 Discussion

The hypothesis of "therapeutic cloning" has been put forth for many years and its fantastic application in the future is an interest for scientists. The most important thing is whether human somatic cell can be reprogrammed and can develop to blastocyst, which has already been proved (Lu et al., 2003; Hwang et al., 2004). However, can oocytes from another mammal reprogram human somatic cell? Sheng's study in recent years proved that human somatic cell cloned embryos could be constructed through interspecies nuclear transfer and an embryonic stem cell could be obtained (Chen et al., 2003). Its feasibility was demonstrated further in our experiment, suggesting that rabbit oocyte is a suitable recipient to support human somatic cell to regain pluripotency and reprogrammability, which is consistent with Sheng's report.

The effects of donor cell type and passage on nuclear transfer were discussed in our study. Results demonstrated that early development rate and blastocyst rate of the granulose cell group embryos were higher than those of the other two, which was consistent with the study on bovine nuclear transfer (Kato et al., 2000). The study on the mechanism of nuclear transfer suggests the cell cycle phase of the donor cell is a key factor in influencing nuclear transfer, and the activity of maturation promoting factor is very high in M II oocyte. If exposed in that circumstance, no matter what phase of the cell cycle the donor nuclei was, DNA replication will begin. G_0/G_1 cell is diploid because their DNA has not synthesized yet, while cells in other cell cycle phase are pluriploid as a consequence of DNA synthesis. Only when G_0/G_1 phase cells are used as donor, the correct ploidy of the chromosome can be proved (Campbell et al., 1993; Cambell et al., 196; Wolf et al., 1998). Because granulose cell proliferates very fast and the G_0/G_1 phase cell rate is high, it is a suitable donor cell type. Unfortunately, granulose cell is difficult to obtain and there is not any granulose cell in a male patient, so its usage is restricted. Fibroblast as donor means lower efficiency compared with granulose cell, but it is easy to obtain. Therefore, in a sense, it is the best kind of donor cell. The effect of passage on nuclear transfer has also become the focus of researchers (Chikara et al., 2000; Arat et al., 2001). It was considered by most people (Kato et al., 1998; Lanza et al., 2000) that passage has a negative effect on nuclear transfer (Liu, 2000). However, no conclusion has been reached on whether passage correlated negatively with nuclear transfer, or only after a certain number of passages, it will influence nuclear transfer efficiency. Our experiment suggested that to a certain extent, passage had no effect on nuclear transfer. But the question remains—what passage or passage range is acceptable?

In nuclear transfer, preparation of the donor cell is an important procedure that is always restricted by many factors. The common protocols for donor cell preparation are (l) isolating cells from fresh tissue (Wakayama et al., 1998); (2) in vitro culture, serum starvation (Wilmut et al., 1997) and no serum starvation included (Cibelli et al., 1998). These protocols are the Herculean labors and moreover, increase the probability of verifying genetic character during passage. Therefore, launching a convenient and efficient donor cell preparation method should have significant effect on nuclear transfer. Refrigeration method was first used by Liu Jilong in bovine cloning in 2000, and the result suggested that not only can it decrease the possibility of chromosome mutation, but it can also simplify experimental procedure. Then, the method is proved to be a convenient and efficient donor cell preparation method (Liu, 2000). This method was applied for the first time in the construction of somatic cell cloned embryos in our experiment and proved to be suitable.

The major methods used in characterization of interspecies cloned embryos are in China (Chen et al., 2003). Some of these methods are restricted by sample quantity, while others are the Herculean labors and easy to be interfered. The FISH technique with human X and Y chromosome specific probe is mature (Munne et al., 1994 ; Delhanty et al., 1997; Laverge et al., 1997). The probe is species specific, and the outcomes are more accurate and reliable. Our experiment further demonstrated that FISH was an ideal method characterizing the nuclear derivation of cloned embryos.

The mitochondrion is an important and unique cell organ. Mammalian mitochondrion has its own genetic material-mitochondrial DNA. A part of (or the whole) donor cell cytoplasm will inevitably be brought into the recipient. Thus, the mitochondria during the development of human somatic reconstructed embryo is very important and remains to be further discussed (Yang et al., 2004).

5 Conclusions

l. Human somatic cell can be reprogrammed by enucleated rabbit oocyte. It is feasible to construct inter-species cloned embryos with human somatic cell as the donor and enucleated rabbit oocyte as recipient.

2. Granulose cell can be used as a donor in nuclear transfer with higher efficiency, but it is difficult to obtain, which makes its use restricted, while skin fibroblast is easy to obtain and should be an ideal donor cell type.

3. To a certain extent, passage of donor cell has no significant effect on nuclear transfer efficiency.

4. Refrigeration is a convenient effective donor cell preparation method in the construction of human somatic inter-species cloned embryos.

5. Single cell FISH is a simple, accurate method in characterizing nuclear chromosome derivation.

Ethical regulations

All experiments in our study were performed in accordance with the "Guidelines on Human Stem Sell Research" issued by the Science and Technology Ministry and Health Ministry of China.

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