Embryonic stem cell as nuclear donor could promote *in vitro* development of the heterogeneous reconstructed embryo

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Abstract **The nucleus of a somatic cell could be dedifferentiated and reprogrammed in an enucleated heterogeneous oocyte. Some reconstructed oocytes could develop into blastocysts *in vitro*, and a few could develop into term normally after transferred into foster mothers, but most of cloning embryos fail to develop to term. In order to evaluate the efficacy of embryonic stem cell as nucleus donor in interspecific animal cloning, we reconstructed enucleated rabbit oocytes with nuclei from mouse ES cells, and analyzed the developmental ability of reconstructed embryos *in vitro.* Two kinds of fibroblast cells were used as donor control, one derived from ear skin of an adult Kunming albino mouse, and the other derived from a mouse fetus. Three types of cells were transferred into perivitelline space under zona pellucida of rabbit oocytes respectively. The reconstructed oocytes were fused and activated by electric pulses, and cultured *in vitro.* The developmental rate of reconstructed oocytes derived from embryonic stem cells was 16.1%, which was significantly higher than that of both the adult mouse fibroblast cells (0%—3.1%, $P < 0.05$) and fetus mouse fibroblast cells (2.1%—3.7%, $P < 0.05$). Chromosome analysis confirmed that blastocyst cells were derived from ES donor cell. These observations show that reprogramming is easier in interspecific embryos reconstructed with ES cells than that reconstructed with somatic cells, and that ES cells have the higher ability to direct the reconstructed embryos development normally than fibroblast cells.**

Keywords: embryonic stem cell, fibroblast, mouse, rabbit, interspecific nuclear transfer.

The technique of nuclear transfer has been used successfully for homogeneous animal cloning, which included cattle[1], sheep[2], mice[3], pig[4], etc. However, the survival rate of cloned animal is very low, since only a few (1%—5%) reconstructed embryos develop into animals surviving to adulthood[1—4], the most of cloning embryos stop development within uterus and some of cloned neonatal animals die in respiratory failure or other abnormal phenotypes. The heterogeneous animal cloning was less reported and it will be hardly to achieve[5,6]. Now, the major issue of cloning technology is how to improve the survival rate of cloning animals. Previously, we have reported that embryos reconstructed by injecting the somatic nucleus of giant panda into the enucleated rabbit oocytes could develop into blastocyts *in vitro*[6]. Bovine oocytes, taken as a universal recipient, could also support the development of embryos produced by nuclear transfer of somatic cells from various animals[7].

There are three kinds of cells, the somatic cells, fetal cells and embryonic stem (ES) cells that are used as donor in intraspecific animal cloning[1—4], but only the somatic cells are used as donor cells in interspecific animal cloning[5,6]. It has been proposed that the nuclear could direct reconstructed embryo to develop normally only when the pattern of gene expression has been "reprogrammed" or returned to the totipotent embryonic state. Inefficient and faulty reprogramming may limit the long-term survival of cloned animals or may be related to their abnormal phenotypes[8]. Mouse embryonic stem cells have unusual karyotypic stability, totipotent, and more resistance to mechanical damage than somatic cells do[9]. ES cells have the unique ability to autonomously direct embryonic development either after nuclear transfer or after injection into tetraploid host blastocysts[3]. It has been found that a significantly higher proportion of clones derived from embryonic stem cell nuclei than the clones derived from somatic nuclei developed to term[10]. In order to explore whether the developmental ability *in vitro* of the heterogeneous cloned embryos derived from embryonic stem cells was superior to that from somatic cells or not, we reconstructed embryos with enucleated rabbit oocytes and nuclei from mouse ES cells, and compared them to embryos reconstructed with two kinds of fibroblast cells, one was derived from ear skin of an adult Kunming albino mouse and the other from mouse fetus somatic cell. We also analyzed the efficacy of ES cells in interspecific cloning, as well as the developmental ability of three kinds of reconstructed embryos *in vitro.*

1 Materials and methods

(ⅰ) Preparation of rabbit oocytes. Mature female Japan Big Eared White rabbits were superovulated with 0.15 mg of follicle stimulating hormone (FSH) (Institute of Zoology, Chinese Academy of Sciences), which was given at intervals of 12 h for 6 times. 12 h after the last FSH injection, 75 IU of human chorionic gonadotropin (hCG)(Insititute of Zoology, Chinese Academy of Sciences) was injected intravenously. 14—15 h after the hCG

injection, ovulated oocytes were flushed from the oviducts with M2 medium (Sigma). Cumulus cells were removed mechanically by pipetting after exposure 0.1% hyaluronidase (Sigma) in M2 medium for 5 min.

(ii) Enucleation of the rabbit oocytes. Oocytes were enucleated by micromanipulation. Oocytes were placed into a small drop of M2 medium containing 10% FBS (Hyclone) and 7.5 μg/mL cytochalasin B(Sigma) under mineral oil. The first polar body (PB1) and approximately 10%—20% cytoplasm beneath the PB1 were removed with an injection pipette. The enucleated oocytes were stained with 10 μg/mL Hoechst 33342 (Sigma) for 10 min and confirmed by a short ultraviolet-light exposure under a fluorescent microscope. Successful enucleated oocytes that did not emit fluorescence were used for nuclear transfer.

(iii) Preparation of mouse ES cells. Mouse ES cell lines were established by the method described in ref. [11]. Kunming albino mice were housed in the animal center of Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. 3.5 d post coitus blastocysts of mice were collected by flushing the uterus with M2 medium after natural mating. The blastocysts were plated onto the feeder layer of STO cell (kindly provided by Dr. Evans), which were treated with 0.1 mmol/L mitomycin C (Sigma) for 2 h after zona pellucida were removed with 5 g/L pronase. The medium was composed of 40% condition medium of BRL, 40% DMEM/F12 (Gibco) and 20% FBS (Hyclone), and supplemented with 0.1 mmol/L nonessential amino acid, 0.1 mmol/L β-mercaptoethanol, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1×10^5 u/L penicillin and 0.1 g/L streptomycin. The inner cell mass was big enough to become the cylinder after cultivation for 4—5 d. The epiblast was picked up and trypsinized, and passaged onto fresh feeder layers, and it was referred to passage zero. 3 d later there appeared some nest-like colonies on the feeder layer. They were picked and passaged every 2—3 d. The karyotype of ES cell used for cloning was 40XY. Histochemistry showed that ES cell expressed alkaline phophatase (AKP) (fig. 1(a)) and stage-specific embryonic antigen (SSEA)-1 (fig. 1(b)). The ES cells at the passages 13 and 14 were used for nuclear transfer after they were trypsinized, resuspended in DMEM/F-12 medium, and placed on a 3.5 cm tissue culture dish (Nunc) for 30 min to remove feeder cells and debris.

(iv) Preparation of mouse fibroblast cells. Adult fibroblast cells were collected from an ear-skin of adult female Kunming albino mouse. In brief, the biopsy area was shaved of fur and the surface was sterilized with 75% alcohol. A small piece of tissue was cut off from the ear and washed several times in PBS with penicillin-streptomycin (Hyclone), then cut into 1-mm cubes and trypsinized for 20 min at 37℃, and dispersed with pipette, and cultured on 35 mm dish (Nunc) containing DMEM (Gibco) and 10% FBS. After subcultivation for 3—4 times, non-fibroblast disappeared and the remainder was all fibroblasts. Serum-starved cells were prepared by exposing fully confluent cell culture to DMEM containing 0.5% FBS for 3—5 d, and non-serum-starved cells were prepared by routine culture. Cell monolayers of serum-starved and non-serum-starved were trypsinized and washed by centrifugation in DMEM with 10% FBS or 0.5% FBS respectively, then incubated at 38℃ in drops medium until use within 1 h. Smooth-membrane-surfaced and more refraction cells were selected for nuclear transfer.

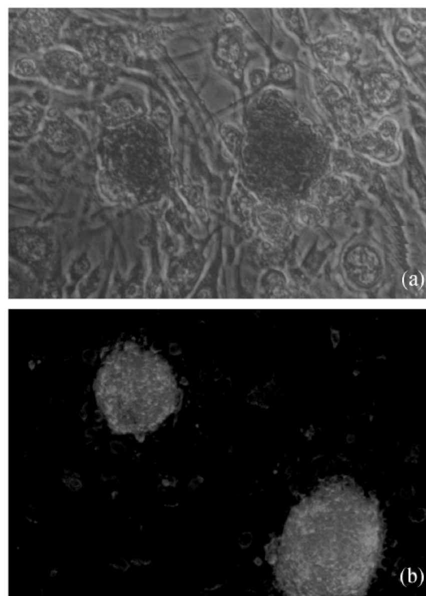

Fig. 1. Morphology and marker expression of ES colonies. AKP (a) and SSEA-1 (b) were positive.

Fetal fibroblasts were obtained from a Kunming albino mouse fetus of 13.5 dpc. The isolated fetus was washed several times in Ca^{2+}- and Mg^{2+}-free PBS. Its head, four limbs and all internal organs were removed and the remnant body tissues were treated and cultured as the ear-skin of mouse did.

(v) Nuclear transfer and embryo culture. Single mouse ES cell was microinjected into the perivitelline space of an enucleated rabbit oocyte for electric fusion. The ES cell-oocyte complexes were moved to fusion solution of 0.3 mol/L mannitol for 3 min balance and treated with 10 μs, 1.8 KV/cm DC, two pulse (BTX 2001 Electro cell manipulates, San Diego, CA), inducing the nuclear-donor cell and recipient oocyte fused together. The reconstructed oocytes were cultured in micro-droplets of M199 (Gibco) containing 15%FBS at 38℃, 5% CO_2 in humid atmosphere. The development of reconstructed embryos was observed under stereomicroscopy every 24 h.

(vi) Chromosome analysis of reconstructed blas-

tocysts. 8 blastocysts were collected randomly after reconstructed embryos were cultured for 4 d and treated with 0.1 μg/mL colchicine (Sigma) for 4 h and with 0.075 mol/L KCL for 40 min at 37℃. After hypoosmotic, the reconstructed embryos were moved to a slide. The subsequence was carried out on slides. Samples were fixed three times with cool Carnoy's solution, each for 15 min, dried in air, and stained with Giemsa. Chromosome analysis of Japan Big Eared White rabbit's somatic cells was performed simultaneously.

(ⅶ) Statistical analysis. The statistical significance of the results in experiments was examined by χ^2 test. Significant difference was determined at $P < 0.05$.

2 Results

The development of reconstructed oocytes *in vitro* was different according to the types of donor cells (table 1). The fused rates of reconstructed oocytes derived from adult fibroblasts, fetus fibroblasts and ES cells were 37.8%—41.8%, 53.5%—64.4% and 62.4% respectively. There was significant difference in the fused rate between adult fibroblast and ES cell ($P < 0.05$), or between adult fibroblast and fetus fibroblast ($P < 0.05$), but no significant difference between ES cell and fetus fibroblast ($P > 0.05$). The clevage rates in three kinds of reconstructed oocytes derived from those cell types were similar to the fused rate. However, the developmental efficiency of blastocysts derived from ES cells (16.1%) was significantly higher than that derived from adult fibroblasts (0% —3.1%, $P < 0.05$) and fetus fibroblsts(2.1%—3.7%, $P < 0.05$). The rate of blastocysts development has no difference ($P > 0.05$) between fetus fibroblast and adult fibroblast. There was no significant difference in the percentage of fused, cleavage and blastocyst development

Table 1 Development of reconstructed oocytes produced from fibroblast and ES cell

Nuclear-donor cell	No. of oocytes	No. of fused (%)	No. of cleavage (% of fused)	No. of blastocysts (% of fused)
Adult fibroblast, starved	153	64(41.8)$^{a)}$	37(57.8)$^{a)}$	2(3.1)$^{a)}$
Adult fibroblast, non-starved	90	34(37.8)$^{a)}$	18(52.9)$^{a)}$	0(0.0)$^{a)}$
Fetus fibroblast, starved	73	47(64.4)$^{b)}$	39(83.0)$^{b)}$	1(2.1)$^{a)}$
Fetus fibroblast, non-starved	101	54(53.5)$^{b)}$	46(85.2)$^{b)}$	2(3.7)$^{a)}$
ES cell	189	118(62.4)$^{b)}$	105(90.0)$^{b)}$	19(16.1)$^{b)}$

a) and b) Data within the same columns with different superscripts are significantly different ($P < 0.05$).

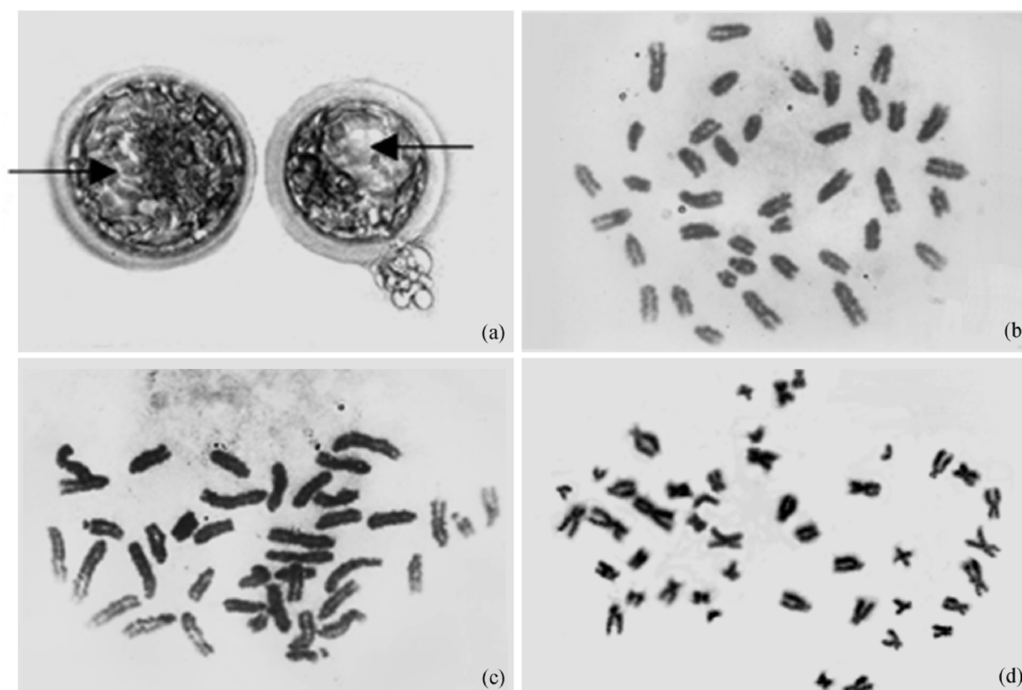

Fig. 2. Interspecific cloning embryos derived from mouse ES cell-rabbit enucleated oocyte developed *in vitro* and karyotype. (a) Reconstructed embryos developed into blastocysts *in vitro*, arrows indicate cavities of blastocysts; (b) the chromosome of mouse ES cell ($2n$=40); (c) the chromosome of blastocyst derived from mouse ES cell and rabbit enucleated oocytes

($2n$=40); (d) the chromosome of Japan Big Eared White rabbit somatic cell ($2n$=44).

between serum-starved fibroblasts and non-serum-starved fibroblasts from adult mouse and fetus mouse ($P > 0.05$).

By analysis of the chromosomes of reconstructed blastocysts, it was demonstrated that the cell karyotype of blastocysts reconstructed from ES cells was 40XY, which was identical to ES cells used for cloning. All of 40 chromosomes were telocentric (fig. 2(b), (c)). The number of chromosome of Japan Big Eared White rabbit cell was 44, which includes telocentric and acrocentric chromosomes (8), metacentric, submetacentric and subtelocenrtic chromosomes (34), and two sex chromosomes (fig. 2(d)).

3 Discussion

In this experiment, the developmental abilities of interspecific reconstructed embryos by injection of mouse ES cell into enucleated rabbit oocytes were analyzed. Fetus fibroblasts and adult fibroblasts of mouse were used as donor cell control. The rates of fused, cleavage and blastocyst development were compared among interspecific embryos reconstructed with all three cells types. Under this condition, the fused rate and the cleavage rate of interspecific reconstructed embryos derived from mouse ES cells were 62.4% and 90.0% respectively, which were similar to that derived from fetus mouse fibroblasts (53.5%—64.4% and 83.0%—85.2%) and were significantly higher than that derived from adult mouse fibroblasts (37.8%—41.8% and 52.9%—57.8%). The blastocyst percent of interspecific reconstructed embryos with mouse ES cells was 16.1%, whereas fetus fibroblasts and adult fibroblasts of mouse were 2.1%—3.7% and 0%—3.1% respectively. It was obvious that the blastocyst percent of embryos reconstructed with mouse ES cells was significantly higher than that derived from fetus fibroblasts and adult fibroblasts of mouse.

Recently, there are many studies on cloning animal with ES cell nuclear[3,10,12]. Wakayama and Mombaerts et al. have found that the cloned mice have a high survival rate when ES cell, even high passage ES cell, was used as donor nuclear in intraspecific animal cloning. Cloned animals with R1-ES cell (an ES cell line established by Nagy, A. et al. in 1993) had a high birthrate of 8.3%. In addition, they also compared the developmental rate of cloned mice with cumulus cell and with ES cell. The former was 1.2%, and the latter was 2.4%[10]. The birthrate of cloned mice with fibroblast of mouse tail-tip was 1.1%[13]. The highest birthrate of cloning animals with ES cells was 33%, the lowest 14%, average 21%, and all of the cloning offspring survived to adult if the ES cells used as donor nuclear were derived from F1 mice [12]. Thus it was apparent that the survival rate and developmental rate of cloned embryos derived from ES cell could be improved greatly no matter what cloning does in intraspecies or in interspecies. This ability of ES cells is not due to a type of fetal cell. The rate of blastcysts of the fetal fibroblasts cloning embryos was 2.1%, which was similar to that of adult fibroblast cloning embryos in our experiment. This mystical ability ES cell possessed might be caused by two reasons. First, the ES cells are highly undifferentiated totipotent cells, so they have unique ability to autonomously direct embryonic development either after nuclear transfer[3,10,12] or after injection into tetraploid host blastocysts[3]. Second, DNA of ES cells had a lower methylation. Some genes of embryonic cells are opened and some genes were closed during embryonic development and cell differentiation. Opening and closing of genes were dependent on the modification of gene methylation or demethylation. The more the cell was highly differentiated, the more its DNA was highly methylated. The more the cell was lowly differentiated, the more its DNA was lowly methylated. When a highly differentiated nuclear was transferred into enucleated oocyte, the reconstructed embryos derived from a highly differentiated nuclear will be reprogrammed. Gene methylation, which happened during embryonic development and cell differentiation, must be removed, and genes that should not be expressed must be methylated. DNA was easy to be demethylated, reprogrammed and required less demethylation when ES cells were transferred into enucleated oocyte[10, 12], so the disadvantage of ES cells was less than that of somatic cells. In a suitable condition of culture *in vitro*, mouse embryonic stem cell lines exhibit unusual karyotypic stability. ES cell lines can be cultured *in vitro* for many passages almost without karyotypic change[9]. It was suggested that ES cell was a fine nuclear-donor in animal cloning. Our research provided a key cue on improving the technology of interspecific animal cloning.

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