Rabbits generated from fibroblasts through nuclear transfer

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Abstract

Somatic cell nuclear transfer offers new opportunities for genetic engineering and genome preservation in mammalian animal species. We show that, in addition to cumulus cells, cultured adult rabbit fibroblasts are also capable of supporting full-term development after nuclear transfer. Nuclear transfer embryos constructed using serum-starved fibroblasts showed a significantly higher developmental rate than non-starved fibroblasts through preimplantation stages. A total of 467 nuclear transfer embryos were transferred into the oviducts of pseudo pregnant mothers. Eight of the 20 surrogate rabbits carried the pregnancy to term and five of them gave birth naturally to a total of nine rabbits. However, all of the offspring died before postnatal day 10. A Caesarean section was performed on three surrogates, giving birth to a total of five rabbits, three of them survived and grew into healthy adults. DNA analyses confirmed that these rabbits were genetically identical to the donor male rabbit. The present study demonstrates that rabbits can be cloned from adult fibroblasts after culture.

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Introduction

Somatic cell nuclear transfer (SCNT) offers new opportunities for genetic engineering and genome preservation in mammalian animal species. Although overall cloning efficiency is low, rapid progress has been made to improve the technology. So far, SCNT has succeeded in generating live animals in many species, including sheep (Schnieke *et al.* 1997, Wilmut *et al.* 1997), cattle (Cibelli *et al.* 1998, Kato *et al.* 1998), mice (Wakayama *et al.* 1998), goats (Baguisi *et al.* 1999), pigs (Betthausen *et al.* 2000, Onishi *et al.* 2000, Polejaeva *et al.* 2000), cats (Shin *et al.* 2002), mules (Woods *et al.* 2003), horses (Galli *et al.* 2003), rats (Zhou *et al.* 2003) and dogs (Lee *et al.* 2005).

The relatively large body size of rabbits makes them the preferred animal species for a variety of fields, including pulmonary and cardiovascular research, as well as drug screenings, toxicity tests and metabolic studies that require frequent blood sampling. Given their frequent use in research, the creation of genetically modified rabbit lines is highly desirable. It has been previously demonstrated in bovines (Cibelli *et al.* 1998) and pigs (Lai *et al.* 2002, Ramsoondar *et al.* 2003) that fibroblasts can be genetically modified and used as nuclear donors to produce animals with specific genetic traits. Theoretically, it should then also be feasible to generate mutant rabbit lines by modifying the genome of rabbit fibroblasts by homologous recombination.

Rabbits were cloned from blastomeres many years ago (Stice & Robl 1988). The development of SCNT technology in rabbits has been the focus of much recent research (Mitalipov *et al.* 1999, Dinnyes *et al.* 2001, Inoue *et al.* 2002, Yin *et al.* 2002) and only female rabbits have been successfully cloned from freshly isolated cumulus cells using modified oocyte activation and embryo transfer protocols (Chesne *et al.* 2002). In the current study, we demonstrate the feasibility of an alternative SCNT protocol to clone rabbits from adult fibroblasts after culture. This may facilitate genetic engineering and genome preservation in rabbits.

Materials and Methods

Unless otherwise noted, all media used were purchased from Gibco Invitrogen. Animal care and the SCNT protocol were approved by the Ethics Committee of Shanghai Jiao Tong University, School of Medicine.

Oocyte collection

Mature White New Zealand female rabbits (approximately 3.5 kg and more than 6 months old) were superovulated by intramuscular injection of 150 IU of eCG (equine Chorionic Gonadotropin, Huafu high-tech Co. Ltd, Tianjin, China) followed, 96 h later, by an intravenous

injection of 100 IU hCG (human Chorionic Gonadotropin, Zhongbao Co. Ltd, Tianjin, China) to induce ovulation. The ovulated oocytes were recovered at 14 h post hCG injection by flushing the oviducts with pre-warmed RD medium (50% RMPI 1640 and 50% DMEM) (Carney & Foote 1991) supplemented with 10% (v/v) fetal cattle serum (FCS, Hyclone Co.) and 10 mmol Hepes/l (Hepes balanced RD, hRD). Cumulus cells were removed by exposure to 100 IU hyaluronidase/ml (Sigma chemical Co.) in hRD medium for 10 min and then pipetted through a fine pipette. Denuded oocytes were washed six times and incubated (38 °C) in RD medium supplemented with 10% FCS, 2 mmol glutamine/l, 223 µmol sodium pyruvate/l and 100 µmol MEM non-essential amino acids/l (modified RD, mRD) and maintained in a humidified atmosphere (5% CO₂) until use.

Preparation of nuclear donors

Fibroblasts were collected through ear-skin biopsy of an adult New Zealand male rabbit as previously described (Dinnyes *et al.* 2001). The skin piece was washed several times in Dulbecco PBS, cut into 1 mm cubes and cultured as explants in DMEM supplemented with 20% FCS (37 °C, 5% CO₂). Fibroblasts developed from explants within approximately 10 days. Cells were then digested with 0.05% trypsin-EDTA, passed to a new dish (passage 1) then expanded further in DMEM with 10% FCS for up to the 13 passages. Some fibroblasts used in the study were frozen in liquid nitrogen. Cells from passages 6 to 13, either fresh or thawed, were used as nuclear donors in the cloning experiments.

Before nuclear transfer, 1.5×10^5 fibroblasts were plated and cultured until cells reached confluency (CC group, confluent culture). Cells were subsequently digested with trypsin and used as nuclear donors. The medium was replaced with DMEM supplemented with 0.5% FCS, 2–5 days before cells were used as nuclear donors (starved cells, SC group). Nuclear donor fibroblasts were routinely resuspended in DMEM with 10% FCS. Cells small in size and with a smooth surface were selected for the nuclear transfer experiments.

Nuclear transfer

A total of 40–80 oocytes were obtained from three rabbits for each nuclear transfer experiment. Oocytes with a normal looking metaphase plate and with the first polar body under the zona pellucida were selected for nuclear transfer. Less than 5% of freshly collected oocytes showed an abnormal size (e.g. out diameter > 200 µm) or color (e.g. opaque in the center). These oocytes were considered unhealthy and discarded. Oocytes were incubated (38 °C, 10 min) with hRD supplemented with 7.5 µg cytochalasin B/ml (Sigma Chemical Co.) before enucleation. The metaphase plate and polar body was

visualized with an inverted microscope equipped with a Spindlevue system (Cambridge Research & Instrumentation Inc.) and was removed with an enucleation pipette (18–22 µm, out diameter; 15–20 oocytes were enucleated within 10 min). The microscope work plate was maintained at 37 °C.

Single nuclear donor cells were inserted into the perivitelline space of enucleated oocytes. The oocyte–fibroblast units were manually oriented in a 1 mm gap chamber of an Electro Cell Manipulator (BTX 830, San Diego, CA) in 0.3 mol Sorbitol/l solution containing 0.1 mmol calcium acetate/l, 0.5 mmol magnesium acetate/l and 1 mg BSA/ml (Sigma Chemical Co.). Oocyte–fibroblast units were subjected to three 20 µsec DC pulses of 3.0 KV/cm and were then incubated with mRD (80 min, 38 °C). To activate the nuclear transfer embryos (nt-embryo) a second set of electropulses was delivered at 16.5 h to 18 h after hCG injection, followed by incubation (1 h, 38 °C) with mRD containing 5 µg cycloheximide/ml (Sigma Chemical Co.) and 2 mmol 6-dimethylaminopurine/l (Sigma Chemical Co.) as previously described (Chesne *et al.* 2002). After chemical activation, nt-embryos were incubated with mRD for 2 h followed by 50 µl of upgraded B2 medium (Laboratoire C.C.D, Paris, France) supplemented with 2.5% FCS. The culture was maintained under mineral oil (Sigma-Aldrich, Inc.) at 38 °C in a humidified atmosphere of 5% CO₂.

Embryo transfer

Pseudopregnant rabbits received 80 IU hCG with a 24 h delay in comparison to the protocol used for oocyte donor rabbits. 8–15 nt-embryos at 2- to 8-cell stage were transferred, through the infundibulum, into each oviduct of the pseudopregnant rabbits.

Microsatellite marker analysis

The nuclear donor rabbit, three cloned progenies and two surrogate mothers were genotyped using the following five primer sets: 5L1C3 (AF421908), ELAMB (M91055), ASICG (AY284844), 6L1F10 (AF421916) and 19L1A4 (AF421948) (van Haeringen *et al.* 1996, Korstanje *et al.* 2003). Genomic DNA was extracted from the blood of all the rabbits.

Statistical analysis

The fusion and development of activated nt-embryos to blastocyst stage was evaluated and data from different treatments were compared with chi-square analysis.

Results

To prepare nt-embryo, a fibroblast was inserted into the perivitelline space and fused to the enucleated oocyte. As shown in Table 1, a total of 178 and 240

Table 1 Pre-implantation stage development of nt-embryos resulting from donor cells from serum-starved and none-starved groups.

Donor cell treatment	Oocytes used	No. (%) fused nt-embryos	No. (%) cleaved nt-embryos	No. (%) blastocysts
CC ^a	178	115 (66)	75 (65) ^c	24 (21) ^e
SC ^b	240	173 (72)	135 (78) ^d	77 (45) ^f

^aCC: confluent cells, none-starved group.^bSC: serum starved cells.^{c–f}Chi-square analyses demonstrating statistical difference between c and d ($P < 0.05$) and e and f ($P < 0.01$).

reconstructed couplets were produced using fibroblasts from the CC group and the SC group respectively. Thirty minutes after electrofusion, 65% (115/178) of couplets from the CC group and 72% (173/240) of couplets from the SC group had fused successfully. The chromatin of the donor cells became condensed to form chromosomes approximately 1 h after electrofusion (Fig. 1A).

The fused nt-embryos were activated by a second set of electropulses 80 min after fusion and subsequent incubation in activation medium for an hour. Pronuclear-like structures appeared (Fig. 1B) at 2 h after chemical activation, in 67% (46/69) of nt-embryos in the CC group and 73% (49/67) of nt-embryos in the SC group. Individual nt-embryos displayed one, two or more pronuclear-like nuclei. Most nt-embryos entered the 2-cell stage 9–14 h after chemical activation, the 4-cell stage within 12–18 h, the 8-cell stage within 20–24 h (Fig. 1C), the morula within 48 h (Fig. 1D) and the blastocyst stage within 72 h (Fig. 1E). Some blastocysts hatched out entirely from the zona pellucida approximately 120 h after activation (Fig. 1F).

The CC and SC groups were compared for performance at each of the pre-implantation stages. As shown in Table 1, a higher percentage of nt-embryos in the SC group entered cleavage (SC:CC=78%:65%, $P < 0.05$) and a significantly higher percentage of nt-embryos in the SC group developed into blastocyst (SC:CC=45%:21%, $P < 0.01$).

Given that the SC group produced nt-embryos with better developmental performance, serum-starved cells were used as nuclear donors in subsequent experiments. As shown in Table 2, of 675 nt-embryos obtained from 945 reconstructed couplets, 494 cleaved (73%, 494/675). A total of 467 nt-embryos at 2–8-cell stage were transferred into the oviducts of 20 pseudopregnant recipients, of which eight carried the pregnancy to term (40%, 8/20). As shown in Table 3, five of the eight surrogate rabbits gave natural birth to nine progenies. All of them died within 10 days of birth. The high death rate appeared to be related to two reasons. First, a proportion of rabbits borne after prolonged gestation experienced dyspnea, the prolonged gestation might have prevented normal postnatal development and function of the organs. Secondly, several surrogate mothers carrying cloned babies had no colostrums in time when the babies were born. The babies tended to die if they did not get milk within 6 h after birth.

In an attempt to improve survival rates, we performed Caesarean sections on the other three pregnant surrogate rabbits at 30 days after embryo transfer and helped the newborn babies to get milk from other mothers. Of the five progenies obtained, four were alive at birth. Although one died on the same day, the other three (one borne on July 1, 2005; two borne on July 8, 2005) lived and grew into healthy male adults (Table 3 and Fig. 2A). The remaining three live rabbits were 7 months

Figure 1 Nt-embryos in pre-implantation stages. (A) Condensed chromosomes (arrow) in the nt-embryo (1 h after fusion). (B) The pronuclear-like structure (arrow) in the nt-embryo (2 h after activation). (C to E) Nt-embryos at the 8-cell, the morula, and the blastocyst stage respectively. (F) A hatched blastocyst. Bar=50 μ m.

Table 2 A summary in development of nuclear transfer embryos *in vivo*.

Sample size	Developmental rate
No. reconstructed couplets	945
No. fused couplets (% from reconstructed)	675 (71)
No. cleaved embryos (% from fused)	494 (73)
No. transferred embryos	467
No. recipient mothers	20
No. recipients delivering (% of total recipients)	8 (40)
No. full-term fetus (% from transferred embryos)	14 (3.0)
Birth weight (g)	55 ± 21 (<i>n</i> = 12)

Table 3 Better survival of cloned rabbits by Caesarean section.

Type of birth way	Natural birth	Caesarean
No. surrogate rabbits	5	3
No. full-term fetus	9	5
No. alive fetus	8	4
No. alive at weaning	0	3

old when this paper was written. One of the cloned rabbits was mated to a conventional female rabbit and the mating produced eight healthy baby rabbits. This result demonstrated that the cloned rabbit was fertile. Microsatellite DNA analysis confirmed that all three cloned rabbits were genetically identical to the nuclear donor male rabbit (Fig. 3 and Fig. 2B).

The body weights of cloned rabbits were in a normal range on average (55 ± 21 g, *n* = 12) as compared to normal newborn rabbits (56.1 ± 5.1 g, *n* = 43), although cloned rabbits displayed more variations. For example, one baby rabbit born at 35 days reached 93 g and another by Caesarean section at 30 days weighed only 20 g.

Primary cultures have been suggested to have less homogenous cell types initially (Kubota *et al.* 2000). Because of this, we utilized cells after at least six passages, when populations grew more vigorous and appeared more homogenous in morphology. We obtained 14 (3%, 14/467) (Table 2) full-term development rabbits using fibroblasts from passages 6 to 13, and

12 were from frozen stored donor cells. The three lived rabbits were cloned from fibroblasts which have been passaged 11 and 12 times. These results demonstrate that the cell culture protocol utilized was suitable for growing nuclear donor cells and that the frozen-thawed processes did not cause apparent damage in the developmental capacity of fibroblasts.

Discussion

In the current study, we demonstrate that adult rabbit fibroblasts can be cultured, expanded and frozen without losing their ability to support the development of nt-embryos, a technology which may potentially be used to modify fibroblast genome by homologous recombination so as to generate genetically altered cloned rabbits.

After the birth of Dolly, it became apparent that somatic cells at the G0/G1 phase could potentially be better nuclear donors (Wilmut *et al.* 1997). Cumulus cells have been used successfully in many animal cloning experiments and 90% of the freshly isolated mouse cumulus cells are naturally in the G0/G1 stage (Schuetz *et al.* 1996, Wakayama *et al.* 1998). Since cumulus cells can only be isolated from female donors to produce female progenies, fibroblasts can be used as an alternative source of nuclear donors and without limitations to age, sex or physiological state.

Serum starvation is an effective way to induce cells into an inactive G0/G1 cell stage. Another method is to maintain confluent cell cultures for a prolonged time period (Boquest *et al.* 1999, Dinnyes *et al.* 2001). In our experiments we obtained higher rates of blastocysts when serum-starved cells were used as donors for nuclear transfer, compared to the rates obtained from nuclear transfer utilizing confluent cells.

In the current study, we collected oocytes 14 h after hCG injection and prolonged the time between fusion and activation to 80 min. This protocol, which may have permitted a longer interaction time between the ooplasm and the nucleus, led to successful blastocyst

Figure 2 Rabbits cloned from adult fibroblasts. (A) Three cloned rabbits (5 month old) resulting from nuclear transfer. (B) The male rabbit whose fibroblasts were used as the nuclear donors in this study.

Figure 3 Microsatellite analyses of rabbit genome. 1, nuclear donor rabbit; 2–4, cloned rabbits; 5 and 6, surrogate rabbits. 5L1C3, ELAMB, ASICG, 6L1F10 and 19L1A4 are the five PCR primer sets used to detect DNA polymorphism in rabbits. The numbers at the top of the figure indicate the sizes of base pairs.

development consistently, providing a variation to that of Chesne *et al.*, who cloned rabbits with oocytes collected 16 h after hCG injection (Chesne *et al.* 2002). Additionally, we utilized a “Spindle View” apparatus to view the oocyte metaphase plate, resulting in better enucleation efficiency.

Chesne *et al.* (2002) found that the pre-implantation development of nt-embryos was delayed by approximately one day compared to normal embryos. Therefore they performed embryo transfer at the 2–4-cell stage using asynchronous recipients mated to vasectomized males 22 h after the donor females. Following the same idea, we transferred nt-embryos at the 2–8-cell stage using asynchronous recipients which were induced ovulation 24 h after the donor females were induced. Eight out of 20 recipients gave birth to 14 (3%) fully developed progenies, of which 12 were alive at birth. These results confirmed that an asynchronous embryo transfer scheme is important for cloning rabbits.

Although a high percentage of the nt-embryos developed to the blastocyst stage in *in vitro* culture, rabbit had a narrow window for implantation *in vivo* that often resulted in low implantation rates of nt-embryos. In addition, prolonged gestation and inappropriate feeding immediately after birth can lead to death of the newborn. As described in this paper, Caesarean section, in combination with appropriate care and feeding of the infants, improved the survival rate of cloned rabbits to 60% (3 out of 5). These data emphasize the importance in caring for the cloned infant rabbits that, like other cloned species, tend to carry physiological defects.

In summary, the present study has demonstrated that rabbits can be cloned from long-term cultured adult fibroblasts. This method may facilitate genome preservation and genetic engineering in rabbits.

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