Nuclear transfer using nonquiescent adult fibroblasts from a bovine ear

LIU Jilong[1], WANG Minkang[1,2], LIAN Li[1], LI Jinsong[1,3], GAO Shaorong[1], HAN Zhiming[1,4], SUN Qingyuan[1], SONG Xiangfen[1], ZHANG Dongmei[5], LI Yihai[5], XU Zhi[5] and CHEN Dayuan[1]

1. *State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing* 100080, *China*;
2. *Yunnan Education College, Kunming* 650223, *China*;
3. *College of Animal Husbandry and Veterinary Science, Yangzhou University, Yangzhou* 225009, *China*;
4. *Department of Biology, Beijing Normal University, Beijing* 100875, *China*;
5. *Tianjin Institute of Animal Husbandry and Veterinary Science, Tianjin* 300112, *China*

Abstract The natural reproduction of mammal is sexual reproduction, which needs fertilization involving sperm and oocyte. Nuclear transfer provided an asexual reproduction method for mammal. Donor cells used in previous experiments of nuclear transfer were mostly from undifferentiated or non-terminally differentiated cells, such as embryonic or fetal cells. However, since Wilmut *et al*. obtained a viable lamb by transfer of an adult sheep somatic cell into an enucleated oocyte, nuclear transfer using adult somatic cell has been successful in several species. Wilmut *et al*. suggested that it was a key factor for the success of somatic nuclear transfer to induce the donor cells into G0 phase ("G0-phase hypothesis"). In order to verify the G0-phase hypothesis, nonquiescent adult fibroblasts from a bovine ear were transferred into enucleated bovine oocytes. The experiments showed that the rate of electrofusion after micromanipulation was above 50%, the cleaving rate was 54.5% and 9.1% of those reconstructed embryos developed to 32-cell stage. These results indicate that for cattle, nuclei from nonquiescent adult somatic cells introduced into enucleated oocytes are at least capable of supporting early development.

Keywords: **nonquiescent phase, adult cattle, fibroblast, nuclear transfer.**

IN 1997, Wilmut *et al*. reported that a viable lamb was produced by electrofusion of a mammary gland-desired cell adult sheep with an enucleated oocyte. It was the first nuclear transfer case using adult somatic cells as nuclear donors. This fact challenged the previous opinion that differentiated mammal somat-

ic cells do not possess totipotency. To recover the totipotency of differentiated somatic cells, Wilmut *et al*. especially emphasized the importance of inducing the donor cells into G0 phase, for reprogramming, by serum starvation method and regarded it as the key factor for their experiments[1,2]. In 1998, Cibelli *et al*. succeeded in nuclear transfer using nonquiescent fetal bovine fibroblasts as nuclear donors[3]. This result seemingly indicated that, to recover totipotency, it was not necessary to induce the donor cells into G0 phase. However, the result of the latter was not enough to judge the G0-phase hypothesis because the donor cells were fetal but not adult somatic cells. Here we used adult nonquiescent fibroblasts from bovine ear as donors in nuclear transfer to test the G0-phase hypothesis.

1 Materials and methods

(ⅰ) Culture of somatic cells. A bovine ear was collected from a local abattoir, cut to about 0.5 cm cubes, submerged in 70% ethanol for 60 s, rinsed by sterile BSS for 3 times and transported to laboratory at 4℃. The tissue was cut finely in laboratory, dissagregated by cold trypsin treatment and cultured in DMEM (Sigma Co., St. Louis, MO, USA) containing 20% FCS (Chuanye Co., Tianjin, China) according to reference [4].

(ⅱ) Oocyte maturation *in vitro*. Bovine ovaries, collected at a slaughterhouse, were kept in DPBS at 25—30℃ and transported to the laboratory within 6 h after slaughter. Oocytes were cultured *in vitro* as described by Wu *et al*.[5].

(ⅲ) Nuclear transfer. Oocytes were denuded of cumulus cells at 22—24 h of IVM by pipetting in CZB medium containing 0.1% hyaluronidase and then placed in CZB medium containing cytochalasin B (7.5 μg/mL) for 30 min.

For nuclear donor preparation, adult fibroblasts from bovine ear at devision or growth phase (nonquiesent phase) were trypsinized at 37℃ for 3—5 min and neutralized by DMEM once the majority of the cells withdrew their pseudopodia and became round.

Oocytes were positioned on the holding pipette with the first polar body at the position of 6 o'clock. The enucleating pipette was inserted under the zona pellucida and the polar body along with the underlying ooplasm was aspirated out. Then the donor cell was injected into the perivitelline space of the enucleated oocyte, touching the membrane of oocyte.

The microinjected oocyte were exposed to M199 (Sigma Co.) containing cytochalasin B (7.5 μg/mL) and 10% FCS for 30 min. They were then placed in Zimmerman medium[6] in an electrical field. Membrane fusion was induced by a square-wave DC pulse (130—140 V/mm for 80 μs).

All the reconstructed embryos were cultured in M199 medium containing 10% FCS at 38.5℃ in 5% CO_2.

Manipulation of the control group was the same as above except no donor cell was inserted.

2 Results

The rate of cell fusion was above 50% (table 1).

The results showed that reconstructed oocytes can cleave and the cleaving rates of 2-, 4-, 8-, 16-, and 32-cell stages were 54.5%, 42.4%, 27.3%, 15.2% and 9.1%, respectively (fig. 1 (d)—(i); table 1). These results suggest that nuclei of nonquiescent, somatic cells from adult bovine are at least capable of supporting early development when introduced into enucleated oocytes.

Table 1 Development of reconstructed bovine embryos

No. oocytes	Group	No. enucleated	No. fused (%)	No. 2-cell (%)	No. 4-cell (%)	No. 8-cell (%)	No. 16-cell (%)	No. 32-cell (%)
112	expt.	64	33 (51.6)	18 (54.5)	14 (42.4)	9 (27.3)	5 (15.2)	3 (9.1)
	control[a)]	19		0				

a) The manipulation of the control group was the same as the experimental group except no donor cell was inserted.

3 Discussion

Previous studies have suggested that adult mammal somatic cells are irreversibly differentiated and do

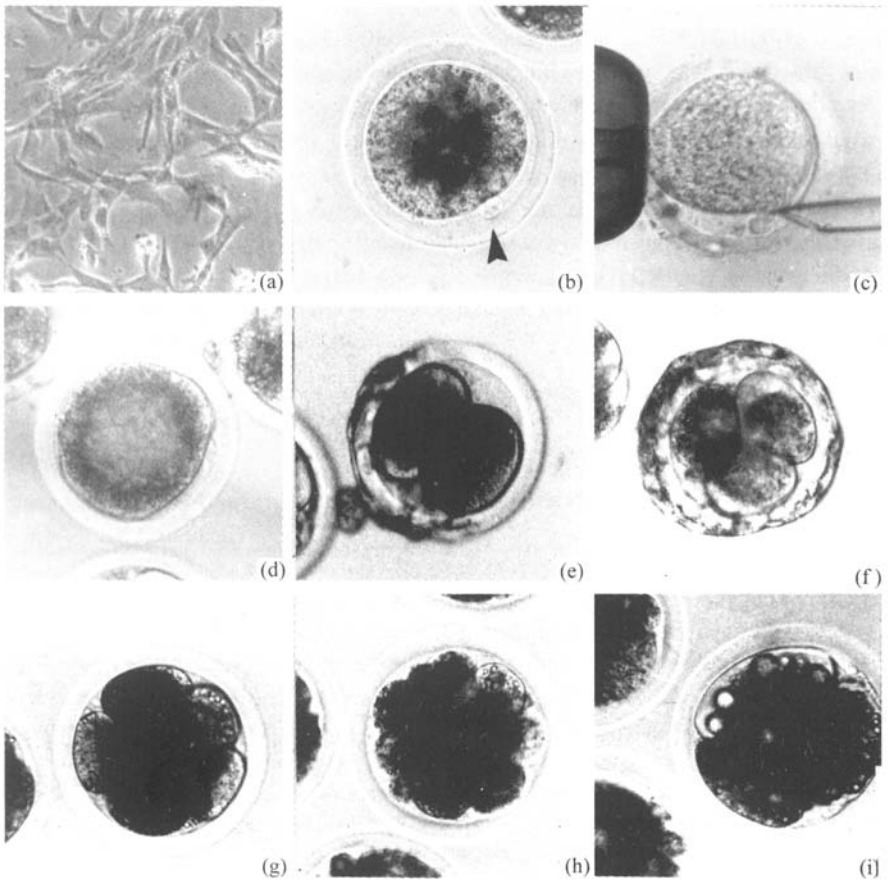

Fig. 1. Cultures of donor (a) and recipient cells (b), nuclear transfer (c) and the development of constructed embryos at 1-cell (d), 2-cell (e), 4-cell (f), 8-cell (g), 16-cell (h) and 32-cell stage (i). The attached bovine ear fibroblasts are at growth phase (a) and the first polar body (arrow, (b)) indicates the maturation of bovine oocyte.

not possess totipotency, so the donor cells used in those nuclear transfer experiments are mainly embryo cells which are undifferentiated. The success of nuclear transfer using terminally differentiated, adult somatic cells as donors indicates that differentiated somatic cells still possess totipotency.

Wilmut *et al*. regarded it as the key factor to induce donor somatic cells into G0 phase. On the one hand, they proposed that the G0-phase nuclei and the MII stage enucleated oocytes in both the "post-activation" and "fusion and activation" methods of reconstruction are coordinated; on the other hand, the chromatin of G0-phase donor nuclei have undergone modification and may be more readily modified by oocyte cytoplasm (dedifferentiation)[1,2]. So, Wakayama *et al*. selected the Sertoli cells and neurons which were at G0 phase and cumulus cells (more than 90% in the G0/G1 phase of the cell cycle) as nuclear donors in their experiments[10]. Kato *et al*. also cloned cow by inducing the donor cells to exit the growth phase[11]. However, Cibeli *et al*. used G1 phase rather than G0 phase fetal-calf-fibroblasts as donors in their nuclear transfer[3]. We froze the somatic cells after culturing for 2 weeks and recovered them one month later. After being subcultured for 4—6 d (when cells did not grow fully), the cells were used as donors in nuclear transfer. On the night before micromanipulation, the medium was refreshed to confirm the donor cells at growth phase (nonquiescent phase). These cells were randomly at G1, S, G2 or M phases. Our experiments show that donor cell at G0 phase is not the necessary factor for somatic cloning. Obviously, it needs further research to judge whether nonquiescent adult somatic cells are capable

of supporting the full-term development of the reconstructed oocytes. In addition, the effects of donor cells at different phases (G0, G1, S, G2 or M phase) on the efficiency of nuclear transfer are still unknown.

Up to now, the donor cells used in adult mammal somatic nuclear transfer are, interestingly, originated from the reproductive system of female animals. For example, the donor cells in the sheep cloning were mammary-derived epithelium[1], those in Kato's bovine nuclear transfer were cumulus cells and oviductal cells[11], and Wakayama *et al*. used cumulus cells for cloning mice[10]. This fact leads to the following two questions. First, when male somatic cells or cells from non-reproductive system are used as nuclear donors, can they support the full-development of reconstructed embryos? Second, does the difference of sex or system affect the efficiency of cloning? Our experiments can partly solve the first problem because the donor cells were from a male cattle and also from a non-reproductive system. As for the second question, systematic and enormous research is necessary.

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