Serial nuclear transfer improves the development of interspecies reconstructed giant panda (*Aluropoda melanoleuca*) embryos

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Abstract **Interspecies somatic nuclear transfer (NT) may provide a new approach for preservation of the endangered rare species. Previous interspecies cloning studies have shown that a nucleus from a quiescent somatic cell supports early development of reconstructed embryos in the ooplasm from another species. In this study, we transferred nonquiescent somatic cells from a giant panda into the perivitelline space of the enucleated rabbit oocytes. After electrofusion (at the rate of 71.6%) and electrical activation, 4.2% of the panda-rabbit reconstructed embryos developed to blastocyst *in vitro*. For improving the development rate of reconstructed embryos, we used serial NT in this study, i.e. blastomeres from reconstructed morulae were transferred into the perivitelline space of the enucleated rabbit oocytes. The fusion rates in the groups of serial Ⅰ, serial Ⅱ and serial Ⅲ were 79.5%, 84.1% and 78.0%, respectively, having no difference with that of somatic group. And the blastocyst rates in serial NT groups were 19.4%, 13.5% and 10.3%, respectively, which are significantly higher than that in somatic NT group. These results indicate that the nuclei from nonquiescent somatic cells can support early development of reconstructed embryos and serial NT can improve the development rate of interspecies reconstructed embryos.**

Keywords: nonquiescent cell, giant panda, rabbit, interspecies nuclear transfer, serial nuclear transfer.

Since the sheep "Dolly"[1] was born, somatic NT offspring have been produced in several species, including mouse[2], goat[3], cattle[4] and pig[5]. The success of somatic NT provides a new way for preservation of the endangered rare species, that is, using interspecies NT to clone endangered rare species. In 1999[6], we reported the production of interspecies cloned giant panda blastocysts by transferring the quiescent giant panda skeletal muscle cells, uterus epithelial cells and mammary gland cells into the perivitelline space of enucleated rabbit oocytes. Dominko et al.[7] reported that those adult somatic nuclei from monkey, sheep, porcine or rat could support early embryo development after being transferred into bovine ooplasm. In 1998, Wells et al.[8] successfully cloned the last surviving cow of the Enderby Island cattle by interspecies NT. In 2000, Lanza et al.[9] have cloned an endangered species (*Bos gaurus*) with normal karyotypic and phenotypic using interspecies NT.

Serial NT, which is a usual approach in intraspecies cloning, has not been used in interspecies NT. Campbell and Wilmut[10] hypothesized that serial NT is of benefit to the development of reconstructed embryos by improving reprogramming of the transferred nuclei in maternal cytoplasm. In this note, we studied the development of reconstructed embryos after transferring nonquiescent giant panda somatic cells into enucleated rabbit eggs and the effect of serial NT on the development of interspecies reconstructed giant panda embryos.

1 Materials and methods

(ⅰ) Culture of giant panda somatic cells. Giant panda peritoneal membranes, collected from a died triplet at Wolong Research and Conservation Center for Giant Panda in 1999, were rinsed by sterilized saline for 3 times, cut into pieces and digested with 0.25% trypsin at 4℃ for 2 h and then at 37℃ for 30 min. The digested cells were cultured and passaged in DMEM/F12 (Gibco) supplemented with 20% FBS (Gibco) according to a previous report[11].

(ⅱ) Superovulation and collection of oocytes. Mature Japanese Big Eared White female rabbits were superovulated with six s.c. injection of 0.6 mg FSH (Institute of Zoology, Chinese Academy of Sciences), twice daily for 3 d. 75 IU of hCG (Institute of Zoology, the Chinese Academy of Sciences) were given i.v. 12 h after the final dose of FSH. At 14—15 h after injection of hCG (post-hCG), mature MII oocytes were flushed from the oviducts with M2 medium (Sigma Chemical Co. St. Louis, MO, USA). The cumulus oocytes complexes (COC) were placed in M2 supplemented with 0.5% hyaluronidase (Sigma Co.) for 3—5 min and cumulus cells were removed by repeated gentle pipetting through fine-bore pipette.

(ⅲ) Preparation of donor cells. To obtain donor cells from adherent cells layer, the fibroblasts from giant panda peritoneal membranes at division or growth phase were digested with 0.25% trypsin at 37℃ for 3—5 min and neutralized by DMEM once the majority of the cells

withdrew their pseudopodia and became round in shape.

(iv) Somatic NT (group of somatic cell). Nuclear transfer was conducted according to the method described in ref. [6]. Briefly, oocytes were preincubated for 15 min in M2 medium with 7.5 μg/mL cytochalasin B (CB, Sigma Co.). The oocyte was hold by a holding pipette, with its first polar body positioned at 2 o'clock under an inverted microscope. The enucleated pipette was inserted under the zona pellucida and the first polar body with approximately 1/4—1/3 of the adjacent cytoplasm containing metaphase Ⅱ spindle was aspirated. The enucleated eggs were stained with 7.5 μg/mL Hochest 33342 (Sigma Co.) for 8—10 s and examined under a fluorescent microscope. Eggs from which all the chromosomes were removed were used for NT. Then a 15—20 μm fibroblast was injected into the perivitelline space of an enucleated oocyte. After injection, the couplets were immediately placed into electrical fusion solution (EFS) as previously reported[12] for 1—2 min. Then they were transferred into a fusion chamber overlaid with EFS. Cell fusion was induced with two direct current (DC) pulses (1.4 kV/cm, 80 μs each, 1 s apart) and examined after the couplets were cultured in M199 with 10% FCS for 0.5 h. The fused oocytes were treated with two electroporations 30 min apart (two 20 μs, 1.4 kV/cm DC pulses, 1 s apart for each) for further activating oocytes.

(v) *In vitro* culture of reconstructed embryos. The panda-rabbit interspecies reconstructed embryos were cultured in M199 with 10% FCS, at 38℃ in 5% CO_2 in air. Embryos were observed for cleavage every 24 h.

(vi) Separation of blastomeres from reconstructed embryos. The reconstructed morulae were obtained 72 h after cultured *in vitro*. Zona pellucida was digested with 0.5% pronase (Sigma Co.) at 38℃ for 3—4 min. After rinsing in M2 medium three times, the embryos were transferred into Dulbecco salt solution, which was devoid of calcium and magnesium ions (Gibco), for 15—20 min. The embryos were then pipetted with a flame-polished narrow-bore pipette in M2 medium to disaggregate blastomere.

(vii) Serial NT (fig. 1 (a), (b), (c)). The blastomeres were used as donor nuclei for NT. The procedures of NT were the same as somatic NT. In serial Ⅰ NT (Group of serial Ⅰ), the blastomeres from somatic interspecies reconstructed morulae were used as donor nuclei. In the serial Ⅱ NT (Group of serial Ⅱ), the blastomeres from reconstructed morulae of the serial Ⅰ NT were used as donor nuclei. In the serial Ⅲ NT (Group of serial Ⅲ), the blastomeres from reconstructed morulae of the serial Ⅱ NT were used as donor nuclei.

(viii) Statistical analysis. Differences between experimental groups were analyzed using Student's *t*-test.

2 Results

The fusion rate of somatic group was 71.6%, having no difference with that of the other groups (table 1). The percentage of reconstructed embryos developed to 2-cell, 4—8-cell, blastocyst in the group of somatic cell were 71.9%, 27.2% and 4.2%, respectively. In the group of serial Ⅰ, the percentage of reconstructed embryos developed to 2-cell, 4—8-cell and blastocyst were 87.1%, 61.3% and 19.4%, respectively, significantly higher than those in somatic group ($P<0.05$). In the groups of serial Ⅱ and serial Ⅲ, the rates of the first cleavage of recontructed embryos were 36.4% and 51.3%, significantly lower than those in somatic group and serial Ⅰ ($P<0.05$).

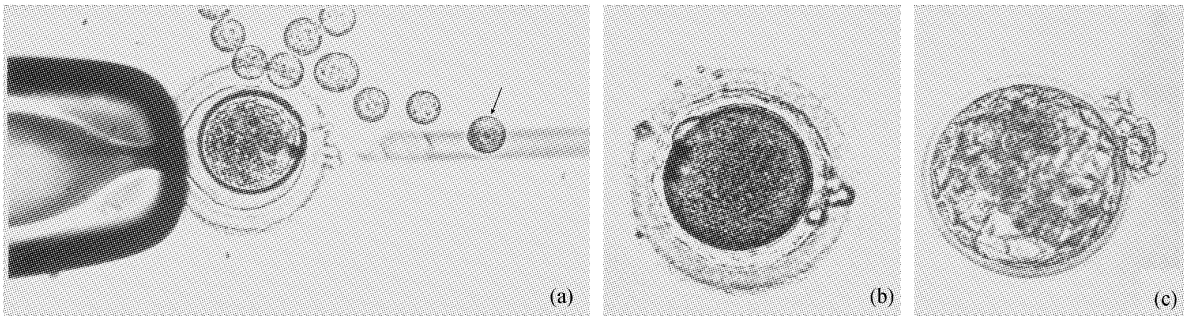

Fig. 1. Serial NT (a), a blastomere fused with an enucleated rabbit oocyte (b) and interspecies reconstructed blastocyst (c). The arrow in (a) indicates the blastomere from reconstructed embryo.

Table 1 Development of Panda-rabbit interspecies serially reconstructed embryos (%)[a)]

Group	No. of NT	No. of fused	No. of 2-cell	No. of 4—8-cell	No. of blastocyst
Somatic cell	134	96(71.6)[a]	69(71.9)[a]	28(27.2)[a]	4(4.2)[a]
Serial Ⅰ	39	31(79.5)[a]	27(87.1)[b]	19(61.3)[b]	6(19.4)[b]
Serial Ⅱ	44	37(84.1)[a]	16(36.4)[c]	10(27.0)[a]	5(13.5)[b]
Serial Ⅲ	50	39(78.0)[a]	20(51.3)[c]	12(30.8)[a]	4(10.3)[b]

a) a, b, c: Within the same column, values with different superscripts were significantly different ($P<0.05$).

However, the blastocyst rates of these two groups were 13.5% and 10.3%, which had no difference from that in the group of serial Ⅰ, but was significantly higher than that in the somatic group.

3 Discussion

The success of intraspecies somatic NT raises a new question for us, i. e. whether somatic nucleus is able to reprogram in the ooplasm from another species and support early development of the reconstructed egg. It has been shown in the previous interspecies cloning studies[6,7] that the nucleus from a quiescent somatic cell could support early development in the interspecies ooplasm. Here, after transferring nonquiescent somatic cells from giant panda into enucleated rabbit oocytes, 4.2% of reconstructed embryos developed to blastocyst *in vitro*. As for intraspecies NT, there are some reports in which nonquiescent cells were used as donors. For example, Liu et al.[13] used nonquiescent adult fibroblasts from a bovine ear as donor nuclei in cattle cloning; the cells used in Cibelli's cattle NT[14] were nonquiescent fetal fibroblasts; and Wakayama et al.[15] used nonquiescent tail tip cells for cloning mice. Our results indicate that the nuclei from nonquiescent somatic cells from giant panda could dedifferentiate in rabbit ooplasm and support early development.

Serial NT is an approach which would be beneficial for reprogramming of the nuclei of differentiate cells and for multiplying the original embryo[16]. Serial cloning was successfully used in somatic NT in some species, such as mouse[17] and pig[5]. In addition, in the bovine[18,19] and goat[20,21] cloning, the serial NT was used for multiplying the number of embryos. In the interspecies serial NT, it appears that serial NT could enhance the compatibility between nucleus and ooplasm from different species. In this study, the blastocyst rates in the groups of serial Ⅰ, serial Ⅱ and serial Ⅲ were 19.4%, 13.5% and 10.3%, respectively, which are significantly higher than that in the group of somatic cells. These data suggest that the nuclei from giant panda somatic cells could be dedifferentiated and their totipotent restored in the rabbit ooplasm and that the nuclei of blastomeres from reconstructed embryos are still multipotent and they are more benefit to the development of reconstructed embryos than those of somatic cells. However, in the groups of serial Ⅱ and serial Ⅲ, the 2-cell and 4—8-cell rates were significantly lower than those in the group of serial Ⅰ, but the blastocyst rates were not different from that in the group of serial Ⅰ. These results imply that the first and second cleavage rates decline after interspecies serial NT, however, the following development is improved once reconstructed embryos pass the early cleavage.

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