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RESEARCH PAPER

Somatic Nuclear Transplantation and Serial Nuclear Transplantation of Human Finger-domain Lacking t-PA Gene in Goat

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Abstract: To research developmental competence of transgenic somatic cell by serial nuclear transplantation, goat cloned embryos were compared with recloned embryos in ability of *in vitro* development. Fetal fibroblasts including human finger-domain lacking t-PA gene was microinjected into the cytoplasm of the MII oocytes. Goat embryos (G0) were cloned by this procedure. A single blastomere from 16-cell to 64-cell goat cloned embryos (G0) was microinjected into the Intracytoplasm of the MII oocytes. Goat embryos (G1) were cloned by this procedure. Goat embryos (G2, G3) were recloned using 16-cell to 64-cell recloned embryos. The developmental time of donor embryo affected the developmental rate of recloned embryos (G1, G2). The result indicates: the cleavage rate of cloned embryos (G0) (76.45±1.17%) was no difference significantly with recloned embryos (G1 G2 G3) (72.18%±1.97%, 76.05%±2.38%, 75.99%±2.84%). The developmental rate of morulae and blastocysts of cloned embryos (47.20%±2.93%, 11.00%±1.42%) were higher than of recloned embryos (34.99%±2.66%, 28.23%±2.00%, 23.34%±1.99%) (3.87%±0.67%, 2.08%±1.66%, 0); The morulae rate (29.57%±1.53%, 24.43%±1.87%), blastocysts rate (1.96%±1.31%, 2.01%±1.34%) of recloned embryos (G1 G2) from 16-cell recloned embryos was lower than that (34.32%±1.31%, 29.76%±1.66%), (3.86%±1.03%, 3.48%±0.34%) from 32–64-cell recloned embryos (P>0.05). In conclusion, nuclear transfer embryos were not recloned mostly; Embryos recloned using 32-cell to 64-cell embryos achieved higher developmental ability compared with using 16-cell embryos.

Key Words: goat, t-PA; transgenic cloned embryos; reclone; developmental rate

As for embryonic nucleus transplantation, there are two advantages about serial nucleus transplantation. First, it is beneficial for differentiated cells to re-programme. Second, it can increase the quantity of the embryo[1]. So far, serial nucleus transplantation has passed through two significantly developmental stages, one is embryonic nucleus transplantation and the other is somatic nucleus transplantation. Serial nucleus transplantation technique has been used to clone domestic animals, such as cattle [2], sheep, goats[3,4] and so on. The record is the 10th passage for serial embryonic clone, and the 3rd passage calf, as well as the 5th passage goat is obtained[4,5]. In addition, 190 embryos have been gained from 1 cattle embryo by serial clone technique. As for somatic nucleus transplantation, somatic clone mouse has been attained by adoption of a technique of serial nucleus transplantation[6–8]. However, the cloning rate of somatic cell clone is pretty low and numerous problems remain to be settled. In the opinion of Campbell and Wilmut[9], donor nucleus is in the milieu of cytoplasm of oocyte for multi-time through serial nucleus transplantation, which is good for re-programme and can improve the developmental rate of reconstructed embryo. Yet, it remains to be further studied that whether serial nucleus transplantation has similar effect on maintenance and recovery of totipotency of mammalian somatic nucleus or not. The study reconstructed the embryo using transgenic somatic cell as a donor nucleus derived from goat fetal fibroblasts containing human finger-domain lacking in t-PA gene by nuclear transplantation and researched on

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serial nucleus transplantation of somatic nuclear transfer embryos so as to prove the developmental potential of transgenic somatic nucleus and improve the efficiency of transgenic embryo in anticipation after through serial nucleus transplantation.

1 Materials and methods

1.1 Reagent and instruments

Micromanipulation System (Nikon), inverted phase contrast microscope (Oltympus), CO_2 incubator (Forma). Cytochalasin B (CB), Ionomycin, 6-DMAP, polyvinyl alcohol (PVA), hyaluronidase, pronase and trypsin were all purchased from Sigma Company. FBS (Hyclone).

All the details of preparation of OM solution, ingredients of SOFaa, monolayer culture of granular cells and preparation of cattle follicular fluid can be referred to in the reference [10].

Cell culture medium: DMEM/F12+10%(*V*/*V*) FBS+10 ng/mL E_2+1%(*V*/*V*) ITS+10 ng/mL EGF.

1.2 Goat ovaries

Goat ovaries, collected from a slaughterhouse located in Xi'an by means of aseptic surgery, were brought to the laboratory within 6 to 8 h in a thermos maintaining the temperature between 20°C and 30°C with physiological saline solution supplemented with 0.32 mg/mL gentamicin sulfate.

1.3 Collection and maturation culture *in vitro* of goat oocytes

The method of slicing was used to collect goat oocytes. The collected cumulus-oocyte complexes were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 for 20 to 22 h. Then, ovarian cumuli around the oocytes were removed with hyaluronidase. Finally, oocytes with first polar body were chosen under a stereomicroscope for further study.

1.4 Enucleation of goat oocytes

Goat oocytes with first polar body were placed into microdrop suitable for micromanipulation supplemented with 7.5 μg/mL cytochalasin B. Each microdrop contained 30 goat oocytes. And then, each oocyte was enucleated by micromanipulation with the micromanipulation system manufactured by the Nikon Company. In the process of enucleation, to begin with, the polar body was placed in the position similar to that of 4 or 5 o'clock when fixing the oocyte. The enucleation needle should be inserted into the oocyte from the position similar to that of 3 o'clock. At last, the polar body, as well as cytoplasm around it was drawn out when the enucleation needle was in close proximity to it. Note that the volume of drawn cytoplasm had better not exceed the limit of one-third of the entire cytoplasm.

1.5 Collection of donor cells

The details of the transfection, identification and culture of goat fetal fibroblasts including human finger-domain lacking in t-PA gene which is used as the donor cell to reconstruct embryo by nucleus transplantation in the primary passage can be referred to the reference [11]. When 80% of the dish area was covered, the cells were centrifuged for 5 min after dealing by means of blood serum starvation for 2 days. Then, the supernatant was extracted, adding 10% PVA into it. Following this, the supernatant was mixed uniformly with a haustorial tube. Finally, cell suspension used to reconstruct nucleus transfer embryo in the primary passage was prepared for further use.

Nucleus transfer embryo (G_0) in the primary passage is the embryo that was reconstructed by injecting goat fetal fibroblasts including human finger-domain lacking in t-PA gene into oocytes in the period of MII. Moreover, embryo G_1 was reconstructed using 16-cell to 64-cell embryo from G_0 as the donor and oocyte in the period of MII was the donor. Embryo G_2 was reconstructed by adopting the same approach as G_1. By analogy, G_3 was cloned. 16-cell to 64-cell stage embryo from G_0, G_1 and G_2 were placed into PBS solution with Ca^{2+}, Mg^{2+} free and 0.25 % pronase, so as to remove the zona pellucida. Then they were scattered to be single blastomere in PBS with tubulus. After that, cell suspension was centrifuged at 1000 r/min for 5 min. And then the supernatant was extracted and 50 μL 10% PVA solution was added. Finally, the mixture was uniformly blended using a haustorial tube. As a result, cell suspension used for reconstructing the serial nucleus transfer embryo was prepared for later use.

1.6 Construction of nucleus transfer embryo

Groups of 15 to 20 denucleated oocytes were placed into the microdrop supplemented with 7.5 μg/mL CB. And then the liquid droplets, respectively containing single donor cell were prepared behind the microdrop by sucking 20 μL to 30 μL donor cell suspension containing 10% PVA, covered with paraffin oil. Both were placed under the micromanipulation system. Nucleus transfer embryo was reconstructed by adopting the intracytoplasm injection method[11].

1.7 Activation and culture *in vitro* of nucleus transfer embryo

Nucleus transfer embryo was activated with Ionomycin and 6-DMAP.

After activation, nucleus transfer embryo was co-cultured with granular cells in the culture medium of SOFaa. During the course of embryo culture, the embryo was washed with embryo culture medium thrice. Then it was co-cultured with monolayer granular cells in the SOFaa culture medium, covered with mineral oil. It was cultured in an incubator at 38.5°C in an atmosphere with 5% CO_2. Three days later, 10% BFF was added into the co-culture system. The state of cleavage needs to be observed and the rate of cleavage needs to be recorded after 2 days. Moreover, it is necessary to observe the embryos which develop to morula or blastocyst between 5 days and 9 days after culture and record the rate of morula or blastocyst to provide the foundation of assessment of embryonic developmental potential.