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Full Length Research Paper

Interspecies embryo reconstruction in Tibetan antelope *Pantholops hodgsonii* by handmade cloning

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Interspecies somatic cell nuclear transfer (iSCNT) offers the possibility of preserving endangered species. Handmade cloning (HMC) has proved to be an efficient alternative to the traditional micromanipulator-based technique in some domestic animal species. This study investigates the possibility of reconstructing the embryos of Tibetan antelope *Pantholops hodgsoni* by HMC and reports the development of cloned embryos. Fibroblast cells derived from ear tissue from a female Tibetan antelope 3 h after death were used as nuclear donors, and iSCNT was performed using goat oocytes matured *in vitro* as recipients. A total of 118 embryos were produced by HMC with a cleavage rate of 70.14% and morula formation rate of 100%. However, no blastocysts were obtained *in vitro*. These results show that somatic cell nuclei of Tibetan antelope can be reprogrammed by goat oocytes. We also demonstrate that HMC is an efficient method of iSCNT using Tibetan antelope nuclear donors. Although the reconstructed embryos did not develop into blastocysts, the morula can be transferred into the womb of a recipient to continue development. Thus, we established an efficient method of iSCNT for producing transplantable embryos in Tibetan antelope by HMC.

Key words: Interspecies somatic cell nuclear transfer, Tibetan antelope, chiru, handmade cloning.

INTRODUCTION

Tibetan antelope or chiur *Pantholops hodgsonii* belongs to the bovidae family of ungulates, and it is the sole specie in the genus *Pantholops*. It inhabits a harsh environment in high-altitude regions of the Tibetan plateau, including the Tibet Autonomous Region, provinces of Qinghai and Xinjiang of China. The United States Fish and Wildlife Service, and the Convention on International Trade in Endangered Species list the Tibetan antelope as endangered due to commercial poaching for its underwool. Interspecies somatic cell nuclear transfer (iSCNT) is one of the few breeding-related technologies that can be used to avoid the loss of genetic diversity observed in a traditional breeding schema and that provides the prospect of saving endangered species from extinction. In addition, iSCNT may offer a potential method for exploring the commercial value of the wool from Tibetan antelope without encouraging poaching. Therefore, an efficient method of iSCNT to produce transplantable embryos in Tibetan antelope is important to protect this species while simultaneously allowing for the future exploration of the commercial value of its wool.

Reconstructing an embryo by iSCNT requires a donor cell from the species to be cloned and a recipient oocyte from a different species. However, owing to the limited

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Abbreviations: iSCNT, Interspecies somatic cell nuclear transfer; **HMC,** handmade cloning; **PBS,** phosphate buffered saline; **EGF,** epidermal growth factor; **DMSO,** dimethyl sulfoxide; **FBS,** fetal bovine serum; **COCs,** cumulus-oocyte complexes; **ZP,** zona pellucid.

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availability of oocytes from wild animals, the cloning of endangered species requires the use of donor oocytes from a related domestic species. The embryos can then be transferred to the womb of the domestic species, where they develop. Some wild mammals have already been cloned using this method (Lanza et al., 2000; Loi et al., 2001; Gómez et al., 2004; Janssen et al., 2004; Kim et al., 2007; Gómez et al., 2008; Oh et al., 2008; Folch et al., 2009), demonstrating that iSCNT is a feasible techniques for increasing the populations of endangered animals. However, although reconstructed embryos often develop into morula and reach the blastocyst stage, embryonic development following iSCNT is slow compared to *in vitro* fertilization and intraspecies SCNT, a critical problem that restricts the efficient production of endangered animals using this technique, as has been demonstrated by a large number of studies on mammals and birds (Hammer et al., 2001; Kitiyanant et al., 2001; Chen et al., 2002; Li et al., 2002; Yang et al., 2003; Ikumi et al., 2004; Lu et al., 2005; Murakami et al., 2005; Wen et al., 2005; Sansinena et al., 2005; Oh et al., 2006; Li et al., 2006; Li et al., 2007a, b; Wang et al., 2007; Zhao et al., 2007; Ishiguro et al., 2008; Kang et al., 2008; Lee et al., 2009; Song et al., 2009; Tao et al., 2009). Therefore, it is important to find a way to make iSNCT more efficient.

Handmade cloning (HMC) is a simple, micromanipulation-free cloning technique that has been used to clone some livestock species (Vajta et al., 2004; Kragh et al., 2005; Du et al., 2007a, b; Shah et al., 2008; Shah et al., 2009). The main advantage of this approach is that micromanipulators are not needed for enucleation and fusion. Instead, nuclear transfer can be used to generate highly efficient enucleation, fusion, and activation (Vajta and Gjerris, 2006). However, this approach has never been applied to iSCNT. Therefore, the aim of the present study is to establish an efficient method of iSCNT using HMC to produce transplantable embryos in Tibetan antelope.

MATERIALS AND METHODS

All of the chemicals used in the experiments were purchased from Sigma-Aldrich Company (St. Louis, MO) unless noted otherwise.

Establishment of the donor cell line

A sample of ear tissue (ca. 1 cm x 1 cm) was isolated from an adult female Tibetan antelope 3 h after death. Before excision, the ear was shaved and washed with soap, then rinsed with physiological saline to remove residual foam. Benzalkonium bromide was used to wash the ear carefully, and then the ear was washed repeatedly with Ca^{2+}- and Mg^{2+}-free phosphate balanced salt solution (PBS). The sample was washed three times with Hepes-buffered TCM-199 medium supplemented with 20% bovine serum (T20; Gibco). Then the biopsy was stored in T20 and immediately transported to the laboratory, where it was washed three times in Ca^{2+}- and Mg^{2+}-free PBS supplemented with 500 IU/ml penicillin (Gibco) and 500 µg/ml streptomycin (Gibco), and then minced carefully in Dulbecco's modified Eagle medium/F12 (D-MEM/F12, Gibco). The minced tissues were cultured in D-MEM/F12 supplemented with 20% v/v fetal bovine serum (FBS), 1 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 40 ng/ml epidermal growth factor (EGF) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 7 to 9 days of culture, the tissue explants were maintained in culture and a fibroblast cell monolayer was established (Figure 1). The cells were further maintained in culture, and then cyropreserved in 10% dimethyl sulfoxide (DMSO), 20% FBS and 70% D-MEM/F12, before being stored in liquid nitrogen. The cells from passage numbers two to six were used as nuclear donor cells for iSCNT. Prior to iSCNT, cells were thawed and cultured in 24-well dishes (Corning). Each well contained 1 ml D-MEM/F12 supplemented with 20% FBS, 1 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 40 ng/ml EGF and the samples were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 3 to 4 days, confluent cells were used as nuclear transfer donors. For nuclear transfer, the medium was removed from one well and cells were repeatedly washed with Ca^{2+}- and Mg^{2+}-free PBS and incubated at 37°C for 5 min in 200 µl trypsin dissolved in PBS. Most of the trypsin solution was then carefully removed and cells were suspended in 1 ml T20 and stored for a maximum of 2 h at room temperature in 1.5 ml Eppendorf tubes.

Preparation of recipient oocytes and iSCNT by HMC

Ovaries were excised from a goat slaughtered at a local abattoir, and transported to the laboratory within 2 h in a vessel containing warm physiological saline. Oocytes were aspirated from antral follicles with a diameter of 2 to 6 mm using a 20 ml disposable syringe with an 18-gauge needle. Cumulus-oocyte complexes (COCs) were selected in DPBS (Gibco) supplemented with 3% FBS. The COCs were washed in T20 and then about 150 COCs were placed in a 35 mm culture dish (Corning) for 20 – 24 h in 2 ml bicarbonate buffered TCM-199 medium supplemented with 0.22 mg/ml sodium pyruate, 10 mM/ml hepes, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10% FBS, 5 µg/ml follicle-stimulating hormone (Ningbo NO.2 Hormone Factory, China), 5 µg/ml luteinizing hormone (Ningbo), and 1 µg/ml estradiol at 38.5°C in a humidified mixture of 5% CO_2, 12.5% O_2 and 82.5% N_2. Then, 20 – 22 h after the start of maturation, COCs were placed into a 1.5 ml Eppendorf tube containing 1 mg/ml hyaluronidase dissolved in 0.5 ml T0 and cumulus cells were removed by pipettes with vortexing. Oocytes with visible degenerative changes or physical damage were discarded. The remaining oocytes were incubated for a further 40 min in 0.5 µg/ml demecolcine at 38.5°C to release nuclear material at the surface of the oocytes. The oocytes were incubated in 2 mg/ml protease dissolved in 500 µl T10 for 10 min at 38.5°C to remove the zona pellucida (ZP). ZP-free oocytes were arranged in a 35 mm culture dish in 2 ml T20 and bisection was performed manually under stereomicroscopic control with blades. After completing the bisection, all demi-oocytes were stained with 10 µg/ml fluorochrome Hoechst 33342 dissolved in TCM-199 for 5 min, washed three times in TCM-199, and then placed in 5 µl drops of TCM-199 in a 60 mm dish (Corning) and covered with mineral oil. Using an inverted microscope and UV light, the positions of demi-oocytes without chromatin staining were recorded. Then cytoplasts were collected under a stereomicroscope and transferred to 1 ml T20 for temporary storage.

For fusion, 30 µl drops of T20, phytohaemagglutinin (1 mg/ml) and fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 m$MCaCl_2$) were combined in a 60 mm dish (Conring) and covered with mineral oil. About 40 prepared cytoplasts were transferred to the first T20 drop and 5 µl of the somatic cell suspension was sedimented to the second T20 drop. Then two cytoplasts were transferred to a phytohaemagglutinin drop for 3 s, and one cytoplast was quickly transferred to the second T20 drop in order to attach a

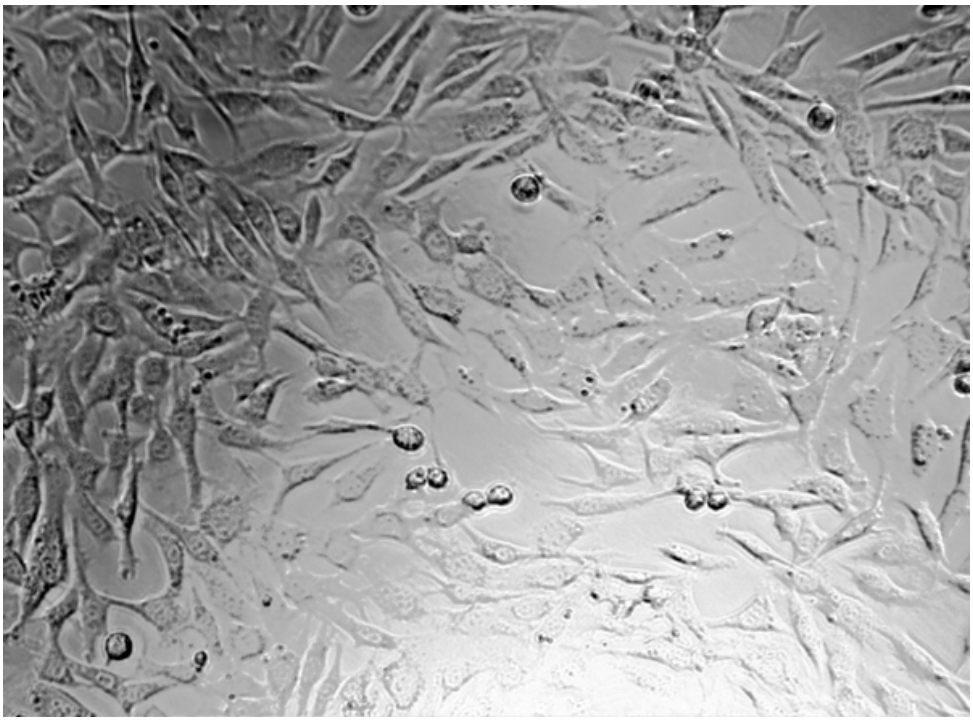

Figure 1. Monolayer of Tibetan antelope somatic cell.

single somatic cell. Following attachment, the cytoplast–somatic cell pair was again transferred to the phytohaemagglutinin drop. The cytoplast–somatic cell pair was conglutinated with another cytoplast in a phytohaemagglutinin drop, and this complex plus one somatic cell were transferred to a fusion drop, and then to a fusion chamber (BTX microslide 0.5 mm fusion champer). The chamber wires were covered with 2 ml fusion medium, and the complex was attached to one of the wires using an alternating current (ac) of 6 V (BTX, ECM 2001, Electro Cell Manipulator). Fusion was performed with a double direct current (dc) pulse of 70 V, each pulse for 20 µs. The complex was then carefully removed and transferred to a 35 mm dish containing 2 ml T20 and then incubated for 15 – 30 min to determine whether fusion had occurred. Fused reconstructed embryos were then transferred to a 35 mm dish containing 2 ml TCM-199 supplemented with 6.294 mg/ml sodium chloride, 0.534 mg/ml potassium chloride, 0.162 mg/ml dipotassium phosphate, 0.251 mg/ml calcium chloride dehydrate, 0.47µl/ml sodium lactate, 0.1 mg/ml magnesium chloride hexahydrate, 2.106 mg/ml sodium bicarbonate, 0.033 mg/ml sodium pyruvate, 0.146 mg/ml L-glutamine, 2% v/v essential amino acids, 1% v/v nonessential amino acids, 8 mg/ml bovine serum albumin, 100 IU/ml gentamycin and 0.01 mg/ml phenol-red (Sof) and incubated at 38.5℃ in a humidified mixture of 5% CO_2, 12.5% O_2, and 82.5% N_2 for 2 to 3 h. Activation was initiated 3 h after fusion. Reconstructed embryos were first incubated in 1 ml TCM-199 containing 5 µM Ca ionophore A23187 for 5 min at 38.5℃. After two subsequent washings in T20, reconstructed embryos were incubated individually (to prevent them from adhering to each other) in 5 µl drops of culture medium containing 2 mM 6-dimmethylaminopurine (6-DMAP), covered in mineral oil and incubated in 5% CO_2, 12.5% O_2 and 82.5% N2 at 38.5℃ for 5 h. Embryos were then washed twice in culture medium, separated into individual four-well dishes (Nunclone) (Vajta et al., 2000), and cultured in 500 µl medium covered with mineral oil at 38.5℃ in 5% CO_2, 12.5% O_2 and 82.5% N_2. Two days after reconstruction, the cleavage rate was recorded; four days after reconstruction, the morula rate was recorded; and eight days after reconstruction, the blastocyst rate was determined under a stereomicroscope.

RESULTS AND DISCUSSION

The use of the iSCNT technique for cloning has great potential as a tool for the conservation of endangered mammal species, as demonstrated by the successful cloning of gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Sansinena et al., 2005), female gray wolf (Kim et al., 2007) and male gray wolf (Oh et al., 2008). In the present study, the donor fibroblast cell line was established from ear skin tissue of a female Tibetan antelope 3 h after death, and morulas were produced from embryos reconstructed by HMC using the antelope's somatic cells and domestic goat oocytes (Figure 2). Loi et al. (2001) generated live mouflon offspring by nuclear transfer of granulosa cells removed from the ovaries of a dead adult female 18 to 24 h postmortem. Oh et al. (2008) produced male gray wolves by nuclear transfer of skin fibroblasts obtained from a 3 year-old male gray wolf 6 h after death. Li and Mombaerts (2007) revealed that chromosome stability and genetic integrity can be maintained in mouse cells even after they are frozen without cryoprotection and that nuclear transfer can be used to rescue the genome of these cells. Thus, we suggest that if cells with intact chromosomes isolated from a dead mammal can be maintained in a stable state, it would be possible to genetically restore that mammal via iSCNT.

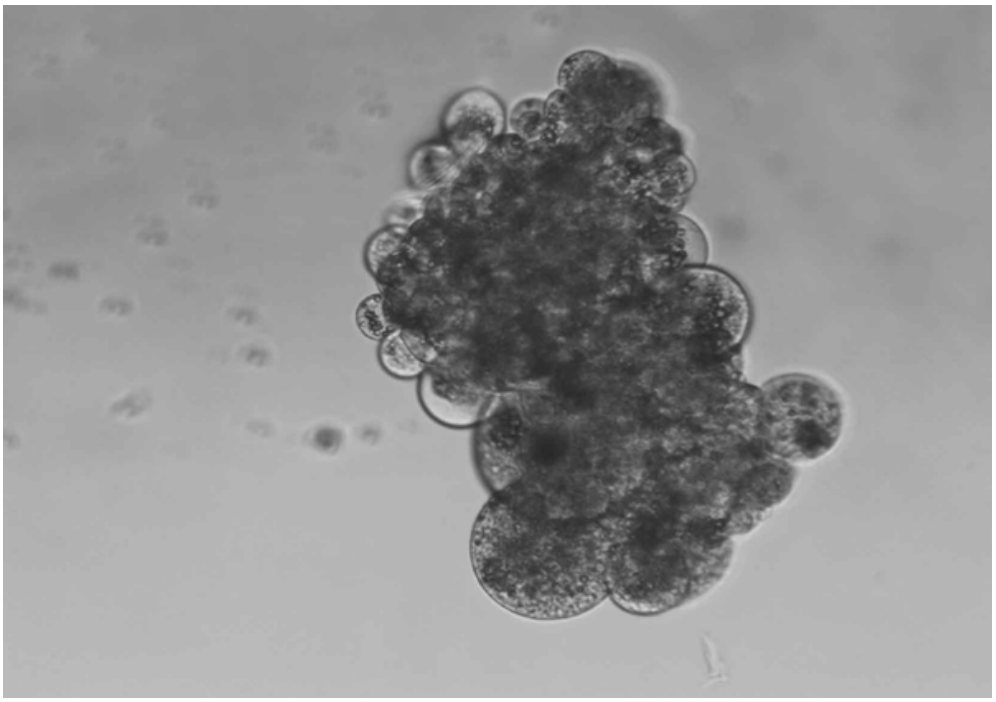

Figure 2. Reconstructed Tibetan antelope-goat morula.

Interspecies SCNT involves recipient oocytes from a different species or a different genus. Viable mammal offspring have never been produced by intergeneric SCNT, although pregnancies have been established (Dominko et al., 1999; Chen et al., 2002; Yin et al., 2006). In the present study, we cloned embryos using Tibetan antelope somatic cells and goat oocytes, which are from different genera. A total of 118 embryos were reconstructed successfully by HMC. Surprisingly, the cleavage rate of cloned embryos was 70.14% and the rate of morula formation from cleaved embryos was 100% while the blastocyst rate was 0%. Our data indicated that the somatic cells from the Tibetan antelope can be reprogrammed by the oocytes of a different genus, namely goat. Although the reconstructed embryos did not develop into blastocysts, the morula can be transferred into the womb of a recipient female. Thus, we established a method of iSCNT to produce transplantable mammal embryos more efficiently compared to previous intergeneric SCNT methods.

Zhao et al. (2007) first conducted iSCNT of Tibetan antelope embryos reconstructed using goat oocytes as a recipient and reported that 66.4% of cloned embryos underwent cleavage, 25.6% developed to the morula stage and 4% reached the blastocyst stage. The factors that influence the success of iSCNT are complex, but in general include technical factors as well as biological factors such as species-specific differences, the characteristics of karyoplasts, and interactions among cytoplasts. The fibroblast cells used for donor cells by Zhao et al. (2007) were derived from a 10-month-old female Tibetan antelope, whereas those used in the present study were from a dead adult female. This may partially explain the differences between our results and those of Zhao et al. In addition, we used HMC to perform iSCNT while Zhao et al. used a micromanipulator. HMC differs from the micromanipulator-based technique in many ways including the procedure of enucleation, the method of fusion, and the use of ZP-free cloned embryos. All of these factors could also help explain the different results obtained in our study. However, based on the morula rates obtained in our study, HMC is a more efficient method than the manipulator-based technique for the antelope–goat model studied herein. However, our embryos reconstructed by HMC did not develop to the blastocyst stage *in vitro*. Therefore, the procedures of HMC and culture conditions of reconstructed embryos must be optimized in future studies.

Conclusion

Goat oocytes are excellent recipients for reprogramming donor cells from Tibetan antelope and HMC is an efficient method for producing transferable Tibetan antelope–goat reconstructed embryos.

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