Dog recloning from muscle fibroblasts in transgenic cloned beagle

Regeneration of an identical transgenic dog

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Abstract— **Dogs (*Canis familiaris*) share many common genetic diseases with humans and development of dog disease models using a transgenic approach has long been awaited. However, due to the technical difficulty in obtaining fertilizable eggs and the unavailability of embryonic stem cells, no recloned transgenic dog has been generated. Here, we attempted the recloning of dogs by nuclear transfer of canine muscle fibroblasts from a transgenic cloned beagle. In order to prepare donor cells for recloning, muscle tissues were collected from a transgenic cloned beagle produced by nuclear transfer of canine fetal fibroblasts modified genetically with a red fluorescent protein (*RFP*) gene. We confirmed that the established muscle fibroblasts expressed RFP under an inverted microscope equipped with RFP specific filter (EX 510-560 nm, BA 590 nm). Fused-couplets (178/218, 81.7%) were chemically activated and transferred into the uterine tube of ten naturally estrus-synchronized surrogates. Two surrogates (20%) maintained pregnancy, but only one subsequently delivered one cloned pup. The present study demonstrated the possibility of recloning of transgenic cloned beagles by nuclear transfer.**

Keywords - reclonig; RFP; muscle fibroblasts; somatic cell nuclear transfer

I. INTRODUCTION

Somatic cell nuclear transfer (SCNT) has been the most successful approach for large animal transgenesis. However, due to the limitation of cell divisions in somatic cells, transgenic cells easily reach senescence and cell division stops following transfection and selection procedure, which results in low efficiency of SCNT. The recloning method is the technique in which cells derived from cloned fetuses or adult clones are used as nuclear donors [1,2]. Therefore, it is capable of extending the life span of donor cells [3]. Moreover, it is an effective technique for multiple genetic modification or gene targeting [4, 5, 6, 7]. However, to our knowledge, no studies have been done on recloning of canine species. Recently, transgenic dogs with *RFP* gene were born by SCNT [8], but one dog died after birth at eleven weeks due to bronchopneumonia. The objective of this study was to assess the feasibility of recloning of a transgenic dog using muscle fibroblasts.

II. MATERIALS AND METHODS

A. Care and use of animals

All animal procedures were performed in accordance with recommendations described in "The Guide for the Care and Use of Laboratory Animals" published by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University. Mixed large-breed female dogs (*Canis familiaris*) between 1 and 5 years of age were used as oocyte donors and embryo transfer recipients in this study.

B. Donor cell preparation for somatic cell nuclear transfer

During postmortem examination, muscle tissue was isolated from the *RFP* transgenic dog and donor fibroblasts were established from that tissue. Fibroblasts were cultured in DMEM, supplemented with 10% (v/v) FBS, 1 mM glutamine, 25 mM $NaHCO_3$ and 1% (v/v) MEM nonessential amino acid solution at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were maintained by culture, passaged, cryopreserved in 10% DMSO and then stored in liquid nitrogen at -196 °C. Prior to SCNT, cells were thawed, cultured for 3 to 4 days until confluent and retrieved from the monolayer by trypsinization for 2 to 5 min.

C. Oocyte Recovery and Somatic Cell Nuclear Transfer

For SCNT, in vivo matured oocytes were obtained by flushing oviducts of mixed breed bitches approximately 72 h after ovulation as previously [9]. In vivo matured oocytes were transported to the laboratory within 10 min, and cumulus cells were removed by repeated pipetting in Hepes-buffered TCM-199 supplemented with 0.1% (w/v) hyaluronidase. Denuded oocytes were placed in Hepes-buffered TCM-199 supplemented with 10% (v/v) FBS, 5 μg/ml cytochalasin B, and 5 μg/ml bisbenzimide (Hoechst 33342). The first polar body and metaphase II spindle were removed using micromanipulators (Nikon-Narishige, Tokyo, Japan) under an inverted microscope equipped with epifluorescence. The enucleated oocytes were placed in bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS to be used for SCNT. A single fibroblast was introduced into the perivitelline space of an enucleated oocyte. Couplets were then placed in a solution of 0.26 M mannitol, 0.1 mM MgSO4, 0.5 mM Hepes and 0.05% (w/v) BSA and fusion was induced using two pulses of direct current of 72 V for 15 μsec with an Electro-Cell Fusion apparatus (NEPA GENE Co., Chiba, Japan). The fused couplets were activated by a 4 min incubation with 10 μM calcium ionophore, followed by 4 h of culture in 1.9 mM 6-dimethylaminopurine [9, 10, 11].

D. Embryo transfer and pregnancy diagnosis

After activation, SCNT embryos were surgically transferred using a 3.5 Fr Tom Cat Catheter (Sherwood, St. Louis, MO) into the ampullary portion of the oviducts of

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eighteen naturally synchronous recipient females. Pregnancies were assessed 23-25 days after embryo transfer, using a SONOACE 9900 (Medison Co., LTD, Seoul, Korea) ultrasound scanner with an attached 7.0 MHZ linear probe.

E. Microsatellite analysis for genotyping

Parentage analysis was performed on the nuclear donor fibroblasts, cloned dogs and surrogate recipients to confirm genetic identity. The isolated genomic DNA samples were dissolved in 50 μl TE and used for microsatellite assay with nine specific markers originally derived from dogs [11]. Length variations were assayed by PCR amplification with fluorescently labeled (FAM, HEX, and NED) locus-specific primers and PAGE on an automated DNA sequencer (ABI 373; Applied Biosystems, Foster City, CA). Proprietary software (GeneScan and Genotyper; Applied Biosystems) was used to estimate the PCR product size in nucleotides.

F. Reverse transcription-polymerase chain reaction

To determine the level of the *RFP* transcript, reverse transcription-polymerase chain reaction (RT-PCR) was performed. Total RNA was prepared and treated with DNase I. The RNAs were reverse transcribed by the first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). PCR was performed on the *RFP* transcript produced by RT. The *RFP*-F forward primer (5′-CGTGAAGCTGAAGGTGA-3′) and *RFP*-R reverse primer (5′-CTCGTACTGCTCCACGA-3′) were used. The primer set *RFP*-F and *RFP*-R yielded a 517 bp amplicon after amplification of *RFP* cDNA. For the ß-actin internal transcript control, the beta-actin-F forward primer (5′-TGCCTTGAAGTTGGAAAACG-3′) and beta-actin-R reverse primer (5′-CTGGGGCCTAATGTTCTCACA-3′) were used. Primer set beta-actin-F and beta-actin-R yielded a 153 bp amplicon after amplification of beta-actin transcript.

G. RFP *expression in recloned* dog (stillborn)

Recloned dog (stillborn) expressing DsRed was identified using a GFsP-5 head light source (Biochemical Laboratory Services). The red fluorescence was produced by illumination with 540 ± 20 nm and detected by an emission filter with a maximal transmittance wavelength of 600 ± 25 nm.

III. RESULTS

Total 218 enucleated oocytes were used for fusion with donor somatic cells which resulted in the fusion rate of 81.7%. After manipulation, 174 recloned embryos were transferred into oviducts of ten estrous synchronized surrogate dogs. Two recipients were pregnant. One recipient aborted at around one month while the other maintained full term development. However, the recloned dog was delivered dead on Day 62 after embryo transfer. Birth weight of the dog was 530g and ubiquitously expressed red fluorescence in the whole body. The expression of *RFP* gene was confirmed by RT-PCR. Microsatellite analyses indicated that genome of the dog was from donor transgenic dog.

IV. CONCLUSION

In conclusion, this study demonstrated that a transgenic dog could be recloned from muscle fibroblasts of transgenic dogs. Further study is needed for the generation of viable recloned transgenic dogs.

TABLE I. IN VIVO DEVELOPMENT OF CANINE RE-CLONED EMBRYOS

Nuclear donor cells	Total
Fusion attempted	218
Fused embryos (%)	178 (81.7)
Embryos transferred to recipients	174
Recipients	10
Pregnancies / recipients (%)	2 (20)
Full term / recipients (%)	1 (10)

TABLE II. SEQUENCE ALIGNMENTS WITHIN 661BASES OF THE HYPERVARIABLE REGION OF MTDNA

Marker	Cell donor	Recloned dog	Oocyte donor 1	Oocyte donor 2	Recipient
PEZ 01	114/122	114/122	114/126	114/126	118/118
PEZ 05	101/105	101/105	97/109	101/101	101/101
PEZ 06	199/199	199/199	175/183	179/191	175/183
PEZ 08	223/231	223/231	231/235	219/231	223/243
PEZ 12	265/277	265/277	269/277	277/277	261/293
PEZ 20	175/179	175/179	175/183	175/179	174/175
FH 2010	228/232	228/232	232/236	232/236	232/236
FH 2079	273/273	273/273	269/273	269/273	269/269

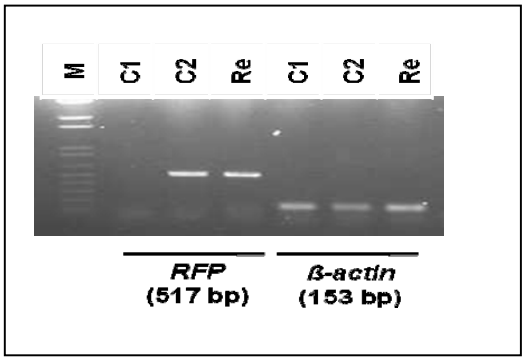

Figure 1. The transgene (RFP) was detected by reverse transcription-polymerase chain reaction. Re; Reclone dog derived from RFP transgenic dog, C1; non-transgenic cloned beagle , C2; RFP transfected fibroblasts.

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