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Advancement and Application of Somatic Cell Nuclear Transfer Technique in Dog

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Abstract

The cloning of canids was succeeded in 2005, several years after the birth of Dolly the sheep and also after the cloning of numerous other laboratory and farm animal species. The delay of successful somatic cell nuclear transfer (SCNT)was due to the unique reproductive characteristics of the female dogin comparison to other domestic mammals, such as ovulation of immature canine oocyte and a requirement of 25 days for the completion of meiosis within the oviduct (Holst & Phemister, 1971). When the technology for the recovery of in vivo matured oocyte was established, the application of cloning also became possible and cloned dog offspring were obtained. This report summarizes the progress of technical procedures that are required for cloning canids and the application of this technique. The first cloned dog, Snuppy, was achieved using an in vivo-matured oocyte which was enucleated and transferred with an adult skin cell of male Afghan hound. After establishment of a criterion of well-matured oocyte for the improvement of SCNT efficiency, we obtained three cloned female Afghan hound and a toy poodle cloned from 14 year-old aged Poodle using SCNT through this factor. To date, cloned dogs appeared to be normal and those that have reached puberty have been confirmed to be fertile. Through application of canine SCNT technique, first, we demonstrated that SNCT is useful for conserving the breed of endangered animal from extinction through cloning of endangered gray wolves using inter-species SCNT and keeping the pure pedigree through the cloning of Sapsaree, a Korean natural monument. Secondly, we showed possibility of human disease model cloned dog and transgenic cloned dog production through cloning of red fluorescent protein expressing dog. Finally, SCNT can be used for the propagation of valuable genotypes for making elite seed stock and pet dog. In summary, dog cloning is a reproducible technique that offers the opportunity to preserve valuable genetics and a potential step towards the production of gene targeted transgenic cloned dogs for the study of human diseases.

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Introduction

Since the first successful cloning of frog using embryonic cell transfer to enucleated egg in 1952 by Briggs and King, the first live offspring produced from differentiated cell populations were two lambs born in 1995 using cultured embryonic cells as nuclear donors and enucleated unfertilized eggs (metaphase II oocytes (MII)) as recipient cytoplasts[1]. In the following year, offspring were produced using cultured cell populations derived from fetal and adult tissues[2]. Since this time, somatic cell nuclear transfer (SCNT) has been successfully applied to a range of species including cattle[3], mice[4], goats[5], pigs[6], cats[7], rabbits[8], horses[9], rats[10], and ferrets[11] using a range of cell types. Animal cloning has many applications to different fields of biomedicine including xenotransplantation, production of bioreactor, cell therapy, and production of human disease model animals. Despite its various applications to biomedicine, the rate of successful cloning is still low.

Dog is men's best friend and easy to handle and communicate with. Also, dog shows the most similar disease patterns to human except that of mouse. Since the production of several mammals by somatic cell nuclear transfer technique, we successfully produced the world's first cloned dog named "Snuppy" in 2005 using somatic cell nuclear transfer. With the brief communication entitled 'Dogs cloned from adult somatic cells[12] published in the journal Nature, Lee et al, Korean researchers, reported the first successful cloning of two Afghan hounds by nuclear transfer from adult skin cells into enucleated mature oocytes. Since thesuccessful production of Snuppy, the canids SCNT has grown rapidly. Accordingly, the aim of this report is to provide a review of cloned canid production by nuclear transfer, together with illustration and perspective about how canine SCNT technique can be utilized.

Materials and Methods

Care and use of animals

In this study, mixed-breed female dogs (*Canis familiaris*) between 1 and 5 years of age were used as oocyte donors and embryo transfer recipients. The study was conducted in accordance with recommendations described in "The Guide for the Care and Use of Laboratory Animals"published by Institutional Animal Care and Use Committee (IACUC) of Seoul National University. In that regard, facilities for dog care and all procedures met or exceeded the standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University.

Preparation of donor fibroblasts and somatic cell nuclear transfer

Canine fibroblasts were obtained by skin biopsy cultures from a seven-year-old Labrador Retriever. After establishment of fibroblast monolayer derived from the tissue explants, the cells were maintained in culture, passaged,cryopreserved in 10% DMSO and stored in liquid nitrogen. The cells from passage numbers 2 to 6 were used as nuclear donor cells for SCNT. Collection of in vivo dog oocytes was

performed approximately 72 h after ovulation and enucleation was done as described in previous reports[13,14], 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 $MgSO_4$, 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 (Sigma-Aldrich Corp.)[15].

Embryo transfer (ET) and pregnancy diagnosis

Within 4 h after reconstruction, activated embryos were surgically transferred into the oviducts of the surrogate mothers. Recipients synchronized in natural estrus were used. Reconstructed embryos were placed in the ampulla using a 3.5 F Tom Cat Catheter (Sherwood, St. Louis, MO, USA). Pregnancies were detected around 23 days post ET using a SONOACE 9900 (Medison Co. LTD, Seoul, Korea) ultrasound scanner with anattached 7.0 MHZ linear probe. Pregnancy was monitored by ultrasound every 2 weeks after initial confirmation.

Microsatellite and mitochondrial DNA analysis of cloned pups

Parentage analysis was performed to confirm the genetic identity of the offspring. Genomic DNA was extracted from blood samples of the surrogate mothers, cloned pups and trypsinized nuclear donor cells. The isolated genomic DNA samples were used for microsatellite assay with nine canine microsatellite markers and for mitochondrial(mt) DNA analysis. Microsatellite length variations were assayed by polymerase chain reaction (PCR) amplification with fluorescently labeled 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. For the mtDNA analysis, oligonucleotide primers were synthesized based on the complete nucleotide sequence of canine mtDNA (GenBank accession no. U96639)[25]: forward, 5′-CCTAAGACTTCAAGGAAGAAGC-3′ reverse, 5′-TTGACTGAATAGCACCTTGA-3′. PCR amplifications were conducted and the products were purified using a Power Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced with an ABI3100 instrument (Applied Biosystems), and their identities with mtDNA were confirmed by BLAST search.

Results

Companion dog cloning

We produced the world's first cloned dog in 2005 from adult somatic cells, named 'Snuppy' which stands for Seoul National University puppy[12]. 'Snuppy'was genetically identical to the donor dog, and his mitochondrial DNA was originated from their

oocyte donor dogs. Since the only viable cloned offspring born in dog was a male, we cloned female dogs by SCNT in 2006[13]. In this study, we found that our efficiency in producing cloned embryos and pregnancies was increased by the use of good quality in vivo matured oocytes. Subsequently, we monitored the growth and health of the three female cloned dogs and evaluated their reproductive capability by artificial breeding with a male cloned dog. Also, we produced, for the first time, a cloned small size breed (toy poodle) using a large size recipient female after nuclear transfer of donor cells of an ageing dog (14 years of age) into an enucleated in vivo mature oocytes of large breeds[16]. These results proved the possibility of the dog cloning in several species.

Dog cloning for conservation

All over the world, similar to other species, canine species have been gradually endangered or extinct. The aim of this study was to produce both female and male endangered gray wolf (*Canis lupus*) and Sapsaree dog, a Korean natural monument, by SCNT for conservation. Donor cells were isolated from an adult female gray wolf and a postmortem male gray wolf. Because of limitations in obtaining gray wolf matured oocytes, we used in vivo matured canine oocytes obtained by flushing the oviducts from the isthmus to the infundibulum. After inter-species SCNT (iSCNT), the reconstructed embryos were transferred to recipient female dogs and we got two female gray wolves in October, 2005, three male gray wolves in August, 2006[14,17]. We confirmed that the DNAs of cloned wolves were identical to the donors by microsatellite analysis. In August, 2007, we produced two cloned dogs from Sapsaree dog, a Korean natural monument, using same protocol by SCNT. Interestingly, a live cloned puppy apparently showed signs of hip dysplasia, which is an inherited and polygenic disease characterized by hip subluxation and laxity and it was also found in the somatic cell donor dog. These results supported the use of SNCT for the preservation of endangered canine species, both within a concerted conservation program, and in extreme situations involving sudden death by iSCNT. Furthermore, it also demonstrated that cloning could be a useful method to study genetic disease.

Cloning of Service dogs

Although viable cloned canids have been successfully produced, the efficiency in canine SCNT has been still low. We focused on synchronization of donor cell cycle as one of approaches to increase the efficiency in canine SCNT. The roscovitine (the cycline-dependent kinase 2 inhibitor) was made a choice to arrest donor cell cycle at G0/G1stage [18]. For this study, canine fibroblasts were obtained by skin biopsy cultures from a drug sniffing dog and cancer sniffing dog which its reproductive tract was removed due to pyometra.Each donor cells were synchronized by treating cells with 15 μg/ml roscovitine for 24 hrs and it was used for producing a reconstructed embryo using SCNT. After embryo transfer, in the case of drug sniffing dog, the four

pregnant surrogate females delivered eleven live pups in October, 2005, and the seven cloned puppies among them are growing healthy to date. Also, we got four cloned cancer sniffing dogs by embryos reconstructed with roscovitine treated cells. All clones were genetically identical to their donor cells, but mitochondrial DNA has originated from their oocyte donor dogs. These results indicate that SCNT has potential abilities for the production of dogs in elite capacity as service dogs. In earlier studies, the success rate is very low. Presently it is improved to around 15% to 30% because of the synchronization of donor cell cycle stage using roscovitine. This advancement in canine SCNT could assist the production of canine transgenic models for research in human and veterinary medicine or biomedical science.

Cloning of transgenic dog using nuclear transfer

In many species, fetal fibroblasts have been primarily used for successfully producing the SCNT-derived offspring, because of its high potential for developmental competence than adult cells [19,20]. To date, success of cloned canine offspring is limited to adult skin fibroblasts because adult cell cloning has primarily been used to preserve their beloved dogs or endangered species [12-14,16,17]. For the first time, we demonstrated that two cloned beagles from fetal fibroblasts have been successfully born by SCNT. The parentage analysis was performed and all cloned pups were genetically identical to the donor dog, and their mitochondrial DNA was originated from their oocyte donor dogs. Base on this result, we gave it a try to produce transgenic cloned beagle using SCNT. In thefirst step, red florescence protein (RFP) gene was introduced into female and male fetal fibroblast donor cells via retroviral vectorinfection and stable transfectants were isolated with hygromycin selection (150 $\mu g\ ml^{-1}$) for two weeks. In the second step, enucleated oocytes microinjected with donor cell expressed RFP and fused by electric stimulation. Lastly, the reconstructed embryos were transferred into recipient mothers. As a result, we demonstrated for the first time that viable transgenic (RFP) female/male cloned beagles were not only successfully born, but also were healthy and expressed the RFP gene all over their bodies. Our results represent a potential step towards the production of gene targeted transgenic cloned dogs by nuclear transfer for the study of human diseases. Another availability of a transgenic animal with a reporter gene is a valuable reagent for cell tracking experiments, since it provides an indelible marking system with high spatial resolution that is visible in live animals [43].

Application of Somatic Cell Nuclear Transfer and Future Perspective

Somatic cell nuclear transfer is an animal cloning technique which is fast developing in several species. Although the applications of SCNT have been demonstrated by many laboratories, the frequency of development and production of cloned animals still remains feeble. Recently in canine, we greatly improved (from 2% to 30%) the cloning efficiency rate using SCNT compare to earlier studies. Practical

application of canine SNCT technique could be expected as follow; (1) the preservation of endangered canid species, (2) production of cloned elite service dog, (3) production of cloned dogs as human disease model using transgenic somatic cell cloning, (4) cloning of pet dogs.

We already accomplished conserving endangered canids and producing elite service dog. With incremental improvement of SCNT method, it could be a very good tool for endangered species preservation efforts and propagation of valuable genotypes. The dog, *Canis familiaris*, has been proposed as an animal model for humans because they have many common genetic diseases. Moreover, dog exhibit 223 genetic diseases similar to those experienced by humans [33], making them one of the important models for various human hereditary diseases. Transgenic mouse models have been invaluable in biomedical research, but their use has been limited by many differences between humans and rodents [21,22]. Recent successes in creating non-mouse transgenic animals [3,23-27] by SCNT have made animal modeling more feasible in alternative non-rodent model of human disease. Dogs 1) have organ sizes comparable to those of humans, unlike the traditional rodent models, 2) generally cohabitate with human beings, minimizing different environmental effects and 3) receive exceptional medical care [28-30]. Indeed, we succeeded in producingtransgenic beagles by nuclear transfer of canine fetal fibroblasts, genetically modified with a RFP gene. But to improve efficiencies of production of transgenic experimental animals by nuclear transfer, more accurate methods for screening genetically modified nuclear donor cells prior to nuclear transfer are needed. In the future, this technology will be much moreimproved than before. Thus, using SCNT method forcloned animal production can offer numerous possibilities to multiple fields. This advancement opens the door for the application of gene-targeting technology to dogs, providing a variety of genetically-modified cloned dogs for research in biomedical science.

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