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Generation of transgenic cloned dogs using adipose-derived mesenchymal stem cells

지방줄기세포를 이용한 형질전환 복제개의 생산

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Generation of transgenic cloned dogs using adipose-derived mesenchymal stem cells

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We accept this thesis as confirming to the required standard

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Declaration

This thesis is submitted by the undersigned for examination for the degree of Doctor of Philosophy to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Hyun Ju Oh

Generation of transgenic cloned dogs using adipose-derived mesenchymal stem cells

Hyun Ju Oh

(Supervisor: Byeong Chun Lee, D.V.M., Ph.D.)
Veterinary Clinical Sciences
Department of Veterinary Medicine, Graduate School
Seoul National University

ABSTRACT

Since the birth of the first cloned dog ‘Snuppy’, valuable canids were produced by SCNT using adult somatic cells. In addition, genetically modified dogs were generated by SCNT using fetal fibroblasts. Fetal fibroblasts are preferred as nucleus donors for SCNT used in producing transgenic dogs because they have excellent proliferative ability, are capable of being genetically modified, and have the ability to produce live offspring. However, the donor transgenic cells become senescent and

unusable because stable transgene-expression, homologous recombination or multiple transfections require a long time for *in vitro* culture. As an alternative to fetal cells, recent reports indicate that some mesenchymal stem cells (MSCs) lines can be maintained sufficiently long enough for homologous recombination events to take place. MSCs can proliferate for many passages in culture and show constant growth. Furthermore, MSCs have the ability to give rise several differentiated cell types. Thus the object of this study was to determine whether canine adipose–derived mesenchymal stem cells (cASCs) can be a suitable donor cell for producing transgenic cloned dogs. In several laboratory animals and humans, ASCs are of considerable interest because they are easy to harvest and can generate a huge number of cells from a small quantity of adipose tissue. ASCs have applications in various research areas, such as cell therapy and tissue engineering especially in bone reconstruction. In order to cASCs in SCNT, this study compared cellular proliferation rate, viability, cellular size and expression patterns of genes related to pluripotency and epigenetic modification between canine fetal fibroblasts (cFFs) and cASCs. The cFFs were established from fetuses of pregnant beagle at the 28th day. The cASCs were isolated from subcutaneous adipose tissue collected from the inguinal region of a healthy dog. The cASCs were characterized through flow cytometry to be positive for CD29, CD44, CD73, CD90 and CD105, but negative for CD31, CD34 and CD45. Proliferation pattern, cellular viability as well as cell size at each passage of cFF and cASC were compared when the culture reached confluence. In addition, real time-PCR was performed to investigate different mRNA transcripts expression in both cell lines.

Moreover, the cASCs were evaluated as a potential donor cell using interspecies SCNT (iSCNT); cASCs were cultured in two different culture media (RCMEP or DMEM) and used for iSCNT. Next, to generate transgenic cloned dog, cASCs were established from a transgenic cloned beagle produced by nuclear transfer of canine fetal fibroblasts modified genetically with a red fluorescent protein (RFP) gene. The cASCs expressed RFP gene and cell-surface marker characteristics of MSCs, including CD29, CD44 and thy1.1. Furthermore, the cASCs underwent osteogenic, adipogenic, myogenic, neurogenic and chondrogenic differentiation when exposed to specific differentiation-inducing conditions.

Isolated cASCs were used for SCNT and after embryo transfer into recipient, RFP-expressing transgenic recloned beagle pups (Magic) were produced by nuclear transfer of cASCs derived from a transgenic cloned beagle (Ruppy1). Another purpose of this study is to determine the degree of genetic identity between the cloned (Ruppy1) and recloned (Magic) dogs and evaluated whether the RFP expression and CMV promoter methylation of these two transgenic dogs are age-dependent. To produce a transgenic dog that expresses neuron specific transgene, human synapsin 1 promoter as primarily neuron selective was chosen. Synapsin 1-RFP (SYN1-RFP) was introduced into cASCs *via* lentiviral vector infection. The SYN1-RFP cells were injected into enucleated *in vivo* matured dog oocytes and fused by electric stimulation. The fused-couplets were transferred into the uterine tube of five naturally estrus-synchronized surrogates.

As results, the cFFs and cASCs differed in the number of generation but not in doubling times at all passages. The mean cell size of cASCs was significantly smaller than that of cFFs. Cellular viability and apoptosis were significantly lower in cASCs when compared to passage-matched cFFs. The level of HDAC1 transcript in cASCs was significantly higher than in cFFs, but expression of DNMT1 was not different between the two groups. OCT4 and SOX2 transcripts showed significantly higher expression in cASCs than in cFFs. Thus, canine adipose-derived stem cells (cASCs) are promising as donor cells for SCNT. With this in mind, cASCs were evaluated as a potential donor cell using interspecies SCNT (iSCNT). RCMEP cultured cells contained significantly higher amount of SOX2, NANOG, OCT4, DNMT1 and MeCP2 than DMEM cultured cells ($P < 0.05$). However, there was no significant difference in the rate of development to blastocysts between the two groups. Thus, these results showed that altering gene expression levels in donor cells by changing the culture medium did not influence subsequent *in vitro* development of cloned embryos. In SCNT for generating transgenic cloned dog, one dog among five (20%) maintained pregnancy and subsequently gave birth of two healthy cloned pups. The present study demonstrated for the first time the successful production of transgenic cloned beagles by nuclear transfer of cASCs derived from a transgenic cloned beagle. Moreover, the degree of genetic identity between the cloned and recloned dogs showed that both cloned dogs carried a single copy and same integration site of the RFP gene. The transgene protein quantity of both transgenic dogs, showed no significant difference in the relative RFP expression between the 1-year-old Ruppy1 and the 1-year-old Magic.

Also, transgene protein levels increased with aging of the two dogs, while promoter methylation status decreased with age. Gene expression and promoter methylation showed similar opposite profiles during growth of the two transgenic dogs. Lastly, neuron specific transgene-expressed dogs were generated by SCNT and three cloned pups (SYN1-RFP A, SYN1-RFP B, and SYN1-RFP C) were produced by natural delivery or C-sec. One of them is still alive, healthy and does not show any abnormalities.

In this thesis, cASCs have superior proliferation patterns, epigenetic modification and pluripotency ability compared to cFFs and as well as capable of producing transgenic dogs by SCNT. Furthermore, cASCs can become a valuable resource to provide an unlimited supply of identical nuclei and to produce a neuronal degenerative disease model dog.

Key words: somatic cell nuclear transfer, canine adipose-derived stem cells, transgenic cloned dog, recloning, synapsin 1 promoter

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LIST OF ABBREVIATIONS

ASC	**Adipose derived mesenchymal stem cell**
MSC	**Mesenchymal stem cell**
BSA	**Bovine serum albumin**
cDNA	**Complementary DNA**
CMV	**Cytomegalovirus**
DMEM	**Dulbecco's modified eagle's medium**
DNA	**Deoxyribonucleic acid**
mtDNA	**Mitochondrial DNA**
MII	**Metaphase II**
PB	**Polar body**
PCR	**Polymerase chain reaction**
SCNT	**Somatic cell nuclear transfer**
DPBS	**Dulbecco's phosphate buffered saline**
ET	**Embryo transfer**
FBS	**Fetal bovine serum**
IVC	***In vitro* culture**
P4	**Progesterone**
RFP	**Red fluorescent protein**
SYN	**Synapsin 1**
RT	**Reverse transcript**

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***** Both authors contributed equally

PART I

GENERAL INTRODUCTION

1. Literature review

1.1. Canine mesenchymal stem cells

The application of stem cells has rapidly emerged for regenerative medicine and tissue engineering in veterinary medicine. A tissue or organ can renew itself because undifferentiated stem cells can differentiate to yield the major specialized cell types of the tissue or organ [1]. Stem cells can be classified as embryonic or adult stem cells, according to a developmental stage be recovered [2-4]. Embryonic stem cells are derived from the inner cell mass of the blastocyst and adult mesenchymal stem cells (MSCs) originate from particular three germ layers [2-4]. Although embryonic stem cells are theoretically highly beneficial, there are various limitations as cell regulations, ethical considerations, and genetic manipulation to their use imposed. In contrast, MSCs are more easily accessible, with neither ethical considerations nor immunoreactivity. Thus MSCs as adipose-derived mesenchymal stem cells (ASCs) or bone marrow-derived stem cells (BMSCs), are getting the spotlight in the fields of stem cell research and regenerative medicine [1, 5].

1.1.1. Sources of canine mesenchymal stem cells

Adult stem cells can be obtained from mesodermal tissues (bone marrow, trabecular bone, synovium, adipose tissue, muscles and cartilage), endodermal tissues (thymus and liver) and ectoderm tissues (skin, hair follicle and dental pulp) [6]. Many

canine research and clinical articles have been published using ASCs or BMSCs, because these are well established and characterized [7-9]. BMSCs are not only be obtained easily and non-invasively, but also be considered the best standard for use in tissue regeneration among MSCs [7-10]. Nonetheless, ASCs derived from adipose tissue have been recently considered a highly attractive option compared to BMSCs, because of the ease of tissue collection and the availability of adipose tissue in the body [11]. In addition to, the fact that stem cell yields are greater from adipose tissue than from other stem cell reservoirs is a significant factor in their suitability for use in regenerative medicine [11].

1.1.2. Characterization of canine mesenchymal stem cells

Human MSCs are characterized by minimally three criteria according to the International Society for Cellular Therapy: (1) plastic adherence when maintained in standard culture conditions, (2) expression of a specific cell surface antigen marker (3) at least tri-lineage differentiation potential [4, 12]. However, unlike human MSCs, there are no available uniform characterization criteria in specific for MSCs from animal origin and especially from canine origin [4, 13]. Nevertheless, some criteria as for human are available for the dog [14, 15]. Recently, undifferentiated canine MSCs can be characterized morphologically, immune-phenotypically and by their gene expression.

Morphologically, MSCs are identified as spindle-shaped cells that grow on plastic dishes in a monolayer [16]. Immunophenotyping has not been universally well

characterized, but this have performed using specific cross-reaction antibodies to indicate surface antigen expression [4, 12]. Several studies have demonstrated the simultaneous expression of several cell surface positive markers (CD29, CD73, CD105 and CD44, Thy-1 CD90 glycoprotein and MHC-I), and negative markers (CD34, CD45, CD14, CD117, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and CD146) either in BMSC or in ASC [4, 16, 17]. In human MSC research, gene expression is frequently used to characterize undifferentiated MSCs and the most commonly studied genes with positive expression are the pluripotency-associated transcription factors NanoG, OCT4 and SOX2 [18]. In line with human MSCs, canine MSCs also appear to be positive for these three gene expression markers [17, 19]. Characterization of these MSCs at the mRNA level is a valuable alternative when no monoclonal antibodies are available to characterize them at the protein level [4].

Lastly, the ability of MSCs to differentiate into multiple lineages through osteogeneis, chondrogenesis and adipogenesis has also been demonstrated for characterization of MSCs [4, 12]. Osteogenesis is verified by the polygonal appearance of the differentiated osteoblast containing nodular aggregates that stain positively with von Kossa and by the mRNA expression of osteoblast markers (Runx2, Osteonectin, Osterix and BSP) [15, 17]. Chondrogenesis is characterized by the presence of three-dimensional aggregates, which stain positive for alcian blue indicating the presence of sulphated proteoglycans. Expression of COL2A, Aggrecan, COMP, COL10A and Sox9 can be detected in the differentiated cells [16, 20]. Adipogenesis is characterized

by the appearance of round-shaped cells with Oil Red O staining of lipid droplets [15]. Adipose-induced cells express PPARg2, CEBPa, FABP4 and LPL [15, 20].

1.1.3. Therapeutic applications of canine mesenchymal stem cells

The main purpose of canine MSC is the conduction of experimental animal studies in which the dog served as a preclinical model for humans. In 1998, the cultured autologous BMSCs were examined the effect on the healing of generated defects in canine femurs. The group treated with a carrier loaded with MSCs showed substantial new bone formation, while the untreated femurs demonstrated atrophic non-union. This result hold potential to provide an alternative strategy to autologous bone-grafting [9]. MSC-based therapies in dogs have mainly been focusing on bone and cartilage repair and inflammatory diseases such as osteoarthritis. Nevertheless, an important share of musculoskeletal problems in dogs consists of tendon/ligament injuries, which are comparable to the human. Since natural repair mechanisms often do not deliver functional recovery or current therapeutic strategies show minimal effectiveness, MSC-based therapies can be considered as potentially revolutionary in this field. Therefore, a challenging future prospect involves the research on MSC-based therapy for these canine tendon/ligament injuries. Since dogs are considered to be a superior animal model for humans, so not only dogs but also human can benefit from state-of-the-art research in this exciting field.

1.2. Canine somatic cell nuclear transfer

1.2.1. Somatic cell nuclear transfer

SCNT in mammals is an assisted reproductive technique to produce an animal from a single cell nucleus using an enucleated oocyte. Nuclear transfer (NT) only became technically feasible in amphibians around 1950 [21] and in mammals about 30 years later [22]. Finally, the first successful mammals cloning was reported that a sheep named Dolly was obtained by cultured somatic cells from an adult ewe in 1997 [23]. Since then, a number of mammals have been produced by SCNT [24-29]. In addition, because somatic cells can be proliferated and genetic modified *in vitro*, this technique has been applied to different fields of biomedicine including xenotransplantation, production of bioreactor, cell therapy, and production of human disease model animals [30, 31].

1.2.2. Dog cloning

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 canine SCNT was due to the unique reproductive characteristics of the female dog in comparison to other domestic mammals, such as ovulation of immature canine oocyte and a requirement of 2–5 days for the completion of meiosis within the oviduct [32, 33]. When the technology for the recovery of *in vivo* matured oocyte was established, the application of cloning also

became possible. Finally, the world's first cloned dog was produced in 2005 from adult skin cell of male Afghan hound, named 'Snuppy' which stands for Seoul National University puppy [34]. 'Snuppy' was genetically identical to the donor dog, and his mitochondrial DNA was originated from their oocyte donor dog. From that birth onwards, canine SCNT technique has been developed for companion dog cloning [34, 35], expanding the elite pool of canids[36], conservation of endangered canine species [37, 38] and generation of transgenic cloned dog for animal model research [39]. Nonetheless, the low efficiency of canine SCNT was a major obstacle to extensive use of this technology. In order to overcome the problem, numerous factors affecting the NT have been studied; cell cycle of donor cell [40], status of oocyte maturation [35], fusion condition [41], condition of oocyte donor and recipient dog [42].

1.2.3. Donor cell and SCNT

Most of efforts to improve the efficiency of SCNT have been focused on donor cells. First, G_0 or G_1 phase of donor cell cycle is a major factor because it facilitate nuclear reprogramming in cloned embryo [43]. The cell cycle synchronization of donor cells can be induced by serum starvation, contact inhibition, and some chemical treatment including roscovitine [43, 44]. In dogs, contact inhibition and chemical treatment are available, and roscovitine treatment (15μg/ml for 24 h) improved cell cycle synchronization and cloning efficiency [40]. Another important factor related to donor cell is cell type; until now, various cell types, such as fetal fibroblasts [40, 45, 46] and adult fibroblasts [35, 40, 41, 47-49], have been used as nuclear donors for

producing cloned dog. The general purpose of canine SCNT is to conserve the companion dog, the elite working dog and the endangered canine species, adult fibroblasts are used mainly as donor cell. On the other hand, fetal fibroblasts are preferred as nucleus donors for SCNT used in producing transgenic animals because they have excellent proliferative ability, are capable of being genetically modified, and have the ability to produce live offspring [26, 27, 40, 50] [26, 27, 40, 50]. Recently, transgenic puppies were born following nuclear transfer from canine fetal fibroblasts transfected with the exogenous genes [40, 46]. Although there is difference in SCNT efficiency between canine fetal fibroblasts and adult fibroblasts, they can be reprogrammed and selected as a donor nucleus for the SCNT [51].

1.3. Transgenic cloned dogs using SCNT

The dog has been proposed for human disease model because they have many common genetic diseases. Moreover, dog exhibit 359 genetic diseases similar to those experienced by humans (http://omia.angis.org.au, accessed April 2015), making them one of the important models for various human hereditary diseases. In addition, 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 [40, 52, 53]. For producing transgenic dog, the use of pronuclear injection or homologous recombination are needed. However, these methods are highly inefficient in large animals because of the limitation of random

integration of highly variable expression and unavailable of embryonic stem cells [54]. Recently, SCNT technique using transgenic donor cells generated transgenic large animals [24, 25], this technique would be feasible to produce genetically modified dog.

1.3.1. Transgenic dogs by cloning

Two kinds of transgenic dogs were born following nuclear transfer from fetal canine fibroblasts transfected with the Red fluorescent protein (RFP) gene [40] or Green fluorescent protein (GFP) conditionally expressed by doxycycline administration (Teton) [46]. In addition, transgenic cloned dogs produced exhibited normal reproductive ability, and the foreign gene was successfully inherited to their offspring as well as stable insertion of the transgene into the genome [46, 55]. Therefore, canine SCNT technique could be applied to produce human genetic disease model.

1.3.2. Transgenic dogs by recloning

The production of transgenic animals through a combination of genetic engineering and SCNT has grown, however, there are limitations in the use of these technique [56]. First, to establish transgenic donor cells for SCNT, transfection and selection procedures must be performed. During this procedures, the donor cells easily become senescent and the number of transgenic cells that can be used for SCNT are limited [57]. Second, mosaic pattern of transgene integration or a heterogeneous number of transgene copies integrated at different chromosome locations disturb

homogeneity among transgenic animals and increase variegation of transgene expression with aging which complicate interpretation of the developmental regulation of transgenes [58]. The strategy to overcome these obstacles is to reclone the first generation of transgenic animals. Several studies have demonstrated that the rejuvenated cells from cloned animals can be used to produce recloned offspring in several species including cattle [26, 59], pig [60] and cat [61]. These reports indicated that life-span of the donor cells can be elongated infinitely using recloning technique and transgenic animals can be produced as much as desired. In dog, recloning of the world's first transgenic cloned dogs expressing RFP was tried to overcome cellular senescence and to produce more advanced transgenic dogs [62]. To establish a cloned transgenic cell line, donor cell from the male dogs who was died at 11 weeks after birth was harvested. Embryos reconstructed by nuclear transfer using the transgenic cell were transferred into recipients, one puppy was produced and confirmed as a recloned dog [62]. Although the recloned puppy was stillborn, recloning technique can be used for establishing a rejuvenated fibroblast cell line and propagating the recloned transgenic dog.

1.4. Canines as Human Disease model

Domesticated dogs are increasingly being identified as good models for a variety of biomedical research fields as they have a number of unique advantages over other commonly used experimental animals. The most interesting thing is that dog share

their habitat with their owner and they generally receive a good level care including highly-trained healthcare. This modeling of the human condition offers valuable opportunities for researchers to examine complex problems such as environmental contributions to diseases, aging and its effect on disease susceptibility and progression, and the effects of long-term treatment therapy protocols [63-65].

Another advantage is that over the past several centuries, domestication and selective breeding of dogs has resulted in nearly 400 distinct populations and thus the most naturally occurring genetic diversity in any one species besides humans. Careful breeding for trait selection has inadvertently resulted in breed-specific disease susceptibilities and approximately 400 naturally-occurring, inherited diseases have been identified in dogs. Most predicted canine genes have known as human homologs and many of these heritable canine diseases have been associated with mutations in canine and human homologous genes. The enormous genetic diversity of canine breeds (many of which have extensive pedigree information) and the broad range of spontaneously-occurring canine diseases afford researchers opportunities to examine genetic etiologies and explore the possibility of gene therapies. Unfortunately, this genetic diversity is also the main disadvantage of canine models as there are breed-specific differences in physiology and metabolism, especially idiosyncrasies in pharmacodynamics and pharmacokinetics, which can introduce complications when interpreting or translating the results. However, increasing research in this area is expanding on our current understanding of these issues [63-65].

Finally, due to the rapid aging of dogs, there is a shorter duration for disease

development and progression. This is an enormous advantage in the context of drug development as clinical trial study times are significantly reduced. Potential for rapid study times along with reduced regulatory guidelines and the increasing acceptance of canine models of human diseases by regulatory bodies is resulting in the growing use of dogs as models for translational medicine. Canine models have served to advance human medicine in a number of areas and have been instrumental in some, such as narcolepsy, hemophilia, retinal degeneration, and muscular dystrophy [63-65].

1.4.1. The dog as Alzheimer's disease model

There are significant breed-specific differences in canine longevity; smaller breeds having longer lifespans and larger breeds having shorter lifespans. Generally, the beagle has been selected as the main breed used for aging studies as its median lifespan is 12 to 14 years, and beagles over 9 years of age are considered "old" representing humans aged 66 to 96 years. Both cognitive and neuropathological changes in aged Beagles have been well documented and seem to closely approximate human clinical observations in many aspects. These features make dogs, and in particular beagles, well-matched for studies of human aging and age-related conditions such as Alzheimer's disease (AD) [66].

Like humans, aged dogs naturally suffer from AD that is characterized by the deposition of significant amounts of Amyloid β (Aβ) protein and the development of diffuse plaques, the extent of which quantifiably correlate with cognitive decline [67]. However, AD-afflicted dogs do not appear to naturally develop neurofibrillary tangles

as observed in humans. This may be explained by the fact that while the Aβ amino acid sequence is identical in dogs and humans, the Tau amino acid sequence is appreciably different in the two species. It has been suggested that presence of Aβ deposits and plaques and lack of neurofibrillary tangles represents early stages of AD, which may uniquely position the canine AD model for investigations into the possibilities of preventative measures and early interventions. Therapeutic strategies under investigation using canine AD models include antioxidant diets and behavioral enrichment. Both of these regimens have been shown to improve AD pathology when delivered individually and even more so when administered in combination. Other treatment strategies being pursued in dogs include disruption of Aβ processing by anti-inflammatory or statin drugs and immunization against Aβ peptide [66, 68].

1.4.2. Human synapsin 1 promoter

The widespread phenotype diversity within the central nervous system underscores the importance of restricting transgene expression to a specified target cell type [69-71]. Failure to do so results in gene expression in non-target cells that confounds data interpretation and may lead to undesirable side effects. Restricting gene expression to a specified cell population within the brain by using cell-selective promoters remains an attractive approach [72, 73]. In addition, cell-type-specific promoters are advantageous since they are less likely to activate host cell defense machinery and are less sensitive to cytokine-induced promoter inactivation than viral

promoters [73]. As such, improved stability and longevity of gene expression can be expected.

Synapsins are a family of neuronal phosphoproteins that coat the cytoplasmic surface of small synaptic vesicles [74]. This family consists of four proteins, synapsin Ia and synapsin Ib and synapsin IIa and synapsin IIb. Synapsins I and II are generated *via* alternative splicing from two different genes [74]. Molecular cloning of bovine, human, and rat synapsins revealed striking homologies in the amino-terminal 420 amino acids of all four synapsins. The major difference between synapsins I and II lies in the C-terminal domain of the synapsin I isoforms. This domain contains clusters of basic amino acids as well as two recognition sites for Ca^{2+}/calmodulin-dependent protein kinase II [74]. Synapsin I has been postulated to link synaptic vesicles to the cytoskeleton, thus regulating the availability of synaptic vesicles for exocytosis [75]. In addition, a role for synapsin I in the regulation of short term plasticity has been suggested [76]. Mice lacking synapsin I or both synapsins I and II are viable and fertile with no gross anatomical abnormalities. These mice, however, frequently experience seizures, indicating the essential functions of the synapsins in synaptic vesicle regulation [76]. Virtually all neurons express the synapsins [74] and there are no non-neuronal counterparts known for the synapsins, in contrast to the synaptic vesicle proteins synaptobrevin, synaptophysin, and synaptotagmin [77, 78]. In transgenesis, direction and limitation of gene expression to neurons have been demonstrated with the Synapsin I promoter [79-82]. Though being highly specific, transgene expression cassette exhibits retained neuronal specificity and a considerably elevated level of

expression of two- to three- fold [83, 84]. Thus, restricted expression of synapsins I and II in the nervous system suggests as good tool for investigating the neuron-specific gene expression.

2. General objective

The purpose of this study is to generate transgenic dogs using cASCs which has multi-lineage differentiation potential for further applications to human disease models. This thesis is composed of 5 parts. In part I; as a general introduction, it was explained why I have designed and performed this study. In part II; general methodology used in this study was described. In part III; several properties of cASCs as donor cell were analyzed. Then, iSCNT was performed using cASCs for evaluating its potential as donor cell. Finally, transgenic dogs were generated by canine SCNT using cASCs in part IV. In part V; a final conclusion of this study was described.

PART II

GENERAL METHODOLOGY

1. Chemicals and materials

All chemicals were obtained from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise stated.

2. 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.

3. Preparation of donor fibroblasts and somatic cell nuclear transfer

Canine fibroblasts were obtained by skin biopsy cultures from cell donor dogs. 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 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 (Sigma-Aldrich Corp.).[15].

4. Embryo transfer 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 an attached 7.0 MHZ linear probe. Pregnancy was monitored by ultrasound every 2 weeks after initial confirmation.

5. Microsatellite and mitochondrial DNA analysis of cloned puppies

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.

PART III

Analysis of canine adipose-derived mesenchymal stem cells

Chapter I. Comparison of cell proliferation and epigenetic modification of gene expression patterns in canine fetal fibroblasts and adipose tissue-derived mesenchymal stem cells.

1. Introduction

Dogs have high potential as animal models for human genetic diseases because they not only exhibit similarities in longevity and physiology but also share a large number of disease types with humans (http://omia.angis.org.au, accessed Feb 2012) [85, 86]. In addition, it has become possible to generate transgenic model dogs that exhibit specific human disease traits [86]. For this purpose, SCNT has emerged as the most suitable technique, because alternatives such as pronucleus injection or germline-transmissible embryonic stem cells are not available, unlike in other species [40, 46] .

Fetal fibroblasts are preferred as nucleus donors for SCNT used in producing transgenic dogs because they have excellent proliferative ability, are capable of being genetically modified, and have the ability to produce live offspring [26, 27, 40, 50]. Using virus-driven, gene-inserted fetal fibroblasts as donor cells, cloned dogs were produced that continuously expressed a red fluorescent protein gene and conditionally expressed a green fluorescent protein [40, 46]. However, the donor cells become senescent and unusable because stable transgene-expression, homologous recombination or multiple transfections require a long time for *in vitro* culture [26, 87]. In addition,

using dog fetal fibroblasts raises an unresolved issue that causes much emotional reaction and heated ethical debate, mainly because of the need to destroy fetuses.

As an alternative to fetal cells, recent reports indicate that some mesenchymal stem cell (MSC) lines can be maintained sufficiently long enough for homologous recombination events to take place [88, 89]. MSCs can proliferate for many passages in culture and show constant growth; furthermore, MSCs have the ability to give rise to several differentiated cell types [90, 91]. In this study, I chose MSCs derived from adipose tissue. The adipose tissue presents some advantages with respect to other investigated stem cell sources because they are easily obtained from lipoaspiration or minimally invasive surgery and can be readily expanded to generate hundreds of millions of cells from a small quantity of fat [92]. Because of these properties, canine adipose derived-stem cells (cASCs) have considerable therapeutic potential for use in several disease processes including treatment of human malignancies. In addition, a recent report demonstrated that cASCs can generate cloned pups when used as donor cells in SCNT [93].

This study comprised a basic experiment to establish criteria for selecting suitable nuclear donor cells for generating transgenic cloned dogs, by comparison analysis of various aspects of ASCs and fetal fibroblasts. The aims of the study were to compare canine fetal fibroblast (cFFs) and cASCs for 1) proliferation rates, viability and cellular size; 2) expression patterns of HDAC1 and DNMT1 genes associated with epigenetic modification and of OCT4 and SOX2 associated with pluripotency and early embryonic development.

2. Materials and methods

2.1. Isolation and culture of fetal fibroblasts

Two fetuses were obtained from pregnant beagles at the 28th day and moved to the laboratory in phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) containing antibiotics. The fetuses were washed three times in PBS, then the heads and other organs were removed and the remains were minced with a surgical blade. The minced embryonic tissues were suspended in PBS and centrifuged at 300 g for 3min. The pelleted tissues were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (v/v) FBS (Fetal Bovine Serum; Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$, and 1% (v/v) minimal essential medium (MEM), 1% (v/v) nonessential amino acid solution (Invitrogen), at 38.0 ℃ in a humidified atmosphere of 5% CO_2 and 95% air. After 7 to 10 days of incubation, a fibroblast monolayer was established from the tissue explants. The cells were maintained in culture, passaged, cryopreserved in 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen. In this study, I used two fetal cell lines for all experiments.

2.2. Isolation and culture of adult adipose-derived mesenchymal stem cells

Adipose tissues were collected from the inguinal region of two dogs, a Beagle and a Doberman. From these, two cASC lines were established as previously described [93]. Briefly, the adipose tissues were digested with 1 mg/ml collagenase I (Invitrogen)

for 60 min at 37 ℃. The digested tissues were filtered through a 100 μm cell strainer and centrifuged at 300 g for 5 min to obtain a cell pellet. The pellet was re-suspended in RKCMP (RNL Bio media for MSC culture, RNL Bio Ltd, Seoul, Korea) containing 5% FBS and then centrifuged at 300 g for 5 min. After centrifugation, the supernatant was discarded, and the pellet was collected. The cells were cultured overnight at 37 ℃ under 5% CO_2 in air, in RKCMP. Cell adhesion was examined under a microscope 24 h later, cell layers were washed with PBS, and the culture medium was changed using fresh RKCMP. The cells were maintained in culture for four to five days until 90% confluence (passage 0), then cryopreserved in 10% DMSO and stored in liquid nitrogen, and it was verified whether these cells had characteristics of ASC by flow cytometry analysis. The cells were cultured and expanded in RKCMP and used for characterization. Two cASC cell lines were used for all experiments.

2.3. Flow cytometry analysis

Trypsinized cASCs (2×10^5 cells) were suspended in 100 μl of PBS containing 5% FBS. Cells were stained with FITC-conjugated CD29, CD73, CD105, CD31, CD34 and CD45 (1:10; BD Pharmigen, San Diego, CA) antibodies and FITC-conjugated CD44 and CD90 (Serotec, Oxford, UK) antibodies. The immunophenotype of cASCs was analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using CELL Quest software.

2.4. Measurement of doubling time and cell viability and size

Cryopreserved cFFs and cASCs were thawed and subcultured at 1×10^5 cells per 35mm tissue culture dishes (Falcon,. Oxnard, California, USA). Growth rate of the cells was calculated using an equation for doubling time. The number of cells counted at each passage when cells were confluent was inserted into the equation. Furthermore, cell viability and cell size at each passage were measured by Countess (Invitrogen) according to the manufacturer's instructions.

$$N = N_0 * 2^x$$

$$\text{Doubling time} = \frac{\text{Total time elapsed}}{\text{Number of generation}}$$

[N = final cell number, N_0 = initial cell number ($1x10^5$), x = generation number of exponential growth, Total time elapsed = time until confluence]

2.5. Total RNA extraction

The cell pellets from passages 3 to 7 of cFFs and cASCs were pooled. Total RNAs were extracted from the cell pellet of each group using the easy-spin™ (DNA-free) Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) according to the manufacturer's instructions with slight modifications where needed. Total RNAs were eluted from all samples following the manufacturer's protocol (iNtRON Biotechnology, Inc.). The eluted total RNAs were calculated by spectrophotometry, equalized for concentration across all samples and immediately stored at -80 °C until used for reverse transcription (RT) and real time polymerase chain reaction (PCR).

2.6. Reverse transcription

Total RNA was subjected to RT for detecting expression of HDAC1, DNMT1, OCT4, SOX2, BAX and BCL2 which are related to epigenetic modification or pluripotency, apoptosis, and expression of b-actin as control. The cDNA was synthesized from 1 μg of total RNA using a SuperScript™ III First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies, Barcelona, Spain) by following the manufacturer's instructions with slight modifications where required. Briefly, total RNAs were taken (5~8 μl) in 0.5 ml PCR tubes and then 1 μl of 50 μM oligo (dT)20 and 1 μl of 10 mM dNTP mix were added. DEPC-treated water was added to the tubes to make 10 μl mixtures, which were incubated at 65 °C for 5 min, and then placed on ice for 2 min. In each tube, 2 μl RT buffer (10X), 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNaseOut™ (40 U/μl) and 1 μl SuperScript™ III RT (200 U/μl) were added and mixed gently. The mixtures were centrifuged briefly and incubated for 50 min at 50 °C. Reactions were terminated at 85 °C for 5 min and chilled on ice, then collected by brief centrifugation and 1 μl RNase H (2 U/μl) was added to each tube and incubated for 20 min at 37 °C. The synthesized cDNAs were then stored at -20 °C until used for real-time PCR. All the products except total RNA were supplied with the kit.

2.7. Real time PCR

Real Time PCR was done according to the instructions (Takara Bio Inc., Shiga, Japan) with slight modification. Primer sequences used for real-time PCR are shown in Table 1. In brief, all primers were standardized using a standard curve. A PCR plate

(MicroAmp optical 96-well reaction plate, Singapore) was made by adding 2 μl cDNA, 1 μl (10 pM/μl) forward primer, 1 μl (10 pM/μl) reverse primer, 8 μl SYBR Premix Ex Tag (Takara Bio Inc.), 0.4 μl ROX Reference Dye (Takara Bio Inc.) and 9.6 μl of Nuclease-free water (Ambion Inc., Austin, TX). For each sample, four replications were made in a plate. The wells were capped by using an optical 8-cap Strip (Applied Biosystems, Carlsbad, CA, USA). The plate was then vortexed and centrifuged briefly in a plate spinner. Real time PCR was done using a 7300 Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

2.8. Statistical analysis

Cell proliferative rates and levels of gene expression from real-time PCR were analyzed by paired Student's t-test (GraphPad Prism version 5, Graphpad Incorporation, San Diego). All data were derived from experiments repeated at least 5 times.

Table 1. Primer sequences used for quantitative PCR

Gene	Primer sequences (5'→3')	GeneBank No.	Product size (bp)
Beta-actin	F-GCTACGTCGCCCTGGACTTC R-GCCCGTCGGGTAGTTCGTAG	NM_001003349	86
HDAC1	F-GCTGCACCATGCAAAGAAGT R-TCGCCGTGGTGAATATCAAT	XM_859623	129
DNMT1	F-CCCAGACCGCTTCTACTTCC R-ACTTGGCTCGCATGTTTGAG	XM_533919	148
OCT4	F-CGAGTGAGAGGCAACCTGGAGA R-CCACACTCGGACCACATCCTTC	XM_538830	114
SOX2	F-CAGACCTACATGAACGGCTCGC R-CCTGGAGTGGGAGGAGGAGGTA	XM_545216	147
BAX	F-ACTTTGCCAGCAAACTGGTG R-AGGAAGTCCAGTGTCCAGCC	NM_001003011	88
BCL2	F-TGAGTACCTGAACCGGCATC R-GTCAAACAGAGGCTGCATGG	NM_001002949.1	100

3. Results

3.1. Comparison of cellular proliferation pattern between canine fetal fibroblasts and adipose-derived mesenchymal stem cells

Canine FFs and cASCs showed similar spindle-shape morphologies in culture (Fig. 1). Canine ASCs were determined by flow cytometry to be positive for CD29, CD44, CD73, CD90 and CD105, but negative for CD31, CD34 and CD45 (data not shown). To investigate cellular proliferation, cells were cultured until they reached confluence and the subcultures were stopped when number of generation became a negative value. Growth curves of both cell lines were typical 'S-shaped' curves (Fig. 2), representing a normal cellular growth pattern. Number of generation and doubling time were measured at each passage. There were significant differences between the two groups in number of generation but not in doubling time at all passages (Fig. 3A and 3B). In the 3-6th passages, cASCs showed higher proliferation rates compared with cFFs. However, the doubling time of cASCs increased more rapidly from the 7th passage compared to cFFs. The cell size and viability of both cell types were measured at each passage and averaged over all the passages from the 3rd to 9th. The mean size of cASCs was significantly smaller than that of cFFs (Fig. 3C, $P < 0.0001$). Cellular viability were significantly higher and apoptotic level was significantly lower in cASCs than cFFs (Fig. 4A and 4B, $P<0.001$).

3.2. Expression levels of genes related to epigenetic modification and pluripotency between fetal fibroblasts and adipose-derived stem cells

The relative expression of genes related to epigenetic modification and pluripotency was analyzed in cell derived from cFFs and cASCs. As shown in Fig. 5A and 5B, DNMT1 expression was similar in cFFs and cASCs, while HDAC1 showed significantly higher expression in cASCs than cFFs ($P<0.05$). As shown in Fig. 5C and 5D, OCT4 and SOX2 expression in cASCs was significantly higher than in cFFs ($P<0.05$).

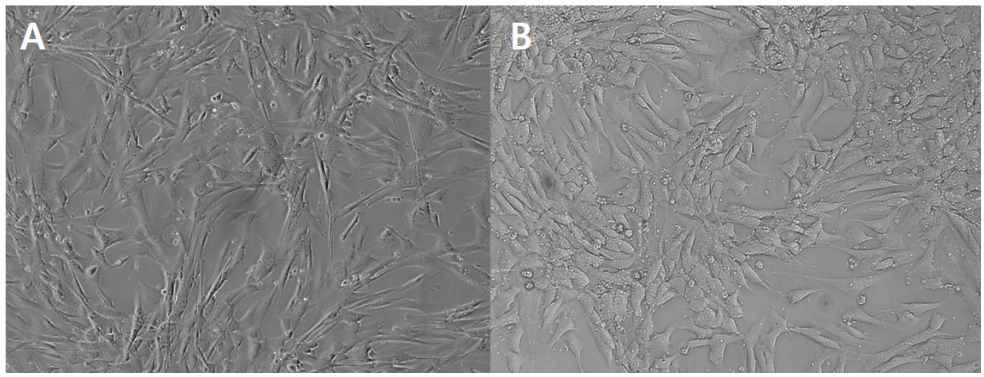


Figure 1. Photographs of cell growth in fourth passage. Morphology of (A) canine fetal fibroblasts (cFFs) and (B) adipose tissue-derived mesenchymal stem cells (cASCs) (magnification 100x).

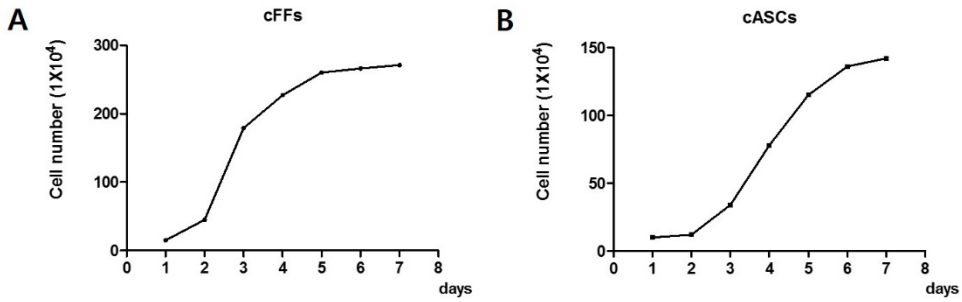


Figure 2. Growth curve of cFFs and cASCs. The sigmoidal curve (lag phase, log phase, plateau) growth pattern observed in cFFs and cASCs at the 4th passage. Equal numbers of cells (1 X 10^5) were seeded in triplicate and aliquots were counted daily during a period of 8 days. (A) Growth curve of cFFs by two fetal cell lines; (B) Growth curve of cASCs by two cASC lines.

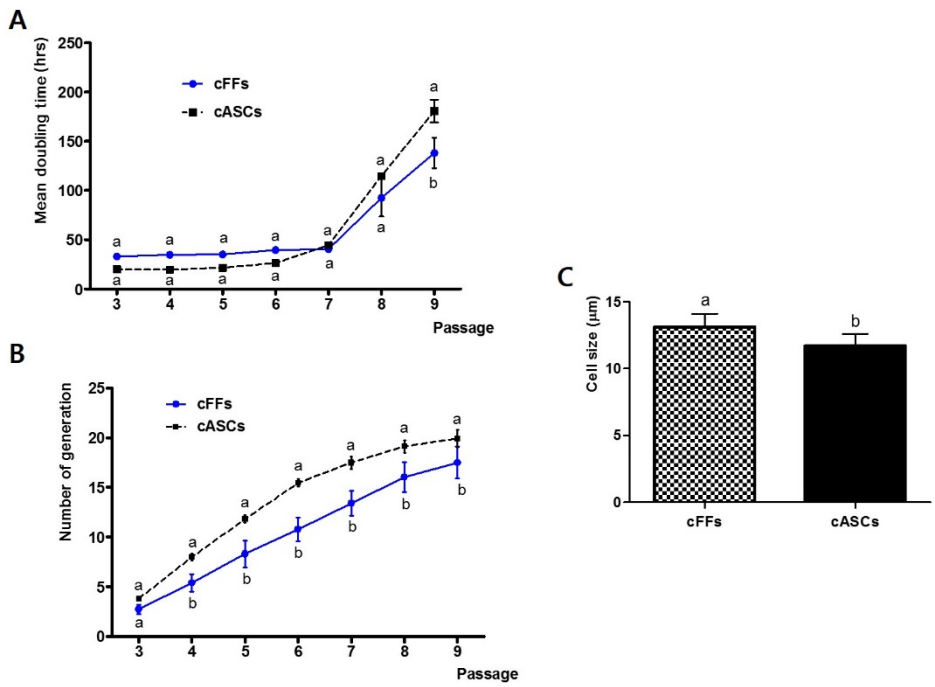


Figure 3. Cellular proliferation pattern and cell size. (A) Doubling time of cFFs and cASCs; (B) The generation number of cFFs and cASCs; (C) Cell size of cFFs and cASCs. Different superscripts (a, b) represent significant differences between groups ($P < 0.05$). Each experiment was performed at least five times using cFFs (pooled data from two fetal cell lines) and cASCs (pooled data from two cASC lines) during the 3–9th passages. Data show mean ± SEM of the two cell lines in each group.

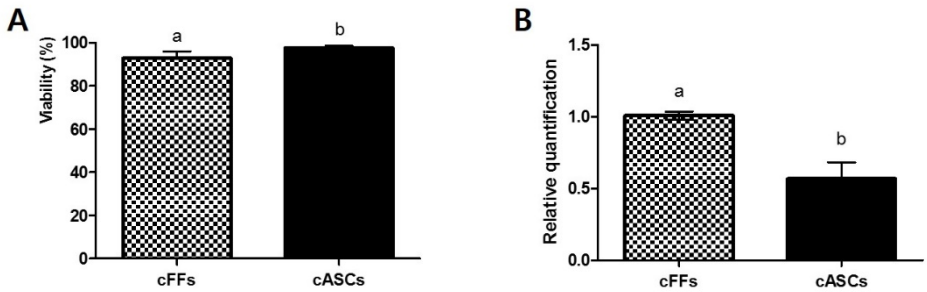


Figure 4. Cellular viability of cFFs and cASCs. (A) Average viability of cFFs and cASCs; (B) Expression profiles of BAX/BCL2 in cFFs and cASCs. Different superscripts (a, b) represent significant differences between groups ($P < 0.05$). Each experiment was performed at least five times using cFFs (pooled data from two fetal cell lines) and cASCs (pooled data from two cASC lines) during the 3–9th passages. Data show mean ± SEM of the two cell lines from each group.

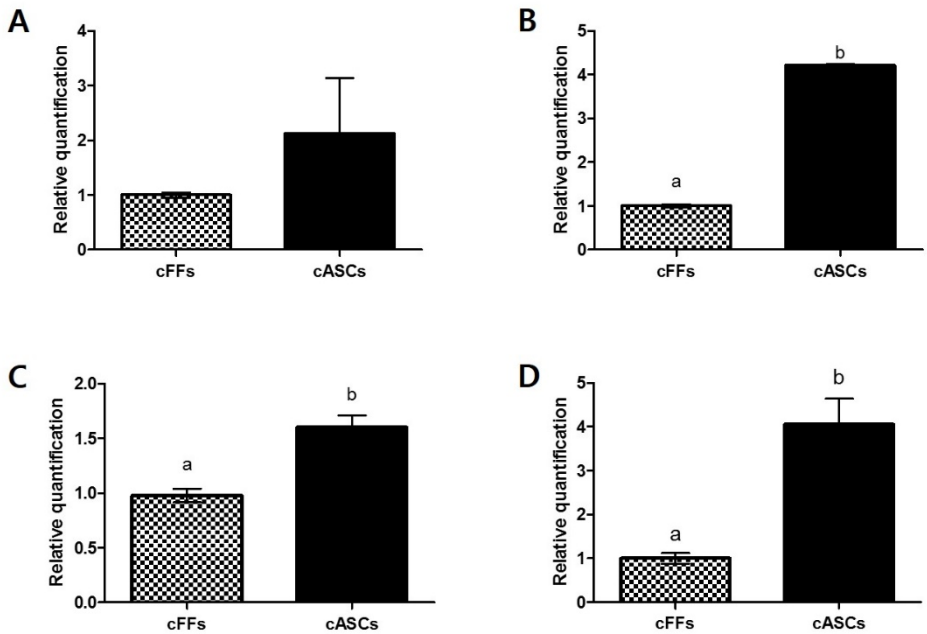


Figure 5. Expression level of genes related to epigenetic modification and pluripotency. Expression profiles of (A) DNMT1 and (B) HDAC1 related to epigenetic modification; Expression profiles of (B) OCT4 and (D) SOX2 related to pluripotency in cFFs and cASCs. Different superscripts (a, b) represent significant differences between groups ($P < 0.05$). Each experiment was performed at least five times. Data show mean ± SEM of the two cell lines from each group.

4. Discussion

The ASC have applications in various research areas, such as cell therapy and tissue engineering especially in bone reconstruction [94], and in animal cloning [93]. Use of these cells has clear advantages such as easy access to subcutaneous adipose tissue, simple isolation procedures, abundant quantities and less ethical concerns [95, 96]. In SCNT research, however, there have been few reports using ASC, compared to FF which are frequently used for SCNT. In this study, I performed comparative analyses of cell proliferation and gene expression between cFFs and cASCs.

First, cFFs and cASCs were assessed during continuous-passage culture for their proliferation patterns and viability. As shown in Fig. 1 and 2, cASCs showed fibroblast-like morphology and sigmoidal growth curves in culture. In line with my results, the same features have been reported in ASC of other species such as horse and human [93, 96-98]. The proportion of live cells was significantly higher and the early apoptotic level was significantly lower in cASCs than in cFFs (Fig. 4A and Fig. 4B). Interestingly, the doubling time of cASCs increased more rapidly from the 7th passage compared to cFFs (Fig. 3A) although cASCs proliferated more actively than cFFs as shown by cumulative population doublings (Fig. 3B). The increased doubling time of late passages is similar to MSC derived from other tissue such as amniotic fluid, amnion and umbilical cord of dogs [99]. It may be that the self-renewal capacity of cASCs is greater than that of cFFs in early passages but decreases with mRNA expression level of pluripotency increasing passage number. It is recommended that

transgenic cell lines be established until the 4-5th passage through selection when cASCs are used as donor cells in SCNT. Generally, cell size was increased when subjected to continuous subculture for transfection [100]. The finding that the size of cASCs was maintained irrespective of passage number might indicate cASCs to be appropriate donor cells.

Secondly, I investigated the expression of epigenetic- and pluripotency-related genes in cFFs and cASCs. Epigenetic changes in DNA methylation and chromatin histone modifications are known to regulate gene expression [101-103]. HDAC1 is an enzyme associated with histone de- or hypo-acetylation. Acetylation of histones by histone acetyltransferases stimulates transcription, whereas deacetylation of histones by HDACs is correlated with transcriptional repression and nucleosomal structure stabilization [104]. DNA methylation is maintained by methyltransferase DNMT1 [105] that controls self-renewal and differentiation of stem cells and is critical for progenitor maintenance and self-renewal in mammalian somatic tissues [106, 107]. In the present study, HDAC1 expression in cASCs was significantly higher than in cFFs, whereas there was no significant difference in DNMT1 between the cASCs and cFFs. The HDAC1/2 multiprotein corepressor complex is essential for pluripotency maintenance in embryonic stem (ES) cells and loss of HDAC1 leads to differentiation of ES cell [108]. In addition, HDAC1 is essential for unlimited cellular proliferation in mouse ES cells [109] . It may be that the proliferative capacity and undifferentiated status of cASCs is superior compared to that of cFFs. Thus, cASCs are recommended as donor

cells for SCNT because they do not become altered during long-term culture for selection after transfection.

With this in mind, I next observed that pluripotent genes in cASCs showed significantly higher expression patterns compared to cFFs. OCT4 and SOX2 are related to pluripotency and are well-known transcription factors that reprogram differentiated somatic cells into ES cell-like pluripotent stem cells [110]. Although OCT4 was considered to be expressed only in ES cells, recent research reported that OCT4 is expressed in bone marrow MSC, equine cord blood cells and even adult human fibroblasts [106, 107, 111]. Similarly, cASCs showed significantly higher expression levels of OCT4 and SOX2 compared to cFFs. It is believed that high expression levels of pluripotency regulators like OCT4 and SOX2 may be essential for “stemness” maintenance of cASCs. Furthermore, high expression levels of OCT4 and SOX2, which are required for embryo cleavage stages and blastocyst differentiation [112, 113], should induce positive effects on reprogramming and development of cloned embryos produced by SCNT using cASC donor cells.

In conclusion, my results demonstrated that cASCs have more stem cell potential compared to cFFs in terms of their proliferation patterns, epigenetic modification and pluripotency ability. I recommend that cASCs are more appropriate than FFs as nuclei donors in SCNT used for transgenesis. Further studies on the efficiency of establishing a stable transgenic cell ling using cASCs and production of transgenic cloned dogs using transgenic cASCs are warranted.

Chapter II. Effect of culture medium type on canine adipose-derived mesenchymal stem cells and developmental competence of interspecies cloned embryos.

1. Introduction

In efforts to improve the efficiency of SCNT in mammals, many studies focused on donor cells have been performed. It is critically important for development of reconstructed embryos that the cell cycle states of the donor cell and the enucleated recipient oocyte are coordinated. In SCNT studies with many species, using donor cells that were synchronized into a quiescent (G0/G1) stage improved cloned blastocyst formation and cloned offspring birth rates [114-117]. Methods such as serum starvation during cell culture or roscovitine are often used to achieve cell cycle synchronization [40, 44].

The type of nuclear donor cell, characterized by its tissue origin and extent of differentiation, is one of the key factors affecting the efficiency of SCNT, but cell selection and treatment are controversial areas. In mice, higher numbers of offspring were produced through nuclear transfer with embryonic stem cells compared to somatic cells [118]. However, *in vitro* development of mouse embryos cloned using hematopoietic stem cells was very inefficient and production of cloned pups was no better than with clones made using other somatic cells such as cumulus, sertoli and

fibroblast cells [119]. In canine SCNT, cloned offspring have been derived using donor cells of several types, which affected the efficacy of cloning [40, 45, 48, 93].

The final factor affecting the SCNT procedure is the complete reprogramming potential of donor cells. Successful reprogramming of donor cells can be influenced by *in vitro* culture conditions including passage number, serum concentration, cell density and chemical treatment [120-123]. Recent studies in mice have shown that treatment of donor cells with chemicals such as trichostatin A changed epigenetic methylation patterns and improved the quality of cloned blastocysts through induced hyperacetylation in mice [124, 125], cattle [126] and pigs [127] SCNT.

To perform this experiment, it is necessary to prepare matured oocyte and donor cell derived from dogs. However, it is difficult to obtain many high-quality recipient canine oocytes because of a limited number of *in vivo* mature oocytes [42] and still low *in vitro* maturation (IVM) rate [128-130]. Therefore, interspecies SCNT (iSCNT) is utilized to perform analyzing the gene expression patterns of donor cells and cloned embryos derived from donor cells with different culture medium. The iSCNT technique has been used widely for evaluating the developmental competence of donor cells, investigating development mechanism of the reconstructed embryos, preserving the endangered animals [131, 132].

Thus, the purpose of the present study was to 1) compare gene expression of canine ASCs grown in two culture medium, 2) analyze *in vitro* development of iSCNT embryos derived from ASCs cultured in two different media and 3) investigate

expression patterns of genes related to stemness, reprogramming and preimplantation development in iSCNT embryos.

2. Materials and methods

2.1. Donor cell culture and preparations

Canine ASCs were prepared as described previously [93]. In brief, cells were isolated from subcutaneous fat tissue collected from the abdomen of a healthy beagle dog under a protocol approved by Seoul National University. Cryopreserved cells at passage 0 were thawed and cultured in two different medium; RCMEP (ASCs culture medium; Keratinocyte-SFM (Invitrogen)-based medium containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, and 5% FBS; RNL Bio Ltd, Seoul, Korea) [133] and Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). Cell adhesion was examined under a microscope 24 h later and cells were washed in phosphate buffered saline (PBS). The respective media were replaced daily. The ASCs at passage 3 were used as iSCNT donor cells. When the ASCs reached 80-90% confluence, they were sub-passaged with each medium. Donor cells were synchronized to the G0 phase of the cell cycle by contact inhibition. Prior to SCNT, cells were disaggregated by 0.25% trypsin-EDTA treatment for 3 min.

2.2. Flow cytometry analysis

The cell fraction at passage 3 was cultured in RCMEP and DMEM until confluence. After trypsinization, detached cells were suspended in PBS at a concentration of 1×10^6 cells/1ml. The cells were stained using specific antibodies: CD29 (1:100, BD Pharmigen, San Jose, CA), CD44 (1:100, Serotec, Oxford, UK) and CD90 (1:100, Serotec) were FITC conjugated. CD31 (1:100, BD Pharmigen), CD 34 (1:100, BD Pharmigen) and CD 45 (1:100, BD Pharmigen) antibodies were phycoerythrin (PE) conjugated. CD29 is rat specific and CD105 mouse antibody. CD34, CD44 and CD90 are canine specific and others are specific to human. The degree of cell surface markers was examined by FACS Calibur (BD Biosciences) using CELL Quest software.

2.3. Oocyte collection and *in vitro* maturation

Bovine ovaries were collected from a local abattoir and transported within 2 h in 0.9% (w/v) NaCl solution at 35 °C. Cumulus oocyte complexes (COCs) were retrieved from antral follicles 2-8 mm in diameter by aspiration with an 18 gauge needle attached to a 10ml syringe. The COCs with evenly-granulated cytoplasm and comprising more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen), supplemented with 10% FBS, 2 mM $NaHCO_3$, and 1% penicillin-streptomycin (v/v). For *in vitro* maturation of bovine oocytes, 30 to 40 COCs were cultured in TCM-199 supplemented with 1 μg/ml follicle stimulating hormone (Antrin, Teikoku, Japan), 10

ng/ml epidermal growth factor, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 1% (v/v) Pen-Strep (Invitrogen), at 39 °Cin a humidified atmosphere of 5% CO_2 in air for 20 h.

2.4. Interspecies somatic cell nuclear transfer

Interspecies somatic cell nuclear transfer (iSCNT) was performed as described earlier [134]. Briefly, *in vitro* matured oocytes were denuded of cumulus cells in Hepes-buffered TCM-199 supplemented with 0.1% (v/v) hyaluronidase. Oocytes with extruded polar bodies were selected and exposed to cytochalasin B (5μg/ml) and Hoechst 33342 (5μg/ml). Metaphase chromosomes were removed under UV light with a fine needle pipette. Enucleated oocytes were randomly divided into two groups for testing canine ASCs cultured in RCMEP and DMEM. Single donor cells cultured in each medium were transferred to an enucleated MII oocyte. A couplet of donor cell-cytoplast complexes was induced to fuse using two pulses of direct current, 35-40 V/cm for 15 μsec each using an Electro-Cell fusion apparatus (NEPA GENE Co., Chiba, Japan). Chemical activation was performed by incubating the reconstructed embryos in modified synthetic oviductal fluid (mSOF) containing 5 μM ionomycin. Cloned iSCNT embryos were transferred into 40μl microdrops of mSOF covered with mineral oil and cultured in an atmosphere of 39 °C, 5% CO_2, 5% O_2 and 90% N_2 for assessing *in vitro* development. On day 2, embryos were evaluated for cleavage to the 2-cell stage or beyond. Blastocyst formation was assessed on day 7. Cleavage rates were recorded on day 2 and blastocysts were selected for further analysis according to International Embryo Transfer Society (IETS) guidelines [135].

2.5. Determination of relative abundance of genes in donor cells and cloned embryos by quantitative PCR

Prior to SCNT, cells were disaggregated by trypsin-EDTA treatment and detached MSCs were rinsed in sterile PBS. RNA was isolated by using the Easy-spinTM Total RNA Extraction Kit (iNtRON Biotechnology, Inc.) from the two groups of ASCs cultured in RCMEP and DMEM. In addition, blastocysts (E-RCMEP, blastocysts derived from RCMEP cultured cells; E-DMEM, blastocysts derived from DMEM cultured cells) were washed three times with PBS and then treated with diethylpyrocarbonate-treated water (Invitrogen) and used fresh. For RNA extraction, blastocysts were pooled in each group. To synthesize cDNA of each sample, reverse transcription was carried out at 50 °C for 50 min using random hexamer and superscript TM III reverse transcriptase (Invitrogen) in a 20 μl reaction volume. Real time RT-PCR (RT-qPCR) was done according to the Takara Bio Inc. guidelines. A total PCR reaction volume of 20 μl was made by adding 2 μl of cDNA, 1 μl forward primer, 1 μl reverse primer, 8μl SYBR Premix Ex Taq, 0.4 μl of ROX Reference (Takara Bio Inc., Shiga, Japan) and 7.6 μl of Nuclease-free water (Ambion Inc., Austin, TX). The reaction was carried out using a 7300 Real Time PCR Cycler System (Applied Biosystems, Foster City, CA). The thermal profile for RT-qPCR was 95 °C for 10min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec, and 72 °C for 40 sec. The forward and reverse primers were designed using the Primer Express 2.0 software program (Applied Biosystems). The sequences of primers and approximate sizes of the amplified fragments are listed in Table 2. The species-specificity of these

primers was validated by testing the same primers on bovine cDNA derived from bovine *in vitro* fertilized embryos. The band was not detected in the PCR product of bovine *in vitro* fertilized embryos using these canine specific primers. The level of all gene transcriptions was normalized to Beta-actin (b-actin) expression levels.

2.6. Statistical analysis

All experiments were replicated at least five times. Statistical analysis was done using GraphPad Prism 4.02 (Graphpad Software Inc, San Diego, CA, USA). The data were analyzed by one-way ANOVA followed by a Bonferroni post-test. Significance was determined when the P value was less than 0.05.

Table 2. List of primers used for Real-Time PCR

Gene	Accession No.	Primer sequence (5'-3')	Product size (bp)
Beta-actin	NM_001003349	F:GCTACGTCGCCCTGGA	173
		R:AGCGGTTCCGCTGCCC	
SOX2	XM_545216	F:CAGACCTACATGAACGGCTCGC	157
		R:CCCACTCCAGGGCGCCCTGCC	
NANOG	XM_543828.2	F:CTCAGTCTCCAGCAGATGC	301
		R:GCCAATCCTGGAGCAGCCACTC	
OCT4	XM_538830	F:CGAGTGAGAGGCAACCTGGAGA	114
		R:CCACACTCGGACCACATCCTTC	
DPPA2	XM_843766.2	F:TACCCATGGCCGAAAAATAG	152
		R:AAATCTTGGCCACCATCTTG	
DNMT1	XM_533919.3	F:GGAGAAACTGAATCTCTTGCAT	115
		R:TAGCCAGGTAGCCCTCCTCT	
DNMT3a	XM_540110.2	F:CAAAGAAGTTTACACAGACATGTGG	178
		R:GCAGAAGTGCCGGAACATCGAGG	
DNMT3b	XM_003433245.1	F:TTGACTTGGTGATTGGTGGA	134
		R:TTTCTTCTCGGCCGGACAT	
MeCP2	XM_003435553.1	F:AAGCCTTTCGCTCTAAAGTGG	150
		R:TGGGAGATTTGGGCTTCTTA	
BAX	AB080230.1	F:TCAAGGCCCTGTGTACCAA	143
		R:ACGTGGGTGTCCCAAAGTAG	
BCL2	NM_001031635.2	F:CAGGGCTGGGTCCTAAGAG	130
		R:TAGCCTGGATGGCAGCTC	
ATPase	NM_001003306.1	F:GCAGGGGATGAAGAACAAG	147
		R:CTCATCCCCTTCCGTGTCTT	
Glut-1	NM_001159326.1	F:GCCTGAGACCAGTTGAGAGC	185
		R:CCCTTTCCTCCCTGCACT	
E-cad	XM_536807.3	F:TGATGAAGAAGGAGGTGGAG	188
		R:GCTGCCTTCAGGTTTTCATC	

3. Results

3.1. Characterization of ASCs cultured in each culture medium type by FACS analysis

The cell surface marker of mesenchymal stem cell marker in ASCs was examined by flow cytometry as suggested in Fig. 6. ASCs cultured in RCMEP were positive for CD29, CD44, and CD90, but negative for CD105, CD31, CD34 and CD45. In the same manner, ASCs cultured in DMEM show identical cell surface marker expression with those cultured in RCMEP.

3.2. Changes in expression of reprogramming- and stemness-related genes in ASCs cultured in different culture media

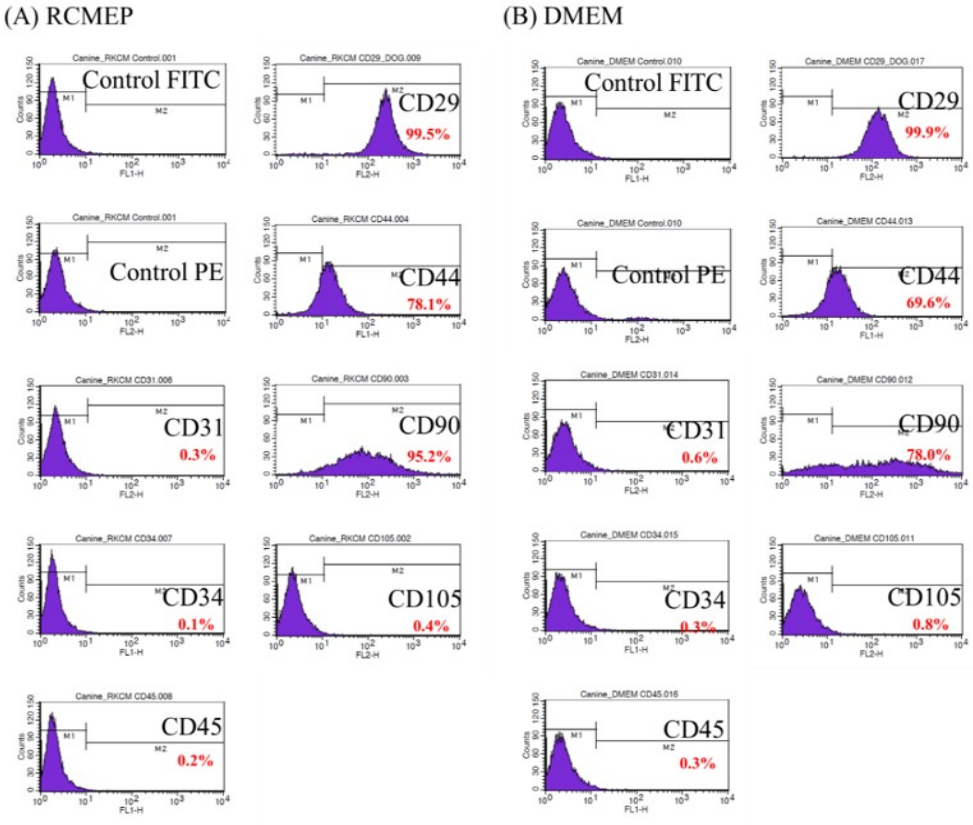
The relative abundance of gene transcripts in ASCs cultured in each medium is shown in Fig. 7. Compared with RCMEP cultured cells, DMEM cultured cells exhibited significantly decreased expression level of SOX2, NANOG, and OCT4 ($P<0.05$). A similar result was observed with reprogramming-related gene expression. DMEM cultured cells showed significantly decreased expression of DNMT1 compared with RCMEP cultured cells. However, there were no significant differences between the two groups of cells for the apoptosis-related genes BAX and BCL2.

3.3. Development of iSCNT embryos derived from donor cells cultured in different medium

As shown in Table 3, when DMEM cultured cells were used as donor cells, the fusion of the reconstructed embryos were significantly higher than those of RCMEP cultured cells (fusion rate, 96.9% vs. 72.7%, respectively, $P<0.05$). However, there was no significant difference in the rate of development to blastocysts between the two groups ($P>0.05$). Hatched blastocyst was not found in both groups.

3.4. Gene expression analysis of reprogramming, stemness and development related genes in iSCNT embryos

Gene expression patterns of SOX2, NANOG, and OCT4 in iSCNT blastocysts (E-RCMEP) derived from RCMEP cultured cells were not different from those produced with DMEM cultured cells (E-DMEM) (Fig. 8). Expression levels of the reprogramming-related genes DNMT1, DNMT3a, DNMT3b, MeCP2 are also shown in Fig. 8. Among these genes, no significant differences were observed except for DNMT1 between embryos produced with RCMEP and DMEM cultured cells. Gene expression patterns of Na/K ATPase, Glut-1, E-cad and BAX/BCL2 in cloned blastocysts were not different between the two groups.



	MSCs cultured in RCMEP	MSCs cultured in DMEM
CD29	+	+
CD44	+	+
CD90	+	+
CD105	-	-
CD31	-	-
CD34	-	-
CD45	-	-

Figure 6. Cell surface staining of ASCs. FACS analysis detecting CD29, CD44, CD90, CD105, CD31, CD34 and CD45 antigen expression of ASCs cultured in (A) RCMEP (B) DMEM.

Table 3. Developmental competence of bovine interspecies somatic cell nuclear transfer embryos derived from donor cells cultured in two medium

Medium Group	Number of embryos enucleated	Number of embryos fused	Number of embryos (percentage, %)		
			Cleavage	Morula	Blastocyst
RCMEP	128	93 (72.7) [a]	70 (75.2) [a]	4 (4.3)	3 (3.2)
DMEM	97	94 (96.9)[b]	77 (87.5) [b]	4 (4.5)	4 (4.5)

[a,b]Different superscripts indicate a significant difference (P<0.05).

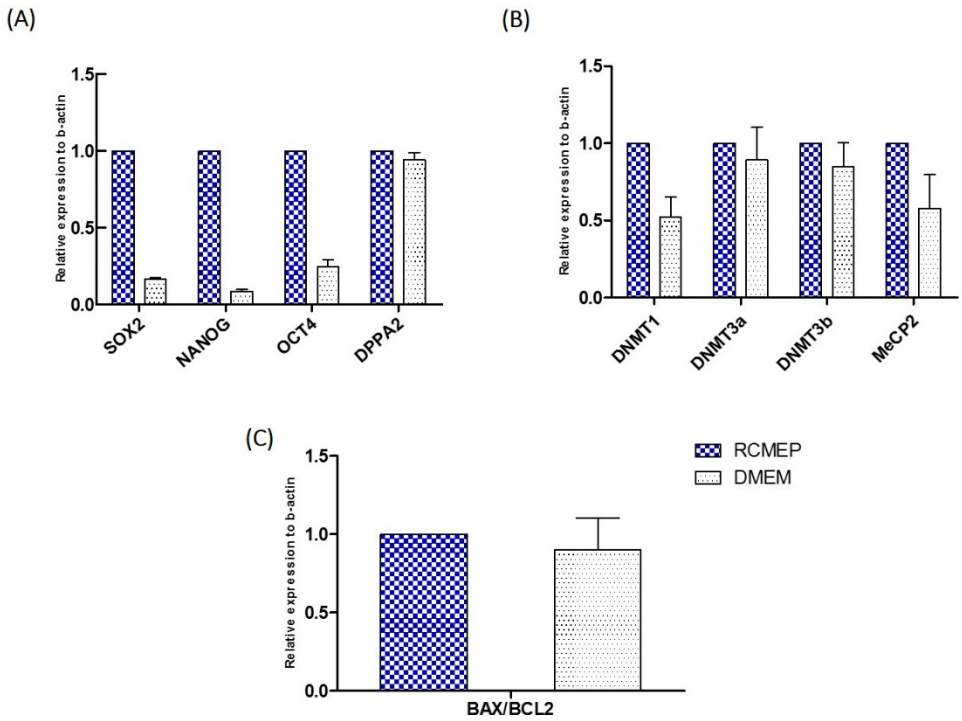


Figure 7. Real-time PCR analysis of ASCs. Relative abundance of transcripts of (A) SOX2, NANOG, OCT4, DPPA2; (B) DNMT1, DNMT3a, DMNT3b, MeCp2; (C) BAX, and BCL2 apoptotic genes in donor cells cultured in RCMEP media and DMEM media. Data presented as mean ± SEM of at least five replicates. Asterisk (*) superscripts indicate a significant difference ($P<0.05$).

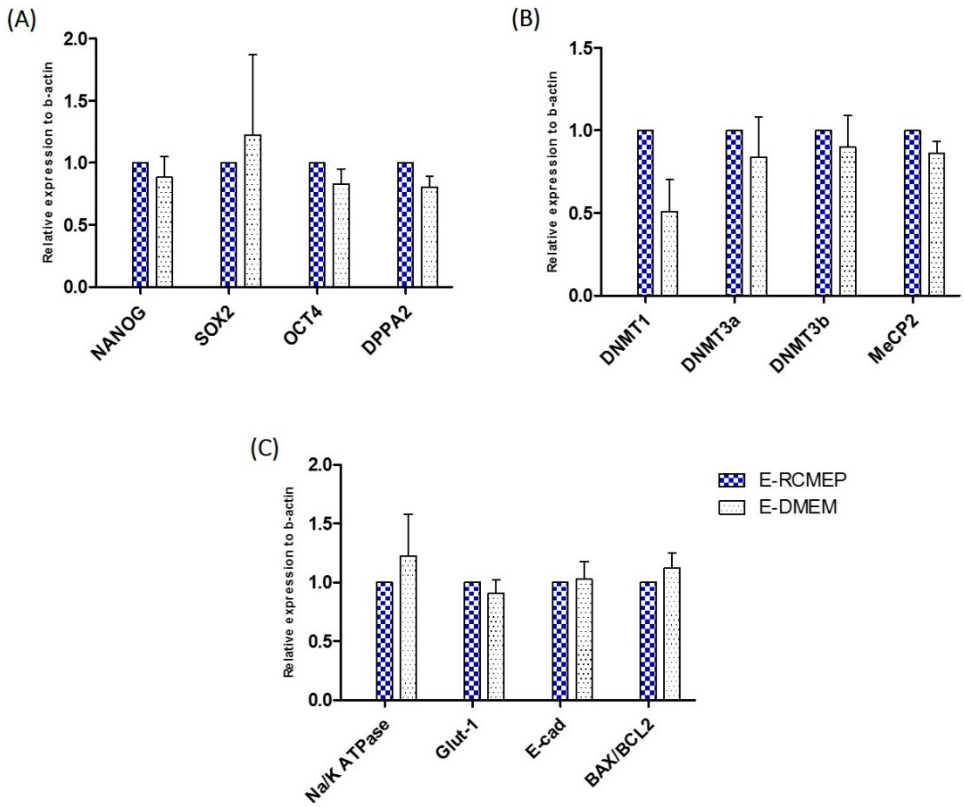


Figure 8. Real-time PCR analysis of iSCNT blastocyst. Gene expression pattern of (A) NANOG, SOX2, OCT4, DPPA2; (B) DNMT1, DNMT3a, DNMT3b, MeCP2; (C) Na/K ATPase, Glut-1, E-cad, BAX/BCL2 expression pattern in iSCNT blastocysts. The figure shows the average relative abundance in cloned embryos. The transcripts of all genes were normalized to that of b-actin. Data presented as mean ± SEM of at least five replicates. Different superscripts indicate a significant difference ($P<0.05$).

4. Discussion

The aim of the present study was to investigate the effect of culture medium for canine ASCs on SCNT efficiency. Because SCNT requires complete reprogramming of somatic donor cells to the totipotent state, expression patterns of reprogramming related genes in somatic cells may affect cloning efficiency. In addition, culture conditions used for donor cells induce different outcomes in preimplantation development of SCNT embryos [136, 137]. In mammals, a direct relationship has been observed between the differentiation state of a donor nucleus and its ability to generate cloned animals *via* SCNT. Therefore, I first investigated the regulatory role of culture medium on stemness maintenance and reprogramming changes in canine ASCs.

The ASCs used as donor cells were characterized by flow cytometry. The cell surface marker of ASCs cultured in RCMEP was identical from ASCs cultured in DMEM. It seems that although intrinsic cell surface marks in ASCs is not easily changeable by culture medium, the gene expression level related with stemness, reprogramming of ASCs can be altered to a certain level by employing culture medium. Because culture medium was maintained only for cell culture during two passages, short term treatment of culture medium may not act to cell surface marker of ASCs. As a general rule, despite of epigenetic marks in somatic cells being stable, they can be altered to a certain level [138, 139]. A decrease in expression of SOX2, NANOG, OCT4 occurred only in DMEM cultured cells. This result indicated that selection of the

proper culture medium can also be employed for inducing differentiation of canine ASCs.

Furthermore, the expression of DNMT1 and MeCP2 genes in DMEM cultured cells decreased compared to RCMEP cultured cells, whereas DNMT3a and DNMT3b genes remained unchanged in both cell groups. DNMT1 mainly acts as a maintenance methyltransferase during the S phase [140], whereas DNMT3a and DNMT3b are mainly responsible for global de novo methylation that establishes new DNA methylation status during the differentiation process [141]. It was suggested that DNMT3a and DNMT3b, which do have some reported maintenance activity, could be compensating and maintaining methylation levels in somatic cells [142, 143] in agreement with the present study. My results showed that the culture medium affected overall expression of reprogramming and stemness related gene expression, except for DNMT3a and DNMT3b. This finding confirmed that gene expression patterns of nuclear donor cells can be changed during culture [144-146].

The *in vitro* development of cloned embryos was monitored and embryonic development using ASCs cultured in DMEM did not differ from development of embryos generated using RCMEP cultured cells. The blastocyst rates of iSCNT are usually below the level of 10% [131, 147]. These data are substantially in accordance with my data showing that 3.2%, 4.5% of embryos cloned with developed to blastocysts.

Thus, an interesting question has been raised whether the altered expression level of stemness maintenance and reprogramming related genes in donor cells is not

sustained during development of cloned blastocysts. If this is true, then artificial or compulsory changes of gene expression in donor cells cannot affect the production efficiency of cloned embryos. In support of this idea, this study provided evidence that changes in transcription levels of stemness maintenance and reprogramming related genes in donor cells are not correlated with regulation of gene expression patterns in iSCNT blastocysts. In agreement with my results, no correlation was found between the expression levels of HDAC1, HDAC2, HDAC3 and DNMT3a and the cloning efficiencies of fetal fibroblasts used as donor cells [148].

In the present study, only DNMT1 showed transcriptional differences between cloned blastocysts of the two culture media groups. It can be conceivable that reprogramming of DNA methylation via DNMT1 might occur during early development. In addition, it is likely that the gene expression discrepancy between donor cells and cloned embryos is due to improper regulation of DNMT function. In fact, it was proved that the epigenetic regulatory transcription level of bovine donor cells is not related to cloning efficiency, and donor cells examined in previous studies were fibroblasts, not ASCs [149, 150]. In a previous study, canine ASCs showed higher DNMT1 expression than canine fetal fibroblasts [151]. So I investigated the impact on SCNT of inducing changes in DNMT expression of ASCs. Although DNMT1 expression by ASCs was differentially down-regulated by the type of culture medium used, it did not have a significant impact on the development of cloned embryos. In cattle, cloned embryos have a tendency to preserve the DNA methylation

patterns inherited from their donor cells [152]. In canine ASCs, it seems that only DNMT1 methylation patterns would be sustained in the cloned blastocysts.

In conclusion, I demonstrated that components of the culture medium can change the expression level of stemness and reprogramming genes in canine ASCs. However, altering gene expression levels in nuclear donor cells by changing the culture medium did not influence subsequent *in vitro* development of cloned embryos.

PART IV

Generation of transgenic cloned dog using canine adipose-derived mesenchymal stem cells

Chapter I. Recloned dogs derived from adipose-derived mesenchymal stem cells of a transgenic cloned beagle.

1. Introduction

SCNT is a useful technology for basic research and production of transgenic animals for biomedical research. The SCNT approach permits the production of transgenic clones using targeted modification of the genome of the donor cells [153]. In spite of successful cloning of several mammalian species using adult somatic cells [23, 34, 154-157], adult fibroblasts are not incompetent for producing transgenic animals as compare to fetal fibroblasts because they have limited ability to proliferate in culture and are refractory to transfection and selection protocols designed to introduce transgenes [158, 159].

As an alternative approach, fetal fibroblasts have been primarily chosen for producing SCNT-derived offspring because of their high developmental competence [159, 160] and their potential for longer term survival and genetic stability in culture, which are required for the duration of the transfection and screening processes [28, 157]. For dog SCNT, fetal fibroblasts were cultured and wild-type and genetically modified beagles were successfully cloned using fetal fibroblasts [40, 45]. In previous study, RFP expressed male cloned dogs were produced using the male fetal fibroblasts with RFP gene [40] . Although they have the same genetic information, their coat color patterns were different from each other. For example, overall black and white pattern in

the body of two puppies are not similar, and showed exclusively discrepancy. It was described as the phenotypic instability and I believe that the main reason for this instability could be inappropriate reprogramming of the donor nucleus [60, 136].

The differentiation status of the nuclear donor cells has been assumed to contribute to the success of cloning since correct epigenetic reprogramming and the resulting changes in transcriptional control are the main processes involved in creating an embryo from a somatic nucleus [161]. In mice, there is evidence to suggest that a less-differentiated cell type can increase SCNT efficiencies compared to terminally differentiated cell types because the former are more easily reprogrammed [136, 161-164]. When mouse ES cells from normally fertilized and SCNT embryos were used as nuclear donor cells, the number of viable offspring was increased compared to when mouse adult somatic cells were used [118, 161, 163, 164]. Besides ES cells, several reports recently showed that adult stem cells from domestic animals used in SCNT are capable of producing viable offspring and can be genetically modified with positive selection [165, 166]. They also are able to proliferate, self-renew and give rise to differentiated daughter cells.

Accordingly, the present study sought to use stem cells as nuclear donors in canine SCNT. Because there is no report of an ES cell line derived from dogs, I decided to employ adipose stem cells derived from inguinal region of a transgenic cloned dog. The aims of the present study were to examine if 1) cASCs can be a suitable donor cell type; 2) transgenic cloned dogs can be recloned using cASCs of transgenic dogs.

2. Materials and methods

2.1. Use and care of animals

In total, 17 mixed-breed female dogs 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 the 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.

2.2. Isolation and culture of canine adipose-derived stem cells

In order to isolate cASCs, adipose tissues were collected from inguinal region of a transgenic cloned beagle produced by SCNT of canine fetal fibroblasts modified genetically with RFP gene [40] . The adipose tissues were washed with phosphate-buffered saline (PBS) and then chopped with scissors in the dish. The chopped samples were digested with 1 mg/ml collagenase I (Gibco, Carlsbad, CA) under gentle agitation for 60 min at 37 °C, filtered through a 100 μm cell strainer and centrifuged at 1,500 rpm for 5 min to obtain the cell fraction. The pellet was resuspended in 5% FBS-containing RKCM (RNL Bio media for MSC culture, RNL Bio Ltd, Seoul, Korea) and then centrifuged at 1500 rpm for 5 min. After centrifugation, the floating non-stem cell adipocytes and supernant were removed and the cell pellet was collected. The cell

fraction was cultured overnight at 37 ℃, 5% CO_2, in RKCM medium. Next day, cell attachment was examined under a microscope, and non-adherent cells were removed by washing with PBS and the medium was changed to RKCM containing 5% FBS. The cell cultures were maintained over four to five days until confluence, and were cryopreserved at passage 0. The cells were cultured and expanded in 5% FBS-containing RKCM and used for characterization and the *in vitro* differentiation experiments at passage.

2.3. *In vitro* differentiation of canine adipose-derived mesenchymal stem cells

2.3.1. Osteogenic induction

At passage two, cASCs from transgenic cloned dogs were plated at 1×10^5 cells/ml in 5% FBS-containing RKCM. The culture medium was replaced with induction medium when cell confluency reached 50%. The osteoblast induction medium was NH Osteodiff medium (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were maintained in culture for 14 days, with 90% of the medium replaced every 3 days. Staining of differentiated cells was done with Alizarin red S staining. The cells were fixed in 70% ethanol for 1 h, washed with PBS and stained with 40 mM Alizarin Red S solution (pH 4.2) for 1 hr. After staining, cells were washed with PBS and then dried at room temperature. The cells were observed a microscope (Axiovert 300, Carl Zeiss, Germany) with a magnification of 100.

2.3.2. Adipogenic induction

At passage two, the cASCs were plated at 1×10^5 cells/ml in 5% FBS-containing RKCM. The medium was replaced with induction medium when cell confluency reached 50%. The adipocyte induction medium was NH Adipodiff medium (Miltenyi Biotec). The cells were maintained in culture for 21 days, with 90% of the medium replaced every 3 days. Differentiated adipocytes were stained with Oil red O. The cells were fixed in 10% formalin for 1 h, washed with PBS and stained with Oil red O solution (60% Oil Red O stock solution; 0.5% Oil Red O in isopropanol and 40% H_2O) for 1 h. The cells were then washed with PBS and observed with a microscope (Axiovert 300) with a magnification of 100.

2.3.3. Myogenic induction

At passage two, the cASCs were plated at 1×10^5 cells/ml in 5% FBS-containing RKCM. The medium was replaced with induction medium when cell confluency reached 50% and maintained for 14 days. The induction medium was SKGM medium containing SKGM SingleOuots (LONZA, Walkersville, MD). After three washes in PBS, cells were fixed with 4% paraformaldehyde for 20 min and incubated in a blocking solution (10% goat and horse serum in PBS) for 2 h. The cells were then incubated with a primary antibody (a human anti-myosin, 1:500 dilution; Chemicon, Billerica, MA) at room temperature for 2 h or overnight at 4 °C. After three washes in PBS, cells were incubated with combinations of AlexaFluor 488-conjugated donkey anti-mouse secondary antibodies (1:1000 dilution; Chemicon), and then stained with

DAPI for nucleic acid detection. The stained cells were observed with an inverted fluorescence microscope (Axiovert 300, Carl Zeiss, Germany) with a magnification of 100.

2.3.4. Neurogenic induction

At passage two, the cASCs were plated at 1×10^5 cells/ml in DMEM containing 10% FBS, 20 ng/ml EGF and 20 ng/ml FGF for 3 days and the medium was replaced with neuronal induction medium. The neuronal induction medium was DMEM containing 10% FBS, 2% dimethyl sulfoxide, 200 μM butylated hydroxyanisole, 1 μM hydrocortisone, 5 μg/ml insulin, 0.5 mM 3-Isobutyl-1-Metylxanthine and 1 mM adenosine 3’5-cyclic monophosphatd sodium salt monohydrate. Induction was terminated after 8 to 10 days of incubation at 37 °C. The cells were fixed with 4% paraformaldehyde for 20 min and incubated in a blocking solution (10% goat and horse serum in PBS) for 2 h. The cells were then incubated with either a human MAP2 antibody (1:200 dilution; Chemicon), a human NSE (neuron specific enolase) antibody (1:10 dilution; Abcam, Cambridge, MA), a human TUJ1 (beta III tubulin) antibody (1:1000 dilution; Abcam) or a human GFAP antibody (1:250 dilution; Chemicon) room temperature for 2 h or overnight at 4 °C. After three washes in PBS, cells were incubated with a combination of Alexa Fluor 488- or 555- conjugated donkey anti-mouse or anti- rabbit secondary antibodies (1:1000 dilution; Chemicon), and then stained with DAPI for nucleic acid detection. The stained cells were observed with an inverted fluorescence microscope (Axiovert 300) with a magnification of 100.

2.3.5. Chondrogenic induction

At passage two, the cASCs (2.5×10^5 cells) cultured in 5% FBS-containing RKCM were centrifuged at 500 g for 5 min and then resuspended in 0.5 ml of NH chondrogenic medium (Miltenyi Biotec) containing dexamethasone, ascorbate, insulin-transferrin-selenium, penicillin, sodium pyruvate, L-praline, L-glutamine and TGF-β. The cells were centrifuged again at 500 g for 5 min to form pellets. The pellets were maintained in culture using polypropylene tubes for 14 days, with 50% of the medium being replaced every three to four days. Differentiated cells were fixed in 10% formalin. After fixation, histological sections (5 μm) were deparafinized, hydrated in distilled water and stained with 1% Toluidine blue for 15 min. The sections were washed in distilled water, dehydrated through 95% and 100% alcohol, cleared in xylene for 3 min and mounted with a cover slip. The stained cells were observed with an inverted fluorescence microscope (Axiovert 300) with a magnification of 100.

2.4. Flow cytometry analysis

Trypsinized cASCs were suspended in PBS containing 5% bovine serum albumin (BSA) at a concentration of 2×10^5 cells/100 μl. The cells were stained with specific antibodies: CD29 (1:100, BD Biosciences, San Jose, CA), CD73 (1:100, BD Biosciences), CD44 (1:100, Serotec, Oxford, UK) or Thy1.1 antibody (1:100, Serotec) were FITC conjugated. CD31 (1:100, BD Biosciences), CD105 (1:100, BD Biosciences) or CD34 (1:100, Serotec) antibodies were phycoerythrin (PE)-conjugated. The antibodies used for characterization of cASC were selected and validated by the

previous reports on characterization of human and canine MSCs [167, 168]. CD34, CD44 and Thy1.1 are dog-specific antibodies and the other antibodies were derived from humans. The expression of the corresponding cell surface markers was assayed by FACS Calibur (BD Biosciences) using CELL Quest software.

2.5. Preparation of nuclear donor cells

The cASCs were further maintained in culture with 5% FBS-containing RKCM, passaged with 0.25% EDTA-trypsin (Gibco), cryopreserved with RKCM containing 10% DMSO (Sigma–Aldrich Corp., St Louis, MO) and stored in liquid nitrogen. The cASCs from passage numbers two to five were used as nuclear donor cells for SCNT. The cells were thawed, cultured prior to SCNT and then retrieved from the monolayer by trypsinization.

2.6. Somatic cell nuclear transfer, embryo transfer and pregnancy diagnosis

Collection of *in vivo* matured oocytes was performed about 72 h after ovulation as described in previous reports [34, 37, 38]. The oocytes were enucleated using micromanipulators (Nikon Narishige, Tokyo, Japan) under an inverted microscope equipped with epifluorescence. An ASC which was expressed RFP fluorescence under an inverted microscope equipped with RFP specific filter (510-560 nm, BA 590 nm) was introduced into the perivitelline space of an enucleated oocyte using previously reported procedures [34, 40]. After microinjection, couplets were induced to fuse with two pulses of direct current of 72 V for 15 μsec each using an Electro-Cell Fusion

apparatus (NEPA GENE, Chiba, Japan). The fused couplets were activated by 4 min incubation with 10 μM calcium ionophore (Sigma–Aldrich Corp.) and reconstructed by 4 h incubation in 6-demethylaminopurine (Sigma–Aldrich Corp.). After reconstruction, cloned embryos were surgically transferred into the oviducts of naturally synchronous recipient dogs as described earlier [35, 37, 40]. Cloned embryos were placed in the ampullary portion of the oviduct using a 3.5 Fr Tom Cat Catheter (Sherwood, St Louis, MO). Pregnancy was detected between 25 and 30 days after embryo transfer with a SONOACE 9900 (Medison, Seoul, Korea) ultrasound scanner with 7.0 MHz linear-array probe. Pregnancy was monitored ultrasonographically every 2 wks after the initial confirmation and the number of fetal puppies was confirmed by radiography after Day 45.

2.7. Parental analysis for genotyping

Parentage analysis was performed on the nuclear donor fibroblasts, cloned dogs and surrogate recipients to confirm genetic identity. The following nine markers which proven for cloned dogs genotyping in previously study [34, 40] were selected for analysis: PEZ1, PEZ5, PEZ6, PEZ8, PEZ12, PEZ20, FH2010, FH2054 and FH2079. The isolated genomic DNA samples were dissolved in 50 μl TE and used for microsatellite assay with nine specific markers originally derived from dogs [34, 40]. 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.

3. Results

3.1. Characterization of adipose-derived stem cells derived from RFP transgenic cloned dogs by FACS analysis

The cASCs had with a fibroblast-like morphology (Fig. 9A) and were attached to the plastic dish; RFP expression was confirmed by microscopic examination (Fig. 9B). The expression of mesenchymal stem cell markers in cASCs was determined by flow cytometry (Fig. 10). Cells were positive for CD29, CD44 and Thy 1.1, but negative for CD31, CD34, CD73 and CD105.

3.2. *In vitro* differentiation of adipose-derived stem cells derived from RFP transgenic cloned dogs

Differentiation potentials of cASCs were investigated. The cASCs were differentiated into adipocytes, osteoblasts, myocytes, neural cells or chondrocytes. Expression of the RFP gene was confirmed by fluorescence microscopy (Fig. 11A2, 11B2 and 11C2; Fig. 11A2) (x 100). Osteogenic differentiation was confirmed by positive Alizarin Red S staining (Fig. 11A3 and 11A4). Mineralized deposits showed as a red color after staining, which reached nearly 100% cellular differentiation (Fig. 11A4). Myogenic differentiation was revealed morphologically with long, multinucleated cell-forming precursors of myotubes and confirmed by green fluorescence with the FITC-labelled human anti-myosin antibody (Fig. 11B1, 11B4). Chondrogenic differentiation was confirmed by toluidine blue O staining for

proteoglycan, a chondrocyte marker. Lacunae formation with extracellular proteoglycan was observed (Fig. 11C3 and 11C4). Adipogenic differentiation was confirmed by positive Oil red O staining (Fig. 12A3, 12A4 and 12A5). Differentiated adipogenic cells accumulated lipid-rich vacuoles in the cytoplasm stained with Oil red O (Fig. 12A4 and 12A5), whereas the control group was not stained (Fig. 12A3). Neurogenic differentiation was confirmed by expression of neural cell markers (MAP-2, NSE or TUJ1 antibody; green) and astrocyte marker (GFAP; green) (Fig. 12B2, 12B3, 12B4 and 12B5).

3.3. Recloning of RFP transgenic pups using adipose-derived stem cells

In total, 121 oocytes from 12 oocyte donor dogs were recovered and 109 of these oocytes were enucleated for cloning. The cASCs were injected into enucleated oocytes and fused by electric stimulation. The fused couplets (82/109, 75.2%; Table 4) were chemically activated and transferred into the uterine tubes of five naturally estrus-synchronized recipients. One of them (20%) maintained pregnancy until full-term and subsequently two healthy offspring were obtained by natural delivery on day 60 after embryo transfer (Table 5). The two cloned pups were genetically identical to the donor dog (Table 6). Also, their whole bodies were detected strong red fluorescence using a Leica inverted microscope equipped with a Texas red filter set (DsRed filter set; Biochemical Laboratory Services, Budapest, Hungary). 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 (Fig. 13D). Interestingly, even

under bright field illumination, the skin and claws of viable recloned pup appeared reddish and were readily distinguishable compared with a non-transgenic pup (Fig. 13B). Unfortunately, one pup died due to the surrogate mother's carelessness. Autopsy of the dead pup revealed no pathologic or anatomical abnormalities. Red fluorescence was seen in all of the tissues including brain, heart, liver, kidney, lung, testis, muscle, intestine, thymus, spleen, adrenal gland, skin, bone and urinary bladder (data not shown).

Table 4. Somatic cell nuclear transfer using canine adipose-derived mesenchymal stem cells

Replication	No. of oocyte donor dogs	Oocyte maturity	No. of flushed oocytes	No. of oocytes with transferred stem cells	No. of fused couplets
1	2	Mature	20	20	15
2	2	Aging	19	17	15
3	3	Mature	30	26	19
4	2	Mature	23	23	20
5	3	Aging/Mature	29	23	13
Total	12	-	121	109	82 (75.2%) [a]

[a] fused couplets/ cell transferred oocytes (%)

Table 5. *In vivo* developmental ability of cloned embryos derived from canine adipose-derived mesenchymal stem cells

Recipient	No. of transferred embryos	Pregnancy	Size of litter (Birth weight, g)	Viability of offspring
A	12	-		
B	15	-		
C	24	-		
D	20	+	2 (260, 270)	50%
E	13	-		
Total	84	20% [a]	2.3% [b]	

[a] Pregnancies/recipients (%), [b] Size of litter / transferred embryos (%)

Table 6. Microsatellite genotyping of recloned beagles.

Marker	1st cloned dog	2nd cloned dog	Nuclear Donor cell	Oocyte donor -1	Oocyte donor-2	Recipient
PEZ1	114	114	114	118/114	122/118	114
PEZ5	105/101	105/101	105/101	109/101	113/109	101
PEZ6	192/184	192/184	192/184	187	187/185	180
PEZ8	231	231	231	235/227	231	235/219
PEZ12	271/261	271/261	271/261	295/277	269	284
PEZ20	175	175	175	179/175	179	179/175
FH2010	231/227	231/227	231/227	239/231	235/227	235/231
FH2054	153/144	153/144	153/144	170/162	166/149	166/149
FH2079	273	273	273	277/273	290/269	273

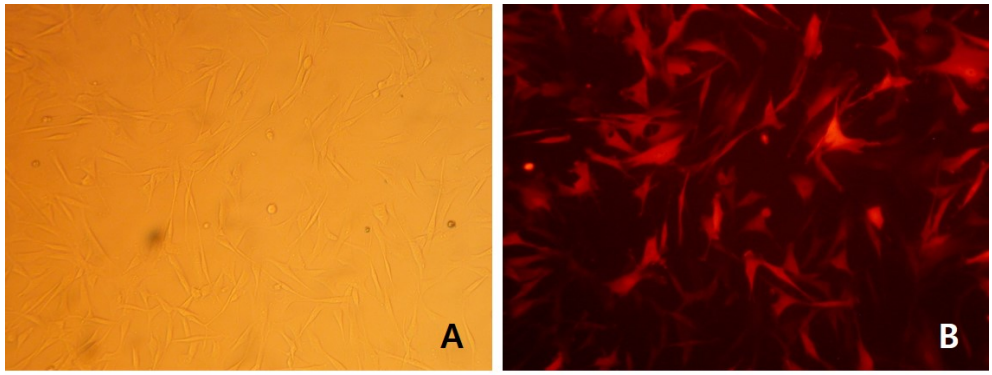
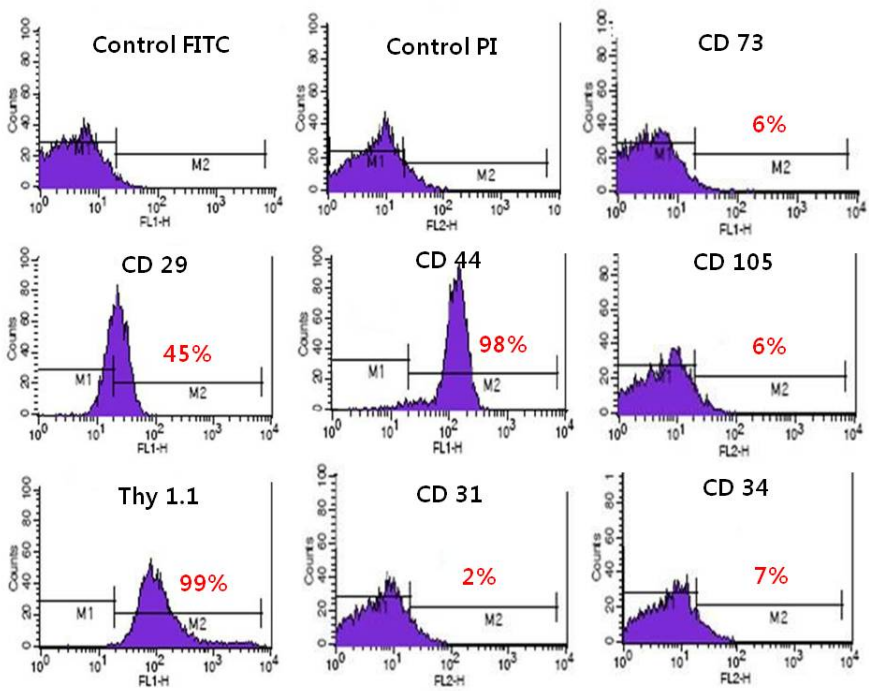


Figure 9. Morphology of canine adipose-derived mesenchymal stem cells derived from an RFP transgenic cloned beagle. (A) Visible light images; (B) Fluorescence images (x 200).



CD marker	Canine Ad-MSC	Human Ad-MSC [26]
CD29	+	+
CD44	+	+
CD71		+
Thy 1.1 (CD90)	+	+
Stro-1		+
CD11		-
CD14		-
CD31	-	-
CD34	-	-
CD45		-
CD73	-	+
CD105	-	+

Figure 10. FACS analysis detecting CD29, CD44, Thy1.1, CD31, CD73, CD105 and CD34 antigen expression. The percentage of cells shows fluorescence intensity with specific antibody staining, as compared to nonspecific fluorescence (control).

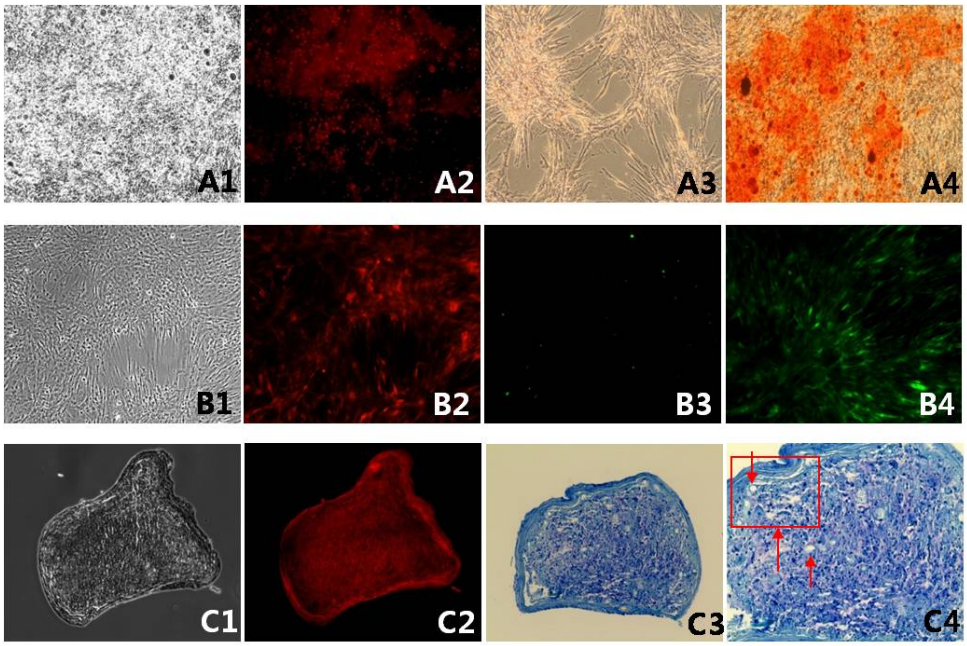


Figure 11. *In vitro* osteogenic, myogenic and chondrogenic differentiation of RFP transgenic dog derived adipose-derived mesenchymal stem cells by immunofluoresence at passage two. The cells transfected with RFP showed (A1) morphology of osteogenic differentiation and (A2) morphology of RFP labeling (x 100). (A3) osteogenic control cultured in normal adipose mesenchymal stem cell medium during 14 days showing negative Alizarin red s staining (x 100). (A4) osteogenic induction during 14 days culture showed morphological changes and mineralized deposits as indicated by positive Alizarin red S staining (x 100). (B1) cells showing morphology of myogenic differentiation and (B2) morphology of RFP labeling (x 200). (B3) the negative control of the myosin immunostaining (x 200). (B4) myogenic differentiation showing expression of myosin as positive immunostaining with myosin antibody (green color) (x 200). (C1) cells showing morphology of

chondrogenic differentiation and (C2) morphology of RFP labeling (x 100). (C3) chondrogenic differentiation 21 days after induction showing lacunae with extracellular proteoglycan formation as evidenced by positive staining with toluidine blue O (x 100). (C4) lacunae as indicated by arrows (x 200). Every experiment was repeated three times.

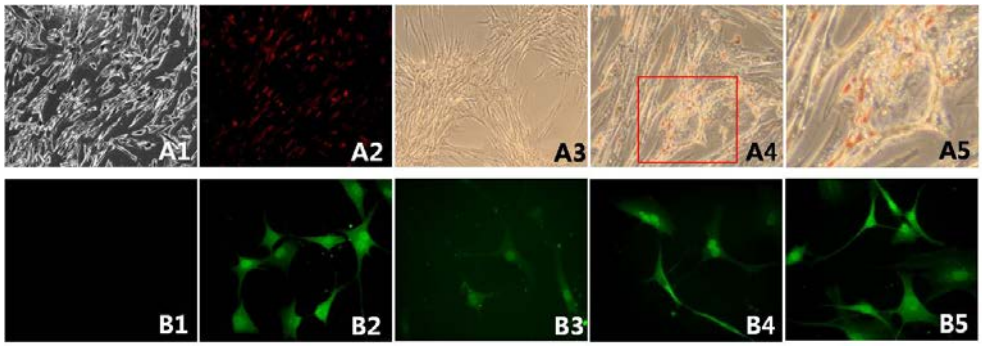


Figure 12. *In vitro* adipogenic and neurogenic differentiation of transgenic dog derived adipose-derived mesenchymal stem cells by immunofluoresence staining at passage two. (A1) morphology of adipogenic differentiation and (A2) morphology of RFP labeling (x 100) is shown. (A3) adipogenic control cultured in normal adipose mesenchymal stem cell medium during 21 days showed negative staining with Oil red O (x 100). (A4) adipogenic induction during 21 days showed morphological changes and accumulation of lipid-rich vacuoles in the cytoplasm as evidenced by positive Oil red O staining (x 100). (A5) lipid droplet deposition (vacuoles) demonstrating positive adipogenic induction. Morphology of neurogenic differentiation 10 days after induction showing large central bodies and neurites (x 400 of a quadrangle in A4). Neurogenic differentiation showing (B1) control, (B2) NSE antibody (green color), (B3) MAP-2 antibody (green color), (B4) TUJ1 antibody (green color) and (B5) GFAP antibody (green color) expression by positive immunostaining (x 400). Every experiment was repeated three times.

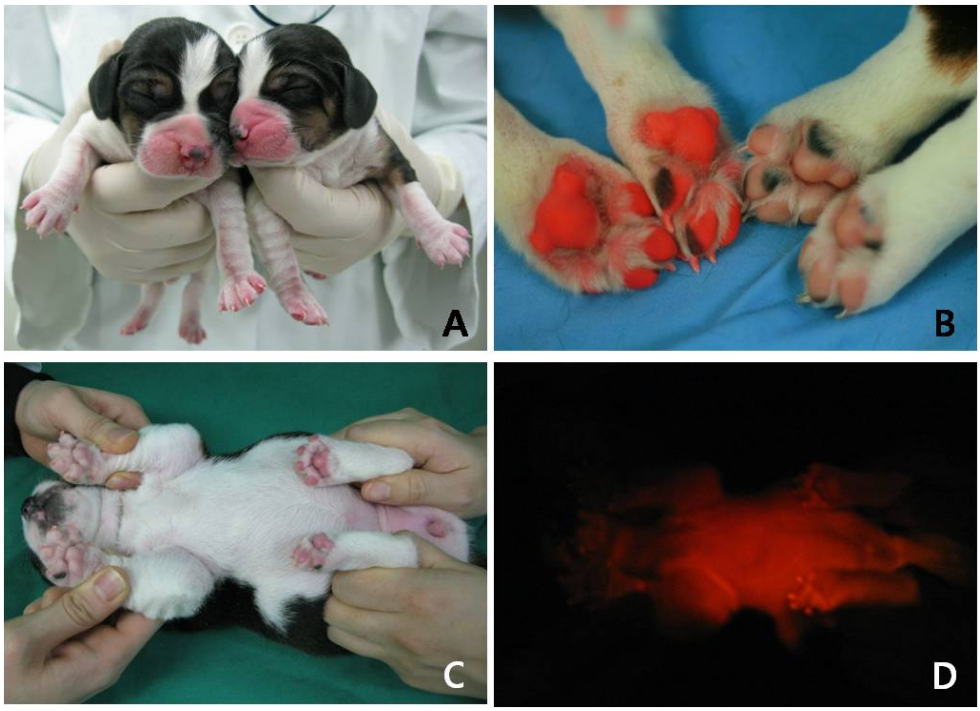


Figure 13. The first dogs recloned by nuclear transfer of adipose-derived mesenchymal stem cells derived from a transgenic cloned dog. (A) The transgenic recloned dog at 2 days after birth. They are named Magic and Stem. (B) Recloned Magic which carries the RFP gene (left) and a non-transgenic puppy (control, right). Notice that the claws and pads of Magic are tinged with red even in bright field illumination; (C) Visible light image; (D) Fluorescence image.

4. Discussion

Adipose tissue is an attractive source for adult stem cells due to its abundance, plasticity in culture and relative simplicity of collection. Recent reports demonstrated that adipose stem cells have the potential to differentiate into other cell types, as well as having the potential for clinical applications [94, 169]. Here, I demonstrated that 1) cASCs from cloned transgenic dogs have the capacity to differentiate into mesodermal and ectodermal lineages *in vitro*; 2) cASCs can be used to generate cloned pups by SCNT, i.e., production of stable, cloned RFP transgenic beagles.

The stem cell characteristics of cASCs established in this study were documented by two lines of evidence. The first line is the result of flow cytometry analysis using cell surface markers of mesenchymal stem cells. The cASC expressed mesenchymal stem cell markers including CD44 and Thy 1.1., but did not express hematopoietic or endothelial markers (CD31 and CD34). The flow cytometric measurements suggest that the primary cultures of cASCs may consist of heterogenous cell populations, e.g. 45% were CD29 positive, 6% were CD105 or CD73 positive and 7% were CD 34 positive. In humans, minimum criteria for characterization of ASC have already been established. The human ASC presented expression of markers CD105, CD73 and CD90, and exhibited no expression of the markers CD45, CD34, CD14, CD11b, CD79a or CD19 [12]. However, in the present study, the cASCs did not react with CD73 and CD105, which is reactive with human ASC. One major reason for the inconsistent pattern of the marker expression between human ASC and cASC could be

due to the use of human CD73 and CD105 antibodies against cASCs. In line with my results, equine ASC did not obtain CD29 reactivity between human ASC and equine ASC [170]. Secondly, the most important criterion to qualify the cASCs as mesenchymal stem cells is their differentiation ability. The cASCs can serve as precursors to a broad spectrum of differentiated cell types. Neupane et al. [20] found that cASCs were able to differentiate along adipogenic and osteogenic pathways. I have assessed the multipotent characteristics of cASCs and showed that they successfully differentiated into osteogenic, adipogenic, myogenic and chondrogenic pathways in the mesodermal lineage and into neurogenic pathways in the ectodermal lineage, under specific culture conditions (Fig. 3 and 4).

It has been hypothesized that the genome of undifferentiated cells, such as stem cells, may be more easily reprogrammed by recipient oocytes during SCNT. The relationship between donor cell differentiation status and nuclear transfer success has been demonstrated in mice. Cloned mouse embryos derived from ES cells showed significantly enhanced survival to term compared with those derived from somatic cell nuclei [162, 164].

Therefore, I hypothesized that cASCs would be superior or at least equal in their suitability as nuclear donors than skin fibroblasts that are routinely used as donors. This possibility was tested in this study along with the purpose of recloning the RFP transgenic cloned dog. In this study, I provide evidence of cASCs' suitability as nuclear donors and the success of recloning the RFP transgenic cloned dog. Although I cannot exactly compare the efficiency between fibroblasts and ASCs, oocyte-donor cell

couplets using cASCs fused at a rate of around 75%, which is similar to earlier reports using adult or fetal fibroblasts [35, 37, 38, 45]. The pregnancy rate, based on the number of pregnant recipients per total number of recipients, was similar to the pregnancy rate (15-30%) reported by previous studies [35, 37, 38, 45]. Here, I report the birth of the first recloned beagles from somatic stem cells and demonstrate the capacity of cASC for *in vivo* development. It is believed that the ASCs do not even compare favorably fibroblasts as nuclear donor and could be useful tool in dog SCNT. In agreement with my results, nuclear transfer using deer antler stem cells [171], porcine fetal somatic stem cells [166], hematopoietic stem cells [172], porcine skin-originated sphere stem cells [173] and porcine neural stem cells [174] have all successfully produced offspring.

Another important outcome of the present study is the successful recloning of an RFP-expressing transgenic cloned beagle by nuclear transfer of cells derived from this animal. The RFP cloned beagle produced in the previous study showed ubiquitous expression in its whole body [40] . The same was found in one of the two recloned beagles produced in this study, which strongly expressed RFP in the whole body and organs. Thus, recloning using cells derived from tissues of a transgenic animal can produce another transgenic clone carrying the foreign gene.

In conclusion, the present study demonstrates that cASCs exhibit multi-lineage differentiation potential and can be a good nuclear donor source for dog cloning. The present study demonstrated for the first time the successful production of cloned beagles by nuclear transfer of cASCs. Furthermore, I have demonstrated that recloning

using cASCs is capable of producing multiple genetic modified clones, and that utilization of canine adipose derived-mesenchymal stem cells may prove to be an excellent cell type for production of genetic disease model.

Chapter II. Age-dependent alteration of transgene expression and cytomegalovirus promoter methylation in transgenic cloned and recloned dogs.

1. Introduction

Transgenic animals have become a powerful tool used as bioreactors for studying pharmaceuticals, the *in vivo* function and regulation of genes as well as generation of models for human disease therapy [175-177]. The production of transgenic animals through a combination of gene operating techniques and SCNT has been consistently growing. Limitations, however, have been imposed to their use for these purposes due to problems in expanding clonal populations of the transgenic animals [56].

Establishment of transgenic donor cells for SCNT requires transfection and selection procedures, the technique necessitates *in vitro* cell culture for a long time, and the donor cells undergo senescence thereby limiting the number of transgenic cell lines that can be used for SCNT [26, 178]. In addition, after introducing a transgene into the donor cell, non-homologous transgene integration or a heterogeneous number of transgene copies integrated at different chromosome locations disturb homogeneity among transgenic animals. The strategy to overcome these obstacles is to reclone the first generation of transgenic animals, and this method has been verified by production of transgenic recloned offspring in several species including cattle [59] and pigs [179].

Recently, I produced a transgenic cloned dog named ‘Ruppy1’ [40] with a RFP gene insertion and also produced ‘Magic’ [93], a recloned dog using an adipose derived-stem cell from Ruppy1. It is expected that the copy number, insertion site and expression pattern of the RFP gene in Ruppy1 are identical to the recloned Magic, but this has not been proven. Therefore, I determined the degree of genetic identity between the cloned and recloned dogs and evaluated whether the RFP expression and CMV promoter methylation of these two transgenic dogs are age-dependent.

2. Materials and methods

2.1. Animals

Two transgenic cloned beagles were used. One is a transgenic cloned dog ‘Ruppy1’ generated by SCNT using a donor cell with an RFP gene insertion [40]. The other dog is ‘Magic’ which was recloned using a stem cell derived from adipose tissue of Ruppy1 [93]. Care of the dogs was conducted in accordance with recommendations described in The Guide for the Care and Use of Laboratory Animals published by Seoul National University.

2.2. Southern blot analysis

Southern blot analysis was performed as previously described [40]. Briefly, genomic DNA was extracted from abdominal skin tissue of two transgenic cloned dogs. Genomic DNA was digested with Hind III and separated on a 1% agarose gel. Then, the fragment of DsRed2 cDNA (682 bp) was amplified with a primer set of 5’-CGCCACCATGGCCTCCTC-3’ and 5’-CAGGAACAGGTGGTGGCG-3’. The southern probe was synthesized using this fragment by the PCR DIG Probe Synthesis kit (Roche, Mannheim, Germany). The resulting probe was labeled with digoxin alkaline phosphatase and purified by agarose gel electrophoresis before hybridization. Detection of labeled DNA on a positively charged nylon membrane was performed using a DIG luminescent detection kit (Roche).

2.3. Western blot analysis

Abdominal skin tissue samples were collected from two cloned dogs at 1 year-old and 4 year-old. The skin tissues were stored at -80 °C until used. Western blotting was performed as described previously [46]. Tissues were lysed in ice-cold protein extraction solution (iNtRON Biotechnology, Inc.). The lysates were centrifuged at 12,000 g for 20 min, and the protein concentrations of the supernatants were assessed using a BCA protein assay kit (iNtRON Biotechnology, Inc.). Equal amounts of proteins of each sample were separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gels. After separation, proteins were transferred to a polyvinylidene fluoride membrane (Amersham, Arlington Heights, IL, USA). The membrane was blocked by incubation in Tris-buffered saline (pH 7.5) containing 0.1% (v/v) Tween 20 and 5% skim milk (MTBST), followed by incubation for 2 h in MTBST containing mouse polyclonal anti-RFP antibody (Abcam, Cambridge, MA) and mouse monoclonal anti-b-actin (Abcam, Cambridge, MA) antibody. After this, the membrane was washed extensively three times in Tris-buffered saline (pH 7.5) containing 0.1% (v/v) Tween 20 and incubated for 1 h in MTBST containing horse-radish peroxidase-conjugated goat antimouse IgG (Pierce, Rockford, IL). Then, SuperSignal West Pico Substrate (Pierce, Rockford, IL) was added and developed after exposing the membrane to X-ray film. The relative quantitation of RFP/b-actin was determined by imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA).

2.4. Identification of transgene integration site on chromosomes

The DNA sequence flanking the transgene integration site was identified using DNA Walking SpeedUp™ Premix Kit-II (Seegene, Seoul, Korea) according to the manufacturer's protocol. Transgene specific primers were used: TSP 1 (5'-TGCACTGTTTGCTGACGCAACCCCCACTGG-3'), TSP2 (5'-TTTCGCCTCGGGCTCAATCACTAGTGAATTCC-3') and TSP3 (5'-AGCAGTTTCTAGAGA ACCATCAGATGTTTCC-3'). The products of third round walking PCR were gel purified (QIAquick PCR purification kit; QIAGEN, Valencia, CA, USA), and the DNA fragments were directly sequenced (Macrogen, Seoul, Korea; http://www.macrogen.com) using a custom-synthesized primer (5'-TCACAGAAGTATGCCAAGCGA-3'). The sequences, except for known sequences, including primers of each product were analyzed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) GenBank (http://blast.ncbi.nlm.nih.gov/).

2.5. Sodium bisulfite modification

Bisulfite modified gDNA was prepared using the EZ DNA Methylation-Gold kit (Zymo Research, USA) according to the manufacturer's instructions. The bisulfite reaction was carried out on 200 ng gDNA and the reaction volume was adjusted to 20 μl with sterile water and 130 μl of CT conversion Reagent were added. The sample tubes were placed in a thermal cycler (MJ Research) and the following steps performed: 10 min at 98 °C, 2 h 30 min at 64 °C, and then tubes were stored at 4 °C. The DNA

was purified using reagents contained in the EZ DNA Methylation-Gold kit (Zymo Research, USA). The converted samples were added onto a Zymo-Spin ICTM Column containing 600 μl of M-Binding Buffer and mixed by inverting the column several times. The column was centrifuged at >10,000 x g for 30 sec and the flow-through was discarded. Then, the column was washed by adding 200 μl of M-Wash Buffer and again centrifuged at >10,000 x g after which 200 μl of M-Desulphonation Buffer was added to the column and allowed to stand at room temperature (20-30 °C) for 15-20 min. After incubation, the column was at >10,000 x g for 30 sec, then washed by adding 200 μl of M-Wash Buffer and again centrifuged at >10,000 x g. The converted gDNA was eluted by adding 20 μl of M-Elution Buffer into the column and at >10,000 x g for 30 sec. DNA samples were finally stored at -20 °C until further use.

2.6. Pyrosequencing analysis

I used the bisulfite pyrosequencing method for methylation analyses of the CMV promoter region. Polymerase chain reaction (PCR) and sequencing primers were designed using Pyrosequencing Assay Design Software v2.0 (Qiagen). The primer sequence is listed in Table 7. PCR reactions were carried out in a volume of 20 μl with 20 ng or more converted DNA, 2 μl of 10X Taq buffer, 5 unit Hot/Start Taq polymerase (Enzynomics, Daejeon, Korea), 2 μl of 2.5 mM dNTP mixture, 1 μl of 10 pmole/μl Primer-S, and 1 μl of 10 pmole/μl biotinylated-Primer-As. Amplification was carried out according to the general guidelines suggested by Pyrosequencing: denaturing at 95 °C for 10min, followed by 45 cycles at 95 °C for 30 sec, then 48 °C for 30 sec,

72 °C for 30 sec and a final extension at 72 °C for 5 min. The PCR reaction (2 µl) was confirmed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. ssDNA template was prepared from 16-18 µl biotinylated PCR product using streptavidin Sepharose® HP beads (Amersham Biosciences, Upsala, Sweden) following the PSQ 96 sample preparation guide using multichannel pipets. Fifteen picomoles of the respective sequencing primer were added for analysis. Sequencing was performed on a PyroMark ID system with the Pyro Gold reagents kit (Qiagen) according to the manufacturer's instructions without further optimization. The methylation percentage was calculated from the average of the degree of methylation at 4 CpG sites formulated in pyrosequencing.

2.7. Statistical analysis

Statistical analysis of the protein levels of Ruppy and Magic tissue samples was performed using GraphPad Prism version 5 (Graphpad Incorporation, San Diego, USA). Statistical significance was determined by one way analysis of variance (ANOVA) followed by Bonferroni *post hoc* testing. Data are presented as means ± standard error of the mean (SEM) and a P-value of ≤ 0.05 was considered statistically significant.

Table 7. Target CpG islands and primers for pyrosequencing

Gene		Primer	Size(bp)
CMV promoter	Forward	5’- GGTGGGAGGTTTATATAAGTAGAGT -3’	98
	Biotinylated-reverse	5’biotin- ATCCCCATATCTTCTATAAAAAT -3’	
	Sequencing primer	5’- ATAAGTAGAGTTAGTTTAGTGAAT -3’	

3. Results

3.1. Analysis of transgene integration

Southern blot analysis revealed that both cloned and recloned dogs have a single copy of the RFP gene that was stably integrated into their genome and found that the RFP gene was stably integrated into chromosome 25 as confirmed by using DNA Walking SpeedUpTM Kit (Fig. 14).

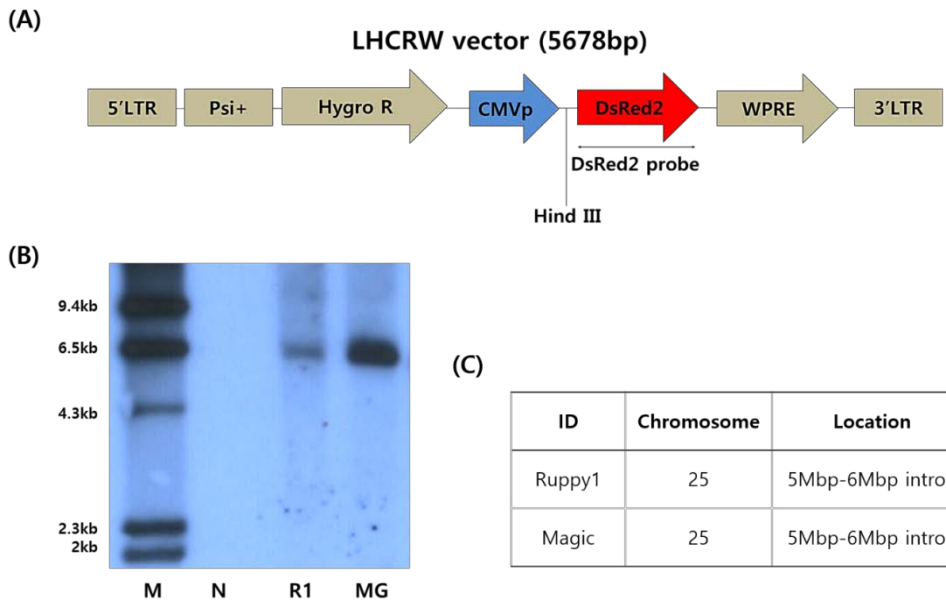
3.2. Comparison of transgene expression in Ruppy1 and Magic

Western blot analysis showed no significant difference in RFP quantity between the 1-year-old Ruppy1 and the 1-year-old Magic but showed proportional relation in transgene protein levels with the age; the RFP levels of Ruppy and Magic at 4years of age was significantly increased (more than 8-fold and 10-fold, respectively) compared with those at 1 year ($p<0.05$) (Fig. 15A, B).

3.3. Methylation status of CMV promoter

Bisulfite modified genomic DNA was prepared using the EZ DNA Methylation-Gold kit (Zymo Research, USA) and converted genomic DNA was used for methylation analyses of the CMV promoter region through a bisulfite pyrosequencing method. The methylation percentage was calculated from the average of methylation at 4 CpG sites. The mean methylation levels of CMV promoter of 1-year-old Ruppy1 and 1-year-old Magic were 26% and 33%, respectively. Interestingly, at 4 years old, the

mean methylation levels of Ruppy1 and Magic were 17% and 25%, respectively which is lower (1.5- and 1.3- fold) compared with those at 1 year-old (Fig. 16A, B).



ID	Chromosome	Location
Ruppy1	25	5Mbp-6Mbp intron
Magic	25	5Mbp-6Mbp intron

Figure 14. Analysis of transgene integration in Ruppy1 and Magic. (A) Southern blot analysis of Ruppy1 and Magic was performed using a Hind III restriction enzyme. (B) The RFP transgene was detected by Southern blot. M, marker; N, negative control; R1, Ruppy1; MG, Magic. (C) The integration position of the transgene. The RFP gene was inserted on chromosome 25 in both transgenic cloned dog.

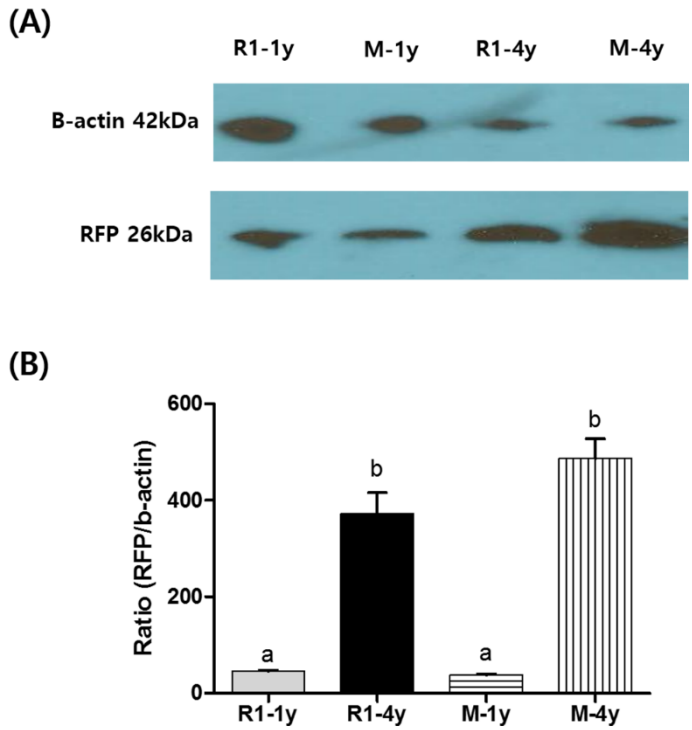


Figure 15. Age-dependent expression of RFP in tissue samples from Ruppy1 and Magic. (A) Western-blot analysis for RFP abundance in each dog at ages 1 and 4 years. (B) RFP expression, normalized to Beta-actin level in each sample. Analysis was replicated three times. Data are presented as the meanstandard deviation. Statistical analysis was performed with. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni posthoc testing, using GraphPad Prism version 5 (Graphpad Incorporation, San Diego, USA). a, b indicates $P \leq 0.05$. R1-1y, 1-year-old Ruppy1; R1-4y, 4-year-old Ruppy1; M-1y, 1-year-old Magic; M-4y, 4-year-old Magic.

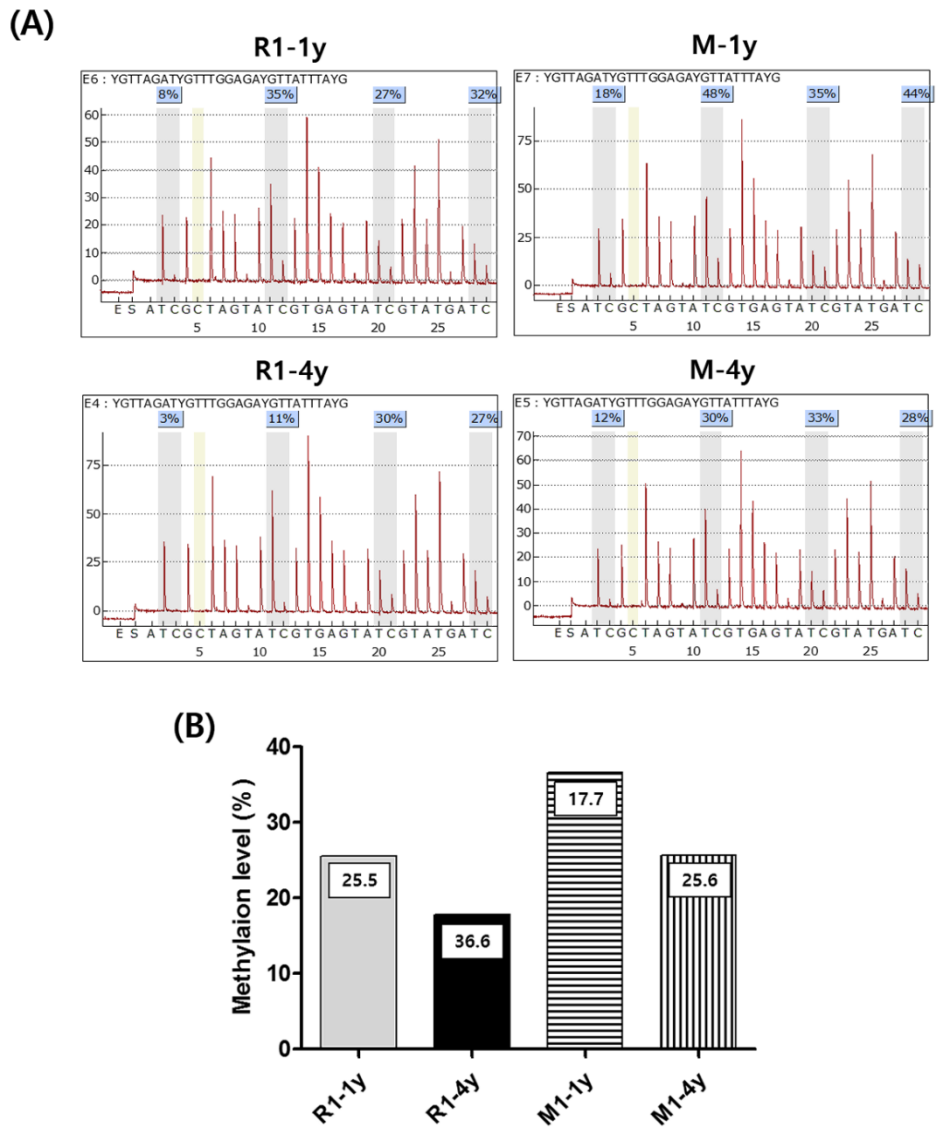


Figure 16. Age-dependent expression of CMV promoter methylation in tissue samples from Ruppy1 and Magic. (A) Representative pyrograms obtained from samples for 4 select cytosine-phosphate-guanine (CpG) sites in the CMV promoter. Each gray shaded column indicates the assayed CpG dinucleotide, and the percentage of methylation at that CpG dinucleotide is indicated above. The yellow shaded boxes are internal

bisulfite-modification control assessments. (B) The average methylation percentages of cytosine-phosphate-guanine (CpG) in the CMV promoter of Ruppy1 and Magic, measured at ages 1 and 4 years. R1-1y, 1-year-old Ruppy1; R1-4y, 4-year-old Ruppy1; M-1y, 1-year-old Magic; M-4y, 4- year-old Magic.

4. Discussion

Recloning, a serial SCNT technique, has been used for the propagation of genetically engineered large animals and for the generation of large animals by multiple gene combination [26, 180]. In the current study, I demonstrated that the copy number and integration site of RFP gene of Magic are identical with those of her nuclear donor, Ruppy1. In line with my results, recloned piglets were produced using as nuclear donors ear cells of transgenic pigs expressing hTPO, and hTPO mRNA was detected in these piglets [179]. In cats, it was reported for the first time that the RFP gene was expressed in a recloned feline [61]. Despite increasingly active use of the recloning technique, there is still insufficient understanding of detail information such as copy number, gene integration site, promoter methylation and age-dependent alteration of transgene expression between transgenic cloned and recloned animals.

Therefore, I next evaluated the methylation status of the CMV promoter for expressing RFP and protein level of the RFP gene with increasing age in two transgenic dogs. In transgenic animals, transgene copy number and DNA methylation status are known to be the main factors incurring silencing of transgene expression [181]. Transgene expression levels in transgenic animals has been especially affected by promoter methylation [182, 183]. Ruppy1, which was used in this study, expressed the RFP gene under the control of the CMV promoter [40]. This promoter has been widely used for transgene expression but is prone to silencing by methylation [183]. Here, my results clearly demonstrated that RFP expression level is associated with

CMV promoter methylation status. In both transgenic cloned dogs, a negative correlation was shown between methylation of the CMV promoter and RPF gene expression.

Interestingly, CMV promoter methylation declined with age while transgene expression increased. Ruppy1 and Magic showed similar patterns of RFP expression alteration with aging. Also, in this study, transgene expression level did not decrease with aging of the transgenic dogs, unlike the previous study. In previous transgenic mice, there is a marked decline in the number of erythrocytes expressing beta-gal with increasing age [58]. GFP-positive transgenic pigs have shown that transgene expression level declined with time. They observed a loss of transgene copy number and increase in transgene promoter methylation with aging in transgenic animals; this may be the cause of gene silencing during development of transgenic cloned pigs and mice [58, 183].

In the present study, I found that 1) a cloned dog and a recloned dog carried the same RFP copy number and the same integration site, 2) the two dogs showed similar alternation of promoter methylation level and gene expression patterns with age. I may conclude that this recloning technique can provide a valuable tool to reproduce transgenic cloned dog to provide an unlimited supply of identical nuclei. In addition, the same integration site and copy number of the transgene may inversely correlate with patterns of promoter methylation. The current results provide a paradigm to study the role of epigenetics in gene expression during aging of transgenic dogs and other mammals.

Chapter III. Neuron-specific expression of the red fluorescence protein in cloned dogs.

1. Introduction

Dogs are being identified as human disease models in biomedical research fields. The most interesting reason is that dog share habitat with their owner and they generally receive a good level care including highly-trained healthcare [63-65]. This offers valuable opportunities for researchers to examine complex problems such as environmental contributions to diseases and its effect on disease susceptibility and progression, and long-term treatment therapy protocols [63-65]. Another thing is that dog has naturally occurring genetic diversity among the species caused by domestication and selective breeding of dogs over the past several centuries. For this reason, the enormous genetic diversity of canine breeds and the broad range of spontaneously-occurring canine diseases afford researchers opportunities to examine genetic etiologies and explore the possibility of gene therapies [63-65].

Especially, the beagle has been selected as the main breed used for aging studies [184]. Like humans, dogs develop Alzheimer's disease (AD) that is associated with cognitive deficits. Deposition of human-like beta-amyloid (Aβ) in the aged canine brain has been well defined [185-187]. Furthermore, there are significant homologies between canines and humans in the amyloid precursor protein (APP) sequence, as well as in many enzymes involved in the processing of APP to form Aβ [188]. These

features make dogs, and in particular beagles, well-matched for studies of human aging and age-related conditions such as AD [66, 67, 189].

Here, neuronal degenerative disease model dog has been generated using a canine transgenic somatic cell nuclear transfer (SCNT) technology with adherence to restrict the transgene expression to a specified neuronal cell type [69-71]. In the present study, Synapsin1 (SYN1) promoter was chosen for direction and limitation of gene expression to neurons [79-82]. The restricted expression of SYN1 in the nervous system has been suggested as a good tool for investigating the neuron-specific gene expression in other species [83, 84]. Thus, the aim of this study is to produce a transgenic dog that expresses neuron specific transgene by human SYN1 promoter.

2. Materials and methods

2.1. Use and care of animals

In this study, mixed-breed female dogs between 1 to 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 Seoul National University.

2.2. Establishment of canine adipose derived-mesenchymal stem cell using Lentivirus vector expressing RFP under the human synapsin 1 promoter

Adipose tissues were collected from the inguinal region of the female beagle and canine ASCs were established as previously described [93]. In brief, adipose tissues were digested with 1 mg/ml collagenase I (Invitrogen) for 2 h at 37 °C and the digested tissues were filtered using a cell strainer and centrifuged for collection of a cell pellet. The cells were cultured overnight at 37 °C under 5% CO_2 in air, in RKCM (R Bio Ltd., Seoul, Korea) containing 5% FBS. A cell layer formed after 24 h, cultured until confluence, then cryopreserved in medium containing 10% DMSO and stored in liquid nitrogen before use for viral infection and SCNT.

In this study, human SYN1 promoter as a neuron-specific gene driving the red fluorescent protein transgene expression was designed. And then, SYN1-red fluorescence protein (SYN1-RFP) was introduced into cASCs *via* lentiviral vector infection. In brief, for making neuron specific transgene expressing cell, lentiviral

vector was constructed. Recombinant lentiviruses packaged with VSV-G (vesicular stomatitis virus G glycoprotein) were cotransfected into 293FT cells by calcium-phosphate precipitation with a viral vector plasmid (pLV-Synp-DsRed2-PGKp-Puro) and Lenti X packaging system purchased from Clontech (Mountain View, CA, USA). Medium was replaced 8 h after cotransfection, and virus-containing medium was harvested 48 h thereafter. Canine ASCs were plated and then infected on the following day with 100 μl of medium containing lentiviral particles (filtered through a 0.45 μm pore-size filter) and polybrene (5 μg/ml). On the next day, cells were incubated in selection medium supplemented with puromycin (5 μg/ml) for 10 days.

2.3. Somatic cell nuclear transfer

Canine *in vivo* matured oocytes were recovered by aseptic surgical procedures 70–76 h after the day of ovulation which was considered when the serum progesterone concentration reached 4.0–9.9 ng/ml. Oocytes surrounded by cumulus cell layers were denuded by repeated pipetting in HEPES-buffered tissue culture medium (TCM)-199 supplemented with 0.1% (w/v) hyaluronidase. The metaphase II spindle of denuded oocytes stained by Hoechst were removed under an inverted microscope equipped with fluorescence beam. One donor cell was injected into the perivitelline space of each enucleated oocyte, then fused with electric stimulation 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 calcium ionophore treatment for 4min, and then cultured in mSOF medium supplemented with 1.9 mM 6-DMAP for 4h.

2.4. Embryo transfer and pregnancy diagnosis

After activation, cloned embryos were immediately surgically placed using a 3.5-Fr Tom Cat Catheter (Sherwood, St. Louis, MO, USA) into the ampullary portion of the oviducts of naturally synchronous recipients. Pregnancy was monitored ultrasonographically between 25 and 30 days after embryo transfer.

2.5. Southern Blot Analysis

Samples for Southern blot analysis were collected from skin tissues of the pups. Southern blotting was performed as previously described [40] . Genomic DNA (20 μg) was digested with BamHI and then separated on a 1% agarose gel. A DNA fragment was used to synthesize a Southern probe using the PCR DIG Probe Synthesis kit (Roche, Basel, Switzerland). The resulting probe was labelled with digoxin alkaline phosphatase and purified by agarose gel electrophoresis before hybridization. DNA was transferred onto a positively charged nylon membrane (Roche), and labeled DNA was detected with a digoxigenin (DIG) luminescent detection kit (Roche).

2.6. Quantification of RFP by ELISA

Quantification of the RFP in the various organs including brain was performed using a RFP ELISA kit (Cell Biolabs, USA). Prior to the assay, the tissue were homogenized in PRO-PREP protein extraction solution (iNtRON Biotechnology, Inc.). Total protein content was determined by using SMART BCA Protein Assay Kit (iNtRON Biotechnology, Inc.) according to manufacturers with the following

modifications: 50 µl of each samples were mixed with 1 ml of working solution. Each samples was incubated at 37 °C for 2 h and then cooled at room temperature. The absorbance was measured for each sample at 562 nm and protein concentration was determined by using BSA standards curves. The ELISA plates were coated with anti-RFP antibody and biotinylated anti-RFP antibody was used as secondary antibody. The RFP standard curve was obtained by 2 fold serial dilutions of 2.5 µg/ml recombinant RFP. Equal amount of total proteins for each sample were examined with the four replications. Absorbance of each sample was read on spectrophotometer at 450 nm as the primary wavelength.

3. Results

3.1. *In vivo* developmental ability of cloned embryos from SYN1-RFP expressing cells

The SYN1-RFP cells were injected into enucleated *in vivo* matured dog oocytes and fused by electric stimulation. The fused-couplets (80/94, 85.1%) were chemically activated and transferred into the uterine tube of five naturally estrus-synchronized surrogates. Three of them (60%) maintained pregnancy and subsequently gave birth to three cloned pups (SYN1-RFP A, SYN1-RFP B, SYN1-RFP C) by natural delivery or C-sec. Unfortunately, the SYN1-RFP A and B that were died at 3 days after birth, but SYN1-RFP C is healthy and does not show any abnormalities (Table 8).

3.2. Confirmation of neuron specific RFP expression

In order to investigate the result in multiple transgene insertions, SYN1-RFP puppies were screened by Southern blot analysis using DNA extracted from skin biopsies. Transgene copy number was estimated by Southern blots analysis. The SYN1-RFP A and B had approximately five and two copies of the transgene integrated, respectively, while an alive SYN1-RFP C has one copy (Fig. 17). At 4-year-old, RFP protein of samples collected postmortem was observed in nervous system including brain, spinal cord and peripheral nerves, but RFP expression was not detected in non-neuronal tissue as heart, kidney and skin (Fig. 18). As expected, expression was observed in Ruppy1 (right) but not in the SYN1-RFP C (left). Interestingly, the

emission of red fluorescence was not detectable in nervous system of SYN1-RFP C while no fluorescence was seen in non-nervous system (Fig. 19).

Table 8. *In vivo* developmental ability of embryos cloned from SYN1-RFP cells

Recipient	Transferred embryos	Pregnancy	Size of litter	Puppy ID	Birth weight (g)
A	19	+	1	SYN1-RFP A	120
B	6	-			
C	16	+	1	SYN1-RFP B	240
D	16	+	1	SYN1-RFP C	310
E	25	-			
Total	82	60%[a]	3.6%[b]		

[a] Pregnancies/recipients (%), [b] Size of litter / transferred embryos (%)

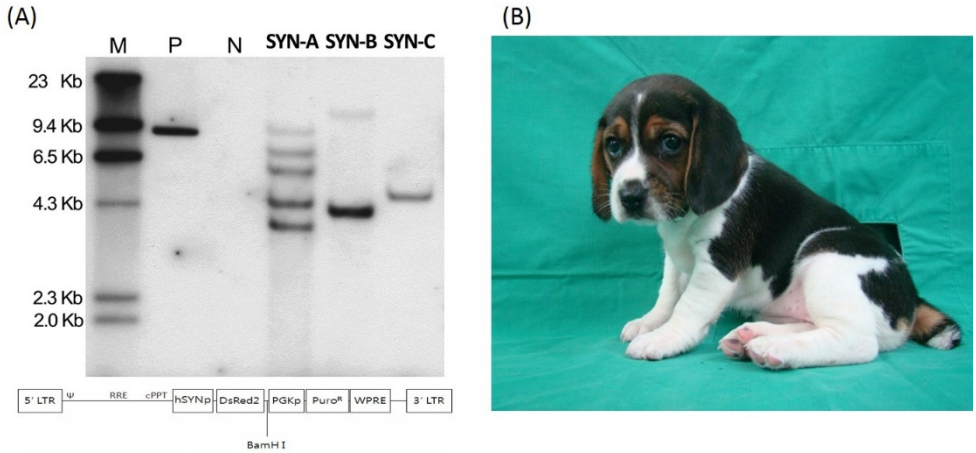


Figure 17. Generation of SYN1-RFP puppies by SCNT using cASCs. (A) Transgene integration of SYN1-RFP puppies by Southern blot analysis; (B) SYN1-RFP C dog with RFP gene under human synapsin promoter by SCNT at 1 month after birth, and named Tung-B. M, marker; P, positive control; N, negative control; SYN-A, SYN1-RFP A puppy; SYN-B, SYN1-RFP B puppy; SYN-C, SYN1-RFP C puppy.

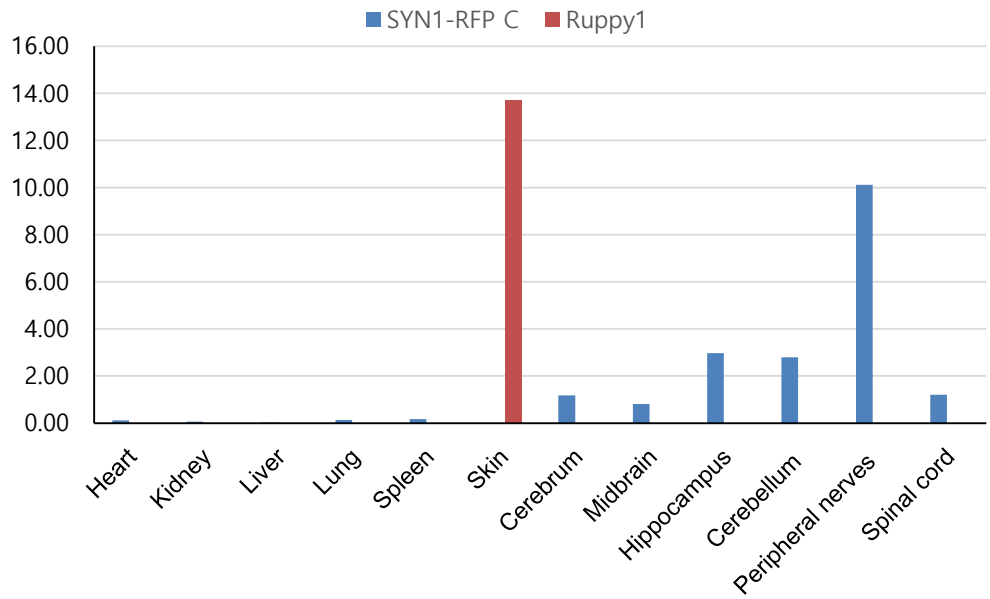


Figure 18. RPF protein using ELISA analysis. Heart, kidney, liver, lung, spleen, cerebrum, cerebrum, cerebellum, midbrain, hippocampus, peripheral nerves, skin and spinal cord of SYN1-RFP C was analyzed. And the skin of Ruppy1 was used as positive control.

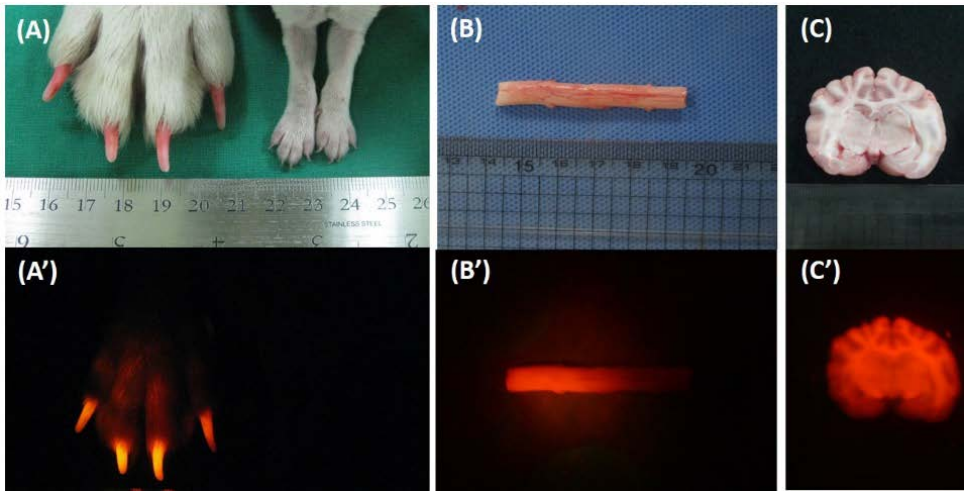


Figure 19. RFP expression pattern in the body and organs of SYN1-RFP C dog. (A, A′) Claws of Ruppy1 which carries the RFP gene under CMV promoter (left) and 1-week-old SYN1-RFP C which carries the RFP gene under synapsin promoter (right); (B, B′) ventral view of SYN1-RFP C spinal cord at 4 years of age; (C, C′) dorsal view of SYN1-RFP C brain at 4 years of age. (A–C) visible light image. (A′–C′) fluorescence image.

4. Discussion

The recent development of canine transgenic SCNT technology has raised new hope for generating human disease model dog [40, 46, 190]. In this study, the human SYN1 promoter was studied and evaluated for use in generating neurodegenerative disease model dogs. The neuron specificity of the human SYN1 promoter has been demonstrated in several studies [82, 191]. After lentiviral transfer of SYN1 promoter construct, a very high specificity for neural expression was seen in rat neostriatum, thalamus and neocortex [82]. Similarly a neuron-specific expression was observed in rat hippocampal/cortical embryonic neurons infected with lentivirus encoding SYN1 promoter [191]. In results of this study, RFP reporter gene under the control of human SYN1 promoter was expressed successfully, consequently, the human SYN1 promoter could be expected to hold the equivalent ability of limiting gene expression strictly to cell populations of neuronal origin when applied in transgenic somatic cell nuclear transfer.

In conclusion, I report here that 1) human synapsin promoter is functional in neural cells of dog brain 2) a neural specific-transgene expressed dog was generated for the first time by transgenic SCNT technique. Furthermore, the SYN-RFP dog has great potential to understand the function of a neuronal degenerative diseases.

PART V

FINAL CONCLUSION

This thesis was conducted to generate transgenic cloned dogs using canine adipose-derived mesenchymal stem cells which has multi-lineage differentiation potential for further application for human disease models.

Firstly, the cellular proliferation rate, viability, cellular size and expression patterns of genes related to pluripotency and epigenetic modification between canine FFs and canine ASCs were compared prior to the SCNT application. It was suggested canine ASC has more stem cell potential compared to canine FFs in terms of their proliferation patterns, epigenetic modification and pluripotency ability.

Secondly, culture medium and potential as donor cell using interspecies SCNT (iSCNT) were evaluated, the cloned blastocyst were derived by iSCNT using canine ASCs. But the altering gene expression levels in nuclear donor cells by changing the culture medium did not influence subsequent *in vitro* development of cloned embryos.

Next, the transgenic beagles by nuclear transfer of canine ASCs were recloned. It was evaluated that the genetic identity between a cloned and a recloned dog and evaluated whether RFP expression and cytomegalovirus promoter methylation of the two transgenic dogs are age-dependent. I concluded that this recloning using canine ASC can provide a valuable tool to reproduce transgenic cloned dog to provide an unlimited supply of identical nuclei.

Finally, SCNT was performed using genetic modified canine ASC for developing novel neuronal degenerative disease models. To generate the dog expressing neuron specific transgene, canine ASCs introducing SYN1-RFP *via* lentiviral vector infection were established, used in SCNT. For the first time, the neural

specific-RFP expressing dog was generated by transgenic SCNT. Furthermore, neuron specific transgene-expressing dog will have great potential to produce a neuronal degenerative disease model. Based on the result in the present study, canine ASCs will be capable of using as an excellent cell type for propagation of genetic modified clones and production of neuronal degenerative disease model dog.

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국문초록

개 지방줄기세포의 분석과
지방줄기세포를 이용한 형질전환 복제개의 생산

오 현 주

(지도교수: 이 병 천)

서울대학교 대학원

수의학과 임상수의학 전공

세계 최초의 복제개 '스너피'가 생산된 이후, 다양한 종의 개과동물들이 체세포복제를 통해 생산되었다. 이후 비글 유래의 태아세포를 체세포복제하여 형질전환복제개가 성공적으로 생산되었다. 태아세포는 우수한 증식률과 유전자조작의 용이성으로 형질전환동물을 생산하는데 유용하게 이용되고 있다. 그러나 외래유전자의 안정적 도입과 다양한 유전자의 도입을 위한 장기간의 체외배양이 필요한 경우에는 세포의 노화나 공여세포로 적합하지 못한 상태에 이르게 된다. 최근에 태아세포의 대안으로 우수한 세포 성장률과 미분화 능력을 지닌 성체줄기세포가

유전자조작을 위해 유용하다는 연구가 보고되어 왔다. 따라서 현재 연구는 개의 지방줄기세포를 이용한 체세포복제 방법을 적용하여 형질전환 복제개를 생산하기 위하여 실시되었다.

사람과 동물에서 지방줄기세포는 채취방법이 간단하며 수득률이 높은 장점으로 세포치료 및 조직재생의학과 같은 다양한 연구분야에서 적용되고 있다. 이러한 지방줄기세포를 체세포복제에 이용하기에 앞서, 지방줄기세포의 특성을 다양한 측면에서 분석하였다. 이를 위하여 세포 증식률, 생존율, 세포크기 및 다양한 유전자의 발현을 지방줄기세포와 비글 태아세포를 이용하여 비교 분석하였다. 이후 지방줄기세포를 이용하여 돼지의 난자로 이종간 체세포복제를 실시하여 복제수정란의 생산 가능성 여부를 검증하였으며, 공여세포로 지방줄기세포를 배양하기 위한 적절한 세포배양액을 선정하였다. 마지막으로 형질전환복제개의 생산을 위하여 이전연구에서 생산된 RFP 가 발현하는 형질전환복제 비글로부터 지방줄기세포를 분리 후, 개 체세포복제를 실시하여 복제개 생산 가능성을 평가하였다. 재복제를 통해 생산된 매직과 세포를 제공한 형질전환복제개 (루피 1)의 유전적 일치성과 나이에 따른 CMV 프로모터와 RFP 발현의 연관성을 분석하였다. 그리고 퇴행성 신경모델개를 생산하기 위하여, 신경계에서만 유전자의 발현을 유도하는 시냅신 프로모터를 이용하여

신경특이적으로 리포터유전자(RFP)가 발현하는 지방줄기세포주를 확립하여 체세포복제를 실시하였다.

결과들은 다음과 같다. 첫째 세포 성장률은 지방줄기세포가 태아세포에 비해 유의하게 높았으며, 세포사멸율과 세포의 크기는 지방줄기세포가 태아세포에 비해 유의하게 낮았다. 또한 전분화능과 후천성관련 유전자 발현에서도 지방줄기세포가 태아세포보다 우수한 결과를 보여주었다. 두번째, 지방줄기세포의 공여세포로의 가능성을 평가하기 위해 실시한 이종간체세포복제에서는 성공적으로 배반포를 생산하여 공여세포로의 잠재력을 확인하였다. 마지막으로 본 연구의 궁극적인 목적인 지방줄기세포를 이용한 형질전환 복제개의 생산이 성공적으로 수행되었다. 이전 연구에서 생산된 RFP 발현 형질전환개(루피 1)의 지방줄기세포를 이용하여 RFP 발현 형질전환 재복제개(매직)를 생산하였다. 뿐만 아니라 루피 1 과 생산된 매직간의 유전적 일치성과 나이에 따른 형질전환유전자의 발현의 변화가 동일한 패턴으로 나타나는 것을 확인할 수 있었다. 그리고 신경특이적으로 유전자의 발현이 조절되는 시냅신 프로모터가 도입된 지방줄기세포주의 생산과 이를 이용한 신경특이적으로 유전자가 발현되는 형질전환복제개를 성공적으로 생산하였다. 따라서 인간의 퇴행성뇌질환과 같은 질병모델개를 생산할 수 있는 새로운 전기를 마련하게 되었다.

결론적으로, 본 연구를 통해 지방줄기세포는 체세포복제를 위한 새로운 공여세포의 대안으로 제시 될 수 있으며, 형질전환 복제개를 생산하기 위한 유용한 세포임을 알 수 있었다. 또한 신경특이적 유전자 발현 기법 및 형질전환체세포 복제기법은 인간 질환모델개 생산을 위한 적절한 방법임을 제시할 수 있었다.

주요어: 지방줄기세포, 체세포복제, 형질전환복제개, 시냅신 1, 신경특이적 유전자 발현 복제견

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