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Production of Transgenic Dogs Carrying Human Phosphoenolpyruvate Carboxykinase and Amyloid Precursor Protein Gene by Nuclear Transfer

사람 Phosphoenolpyruvate Carboxykinase와 Amyloid Precursor Protein 유전자가 도입된 형질전환 체세포 복제 개 생산

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Abstract

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Dog is of immense importance to human society due to its emotional and therapeutic values throughout history. Lately, with the current burgeoning necessity of canine genetic information for reflecting human disease, transgenic dogs carrying human genes associated with a certain disease could provide a therapeutic tool and preclinical model for testing medicines. The aim of the present study was to improve canine cloning technology by studying various factors that affect the pregnancy output and the cloning efficiency in dog cloning and to establish the transgenic cell lines produce the transgenic cloned dogs carrying human phosphoenolpyruvate carboxykinase and amyloid precursor protein gene, and characterize their pathophysiology.

In the first study, 10 breeds providing the donor nuclei for nuclear transfer were categorized into four groups according to their body weight. Reproductive parameters were evaluated in transferring reconstructed embryos from each breed into the surrogates. The number of live pups delivered in the ultra large group (2.4 %) was significantly higher compared to the other groups (1.0 % for large group; 0.9 % for medium group; 1.1 % for small group; $P < 0.05$). There was no direct correlation between the number of embryo transferred and the litter size. The highest litter size was examined in ultra-large breeds. A significantly shorter gestation period was observed in the small breed (58.8±0.3 days) compared to others (59.8±0.1 days for ultra large; 60.7±0.3 days for large, 58.8±0.3 day for medium; $P < 0.05$). The mean BW of cloned pups significantly higher in the ultra-large group (551.4±28.1 g) and the large group (575.4±18.8 g) compared to the other groups (412.7±15.3 g for medium group; 209.2±10.5 g for small group; $P < 0.05$). There was no significant difference in the pregnancy maintenance dependent on the breed size. The meiotic maturation of the recipient oocytes and the passages of donor nuclei do not affect the cloning efficiency. However, reconstructed embryos from the immature oocytes used as recipient cytoplasts had neither attached nor implanted, on the other hand, those from the aged oocytes had maintained a full term pregnancy.

In the second round of the study, in order to establish the type 2 diabetes mellitus dog model, the transgenic cells carrying phosphoenolpyruvate carboxykinase were selected and inserted into the enucleated canine oocytes. Forty-seven reconstructed embryos were transferred into five surrogate mothers resulting in two pregnancies. Three puppies were born and confirmed genetically identical with the donor using the microsatellite analysis. Using PCR, one puppy was confirmed to carry the exogenous phosphoenolpyruvate carboxykinase gene. Relatively high expression of phosphoenolpyruvate carboxykinase gene was observed in the liver biopsy using RT-PCR. No symptoms directly reflected to type 2 diabetes mellitus were observed in the dog.

In the third study, to establishment for Alzheimer’s disease dog model, transgenic cells expressing the human amyloid precursor protein gene containing well-characterized familial Alzheimer’s disease mutations was selected and used for the donor nuclear. Founder transgenic male and female were produced and mated to produce the second generation. The expression of the transgene was confirmed by observing the expression of green fluorescence protein in the body as a visual transgene marker. The presence and expression of the mutated amyloid precursor protein genes were determined in the brain. One transgenic dog showed spontaneous tetanic convulsion that were examined in Alzheimer’s disease-like symptoms such as enlarged ventricles, atrophied hippocampus, and β-amyloid plaques in the brain.

Taken together, the results showed that the efficiency of cloning and fetal parameter after the embryo transfer in the canine could be improved by selecting the appropriate genotype. The transgenic dogs expressing phosphoenolpyruvate carboxykinase and mutant amyloid precursor protein gene could be produced using lipofection-mediated transgenic technique. These findings may contribute to improve the efficiency of dog cloning and to better understand the pathogenesis and/or therapeutic targets of type 2 diabetes mellitus and human Alzheimer's disease.

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Key Words: dog, oocyte, nuclear transfer, type II diabetes mellitus, phosphoenolpyruvate carboxykinase, Alzheimer’s disease, amyloid precursor protein, embryo transfer.

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List of abbreviations

Aβ	β-Amyloid
ABCC8	ATP binding cassette, subfamily C, member 8
AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
ART	Assisted reproductive technology
APOE	Apolipoprotein
APP	Amyloid precursor protein
BW	Body weight
CA	Cornu ammonis
CALPN10	Calpain 10
cDNA	Complementary DNA
CDS	Cognitive dysfunction syndrome
CMV	Cytomegalovirus
Ct	Threshold cycle
CTF	Carboxy-terminal fragment
DG	Dentate gyrus
DMRs	Differentially methylated regions
EGFP	Enhanced green fluorescent protein
FBPase	Fructose-1,6-bisphosphatase
FBS	Fetal bovine serum
G6Pase	Glucose-6-phosphatase
GRB10	Growth factor receptor-bound protein 10
GV	Germinal vesicle
GVBD	Germinal vesicle break down
ICSI	Intracytoplasmic sperm injection
IGF2	Insulin-like growth factor 2

IRES	Internal ribosomal entry site
IVF	*In vitro* fertilization
LOS	Large offspring syndrome
MAPK	Mitogen-activated protein kinases
mhAPP	Mutated human amyloid precursor protein
MI	Metaphase I
MII	Metaphase II
MPF	Maturation promoting factor
MRI	Magnetic resonance imaging
mSOF	Modified synthetic oviductal fluid
NEAA	Non-essential amino acids
Neo^r	Neomycin resistant gene
NFT	Neurofibrillary tangles
PEPCK	Phosphoenolpyruvate carboxykinase
PPARγ	Peroxisome proliferator-activated receptor-γ
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PVDF	Polyvinylidene difluoride
RSV	Rous sarcoma virus
sAPPα	Soluble APP fragment α
SCNT	Somatic cell nuclear transfer
SUR1	Sulfonylurea receptor
SV	Simian virus
T2DM	Type 2 diabetes mellitus

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General introduction

Animal models are imperative biomedical resources for understanding human diseases. Currently, mice are the most widely used animal models due to their economic advantages and advanced transgenic techniques compared to those for other species (Palmiter *et al*., 1986; Stacey *et al*., 1988; Gordon, 1989). However, their phylogenetic distance between humans hampers the application of their model reflecting human diseases. Cloned animals that are relatively close to humans are sheep (McCreath *et al.,* 2000; Denning *et al.,* 2003; Wheeler, 2003), cattle (Donovan *et al.,* 2005; Richt *et al.,* 2007; Houdebine, 2009), pig (Polejaeva *et al.,* 2000), dog (Lee *et al.,* 2005), and non-human primate (Yang *et al*., 2008; Sasaki *et al*., 2009).

Transgenic animals are referred to as animal lineages carrying exogenous DNAs and expressing proteins and phenotypes as being intended. To date, the technique called pronuclear injection is extensively used to produce transgenic animals. However, the major drawback of this technique is that the expression of target genes from the offspring is low (Nottle *et al.,* 2001). After the birth of the first cloned sheep using the technique called the somatic cell nuclear transfer (SCNT) (Wilmut *et al.,* 1997), transferring a nuclear using a donor cell from genetically modified cell lines has been highlighted as an alternative way to produce a transgenic animal. Several transgenic animals have been successfully produced, such as pigs (Lai *et al.,* 2002), cattle (Gong *et al.,* 2004), goats (Keefer *et al.,* 2001), non-human primates (Chan *et al.,* 2001), as well as dogs (Hong *et al.,* 2009).

A domestic dog is the most favorable animal companion throughout the history.

They were also used to guard human properties and provide humans with amusement. In biomedical perspective, dogs provide valuable models of human diseases that can be used to validate and study the disease in a genetic level (Karlsson *et al.,* 2008), because the clinical manifestation of diseases in dogs is similar to that of humans (Head *et al.,* 2002; Lindblad-Toh *et al.,* 2005). As a result of the intensive selection (Sargan, 2004), spontaneous incidences of certain diseases have been examined due to the accumulation of genetic risk factors (Shearin *et al*., 2010). However, many analogous with human diseases are confined to a particular breed or a group of breeds, with underlying genetic variations in loci affecting phenotypic traits. Although dogs are considered as valuable resources for studying human diseases, the progress in basic reproductive techniques for dogs has been slow compared to those for other species. The limitations in the application of assisted reproductive technology (ART) in canine can be attributed to the insufficient consequences from the *in vitro* experiments maturation and the culture of oocytes. The exact mechanisms of acquisition of meiotic competence are not yet known. Despite of a number of *in vitro* attempts to improve meiotic competence of canine oocytes (Hewitt *et al*., 1998; Hewitt *et al*., 1999; Songsasen *et al*., 2002; Rodrigues *et al*., 2003; Rota *et al*., 2004; Hossein *et al*., 2007; Hatoya *et al*., 2009; Sturmey *et al*., 2009), the efficiency is still very meager (Luvoni *et al*., 2003). The first cloned dog has been produced by the SCNT, using *in vivo*-collected oocytes (Lee *et al.,* 2005). Not long after, the successful production of a transgenic dog expressing red fluorescent proteins using the retroviral gene delivery method has been reported (Hong *et al.,* 2009), which was

a stepping stone towards developing transgenic dog models using SCNT.

T2DM and AD are inarguably cause serious health problems for humans that require urgent attention. In near future, the number of T2DM and AD patients will significantly increase as the population ages. More than 350 million people worldwide are currently suffering from diabetes and its complications (Lee-Kubli *et al.,* 2014). Approximately 15 million people are suffering from Alzheimer's disease and this number is expected to more than double in the next generation (Götz *et al*., 2004). T2DM is a heterogeneous disease characterized by persistent hyperglycemia. Insulin is the most paramount hormone that regulates body glucose level acting in predominantly inhibiting the expression of genes encoding the key gluconeogenic enzymes called PEPCK. PEPCK is a key enzyme for catalyzes the rate-limiting step of gluconeogenesis, sequential catalysis from pyruvate to free glucose (Granner *et al*., 1983; Lange *et al*., 1994; Dickens *et al*., 1998; Scholl *et al*., 1999). AD is a multifactorial and polygenic disease in which environmental and genetic factors play a major role. APP is believed to be responsible for the early-onset familial form of the AD, resulting in formation of plaques containing β-amyloid (Aβ) and Tau-containing neurofibrillary tangles (NFT) (Kragh *et al*., 2009). These amyloid plaques and NFTs are the major causes of AD that alter neuronal morphology, which eventually leads to neuronal death (Gómez-Isla *et al*., 1997; Buée *et al*., 2000; Ballatore *et al*., 2007; Meyer-Luehmann *et al*., 2008).

To conquer these two devastating diseases, dogs are accepted as suitable

animal models providing proper phenotypes of these diseases. Dogs may naturally suffer metabolic disorders such as T2DM, as well as cognitive defects such as AD with age (Bain *et al.*, 2001; Neilson *et al.*, 2001; Rand *et al.*, 2004). There are a number of similarities in clinical manifestations of these diseases between dogs and humans. To date, the gene modification technique and the ART are gaining momentum for investigating the functions of a specific gene and its mechanisms associated with disease conditions. The transgenic dog models are extensively used for the mechanistic study in a single gene or mutations on T2DM and AD.

The literature review dealt with the general canine reproductive physiology, the canine artificial reproductive technique, and the genetic background of T2DM and AD. The main study consists of three chapters. The first chapter covers the optimization of the procedures for cloning dogs. The latter two chapters cover the production of canine disease models using the SCNT technique.

Research background

1. Transgenic animals

1.1. Diseases model animals

Generation of transgenic animal models is indispensable for the evaluation of therapeutic strategies and for the investigation of underlying mechanisms of human diseases. In addition, these models reveal the regulatory genes and proteins involved in the development of these diseases. To date, numerous transgenic models have been developed from invertebrate species such as the arthropod fruit fly and the nematode and vertebrates species such as fish, amphibians, birds and mammals (Huss *et al.*, 2008). Among mammals, mouse is the most widely used animal because of advantages such as relatively low maintenance cost, short gestation time, and availability of advanced technologies for gene modification. However, their distant phylogenetic relationship with humans limits their application in determining the pathophysiology of human diseases. Therefore, scientists have been using alternative animal models that are phylogenetically similar to humans, such as sheep (McCreath *et al*., 2000; Denning *et al*., 2003), cattle (Donovan *et al*., 2005; Richt *et al*., 2007; Houdebine 2009), pigs (Lai *et al*., 2002; Kragh *et al*., 2009), dogs (Hong *et al.*, 2009), and non-human primates (Yang *et al*., 2008; Sasaki *et al*., 2009).

1.2. Two major techniques for producing transgenic animals

Transgenic animal has been genetically modified to carry genes from another

species. Exogenous DNA has been inserted into the animal's genome, expressing a protein and a phenotype as being intended. To date, the most widely applied procedures for producing transgenic animals are microinjection of the interest gene(s) into the pronucleus of a fertilized egg and transplantation of transgenic somatic cell into enucleated oocytes (SCNT).

Pronuclear injection is used extensively to produce transgenic animals. After *in vitro* fertilization (IVF), a pronucleus is formed close to the paternal chromosome while a second pronucleus is formed close to the maternal chromosome. Generally, exogenous DNA is introduced into the male pronucleus because it is larger and closer to the oocyte membrane than the female pronucleus. These presumptive embryos are subsequently transferred into the uterus of pseudo pregnant surrogate mothers. Although this technique is reliable, its efficiency is low—only <5 % of the animals born carry the target transgene (Nottle *et al.,* 2001).

It has now been 20 years since Ian Wilmut and his colleagues (1997) reported their article showing that somatic cell from an adult animal could be reprogrammed in enucleated oocyte gave rise to live lamb. This achievement showed that nuclei from mammalian differentiated cells could be reprogrammed by the cytoplasm of oocyte and subsequent full-term development when transferred into surrogate. Cloned animal have the genotype of the cell used as nuclear donor. The cell using as donor cell are genetically modified during culture, resulting offspring will carry the genetic modification. Although only 2-

3 % of cloned embryos survive to term, the technique has significant leap forward over pronuclear injection due to prescreening gene modification in donor cell and therefore every live-offspring carrying transgene.

1.3. Spontaneous canine model

A number of different dog breed exist through the intensive selection. Each breeds an inherited disease with common morphological and behavioral traits shares (Sargan, 2004). Spontaneous incidences of inherited diseases have been observed in different breeds probably due to the accumulation of a few genetic risk factors through drift or selective breeding (Shearin *et al.,* 2010). Recently, polygenetic disease that implicated in the homogeneity of genetic risk factor can mapped with lesser samples, revealing common pathway between human disease and dog disease (Chase *et al.*, 2009). The recent development of a canine high-throughput SNP genotyping array will facilitate to unravel human disease reflecting dog genetics. Therefore, genome-wide surveillance of canine spontaneous disease is a valuable tool for understanding human disease along with the transgenic canine models.

2. Diabetic model

T2DM is one of the most prevalent metabolic diseases. The obesity have

been shown to be a predisposing factor the development of T2DM (Després *et al.*, 2006). The growing public health burden of T2DM across the world is enormous, that requires adequate and strong intervention for prevention or improved therapeutic tools for this disease. Therefore, the search for better understanding of underlying mechanism of human T2DM and the need for new therapy emphasize the role of appropriate animal models mirroring the characteristics of human T2DM

2.1. History of Canine model for T2DM

Diabetes was first recognized in a dog in 1890 by inducing the removal of insulin-producing pancreatic β-cells of islets (v. Mering *et al.*, 1890). To date, several publications have covered canine anatomy, physiology, pathology, and treatment relevant to T2DM. In dogs, chronic diabetes can be induced using several procedures, each having its pros and cons. Ablation of β-cells by using chemical reagents such as alloxan or streptozotocin is a simple procedure compared to the surgical removal of the pancreas (Lenzen, 2008; King, 2012).; however, this procedure has unpredictable effects on the animal, such as damage to other tissues. Alternatively, total pancreatectomy can be considered, but the procedure is time consuming, requires elaborate surgical skills and postoperative care, and eliminates the exocrine and endocrine functions of the obtained animal model. This method is useful for comparing the conditions before and after the onset of T2DM.

2.2. Transgenic T2DM models

Recently, transgenic techniques are gaining momentum for investigation of function of specific gene and its mechanisms probably associated with disease conditions. The transgenic animal carrying transgene relevant to the role of genes on peripheral insulin action, insulin secretion and hepatic glucose production are developed. Furthermore, combination or double knockout mouse models have been produced which clearly show the mechanisms implicated in development of insulin resistance and β cell dysfunction resulting in overt hyperglycemic condition in human T2DM (Porte *et al.*, 2001). The transgenic murine models are used extensively for the mechanistic study in single gene or mutation on T2DM *in vivo*, but generation of transgenic models is highly sophisticated and costly procedure for the production and maintenance.

2.3. Regulation of glucose production

During fasting condition, gluconeogenesis occurring in the liver is the pivotal mechanism for supplying glucose to the organism from precursors such as lactate, gluconeogenic amino acids, and glycerol (Cherrington *et al.*, 1999; Nordlie *et al.*, 1999; Saltiel *et al.*, 2002). In contrast, when plasma glucose levels are high, gluconeogenesis is unnecessary and is consequently stopped. Integration of these events is complex and occurs through various hormonal and nutritional factors. The principle parameters affecting hepatic glucose output are concentrations of

available glucogenic substrates and activities of a few regulatory enzymes.

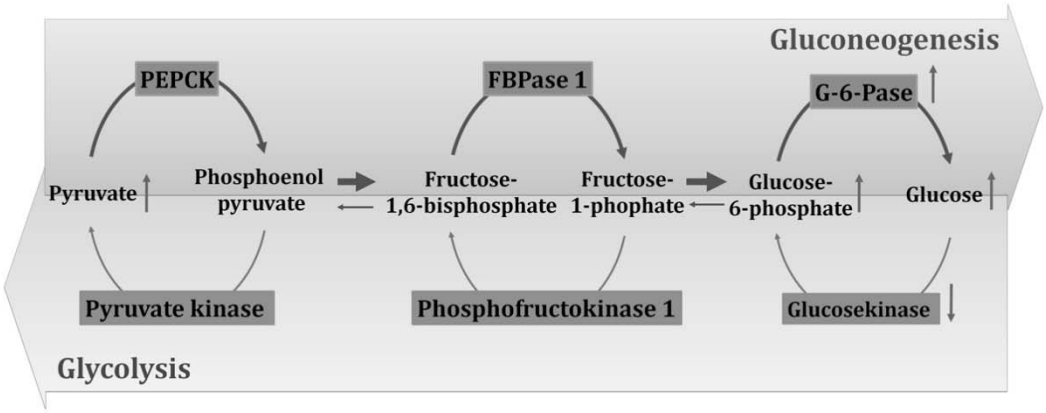

Figure 1. Schematic representation of the glycolytic and gluconeogenic pathways in the liver

Three major enzymes such as PEPCK, glucose-6-phosphatase, and FBPase are engaged in the production of glucose from precursors in the liver.

Insulin is the most important hormone that hampers gluconeogenesis; it acts by predominantly inhibiting the expression of genes encoding the key gluconeogenic enzymes PEPCK, FBPase 1 and G6Pase, as demonstrated in Figure 1. PEPCK catalyzes the rate-limiting step of gluconeogenesis, i.e., the conversion of pyruvate to phosphoenolpyruvate, while G6Pase catalyzes the conversion of glucose-6-phosphate to free glucose. During fasting condition, glucagon induces the expression of genes encoding G6Pase and PEPCK (Granner *et al*., 1983; Lange *et al*., 1994; Dickens *et al*., 1998; Scholl *et al*., 1999).

In the initial stages, insulin resistance characterizes T2DM, thereby leading to an increase in hepatic glucose levels. In patients with T2DM, the rate of hepatic gluconeogenesis is significantly increased, which significantly contributes to fasting hyperglycemia in diabetics, compared to that in control subjects. Therefore, knockout-mice lacking organ-specific insulin receptors in the liver show impaired glucose tolerance and increased hepatic glucose production with elevated G6Pase and PEPCK levels in the liver (Michael *et al.,* 2000).

2.4. Genes implicated in T2DM

T2DM is a heterogeneous diseases characterized by persistent hyperglycemia. The underlying pathogenesis of diseases remains unclear despite the recognition of atypical phenotype. A recent study has identified more than 36 genes that are associated with the risk factors of T2DM (Herder *et al.*, 2011). Genes implicated in the development of T2DM include PPARγ, ABCC8, and CALPN10 (calpain 10). PPARγ is well characterized because of its significance in adipocyte and lipid metabolism. PPARγ decreases insulin sensitivity, thereby increasing diabetic risk. ABCC8 encodes a high-affinity sulfonylurea receptor (SUR1) subunit that is a part of ATP-sensitive potassium channels, which control the release of insulin and glucagon from β-cells. Mutant forms of these genes can influence potassium channel activity and insulin secretion, ultimately resulting in

the development of T2DM. CALPN10 encodes a ubiquitous member of intracellular calcium-dependent cysteine protease.

3. AD model

3.1. Pathophysiology of AD

Impaired memory is one of the most prevalent cognitive features of elderly adults. A subset of these adults have severe cognitive impairment (dementia) defined as AD (Evans *et al.,* 1989). Disorders associated with cognitive senescence are generally recognized as health problems and are a major concern for the health-care system (Forbes *et al.,* 1995). Amyloid plaques and neurofibrillary tangles (NFTs) in the brain characterize AD. Amyloid plaques and NFTs result from the abnormal accumulation of amyloid β (Aβ) peptide and hyperphosphorylated tau protein, respectively, which leads to neuronal damage and neurotoxicity in the AD-affected brain (Alonso *et al.,* 1996). Amyloid plaques are usually formed in the neocortex, hippocampus, and amygdala of the brain. NFTs are formed in the subiculum; cornu ammonis 1 (CA1) region of the hippocampus; entorhinal cortex and neocortex; and several subcortical areas, including the amygdala, nucleus basalis of Meynert, ventral tegmental area, dorsal raphe, olfactory bulb, and some thalamic and hypothalamic nuclei (McKee *et al.,* 2009). Although the clinical features of AD are known, the mechanism underlying the pathogenesis of AD is yet to be elucidated.

Transgenic animal models of AD provide important insights on aging as a developmental process and on the underlying mechanisms of impairments in learning, memory, and cognitive functions.

The first transgenic model of AD was established by the overexpression of amyloid precursor protein (APP) in hopes to reproduce the amyloid precursor. Mutations in APP are implicated in familial AD.Aβ, a degradation product of APP, accumulates in senile plaques and synaptic terminals of the AD brain (Hardy *et al.,* 2002). Several researchers have suggested that overexpression of human APP or fragments of APP cloned using neuron-specific promoters may show an amyloid pathology that resembles the pathology observed in AD. To date, dozens of mouse models have been developed to study amyloid pathology due to the overexpression of APP variants.

The first transgenic AD model expressing wild-type human tau protein was produced in 1995.This model showed hyperphosphorylation and somatodendritic localization of the tau protein without the formation of NFTs (Götz *et al.,* 1995). Overexpression of wild-type human tau isoforms in mice reflected only limited aspects of tau pathology in AD. To date, mice expressing human P301L mutant tau protein, which results in the formation of NFTs, have been widely used to study the pathophysiology of dementia.

3.2. History of canine AD research

Dog may naturally suffer cognitive defect with age that is recognized as numerous aspects of early AD. From the molecular aspects, there are extensive homology for the canine Aβ (100 % similarity), its precursor protein (APP, 98 %), and the enzymes for APP processing (92-100 %) with human counter parts (Johnstone *et al*., 1991). From pathogenetic aspects, in the brain of aged dog, β-amyloid (Aβ) deposits and amyloid angiopathy have been observed, as well as the extended of that deposition has been implicated with the decreased cognitive function (Head *et al*., 2002; Skoumalova *et al*., 2003; Pugliese *et al*., 2006; Rofina *et al*., 2006; Siwak-Tapp *et al*., 2008). In addition, neurodegeneration and oxidative damage have also been observed in aged dog populations (Borras *et al*., 1999; Colle *et al*., 2000; Rofina *et al*., 2006, Bernedo *et al*., 2009). Canine neurology with behavioral medicine is a well-developed field among veterinary sciences, and there are a number of longitudinally accessible records (Overall, 1997). To treatment the preclinical development of AD, almost effective treatment strategy has been applied to the dog, from cholinergic agonists to antioxidant and mitochondrial enzymatic cofactor (Milgram *et al.*, 2002; Araujo *et al.*, 2005; Landsberg, 2005; Siwak-Tapp *et al.*, 2005; Head, 2007). Therefore, these previous observation suggest that dogs are an suitable spontaneous model for studying the mechanisms of AD (Sarasa *et al*., 2009).

Dogs may naturally develop cognitive defects with age that are recognized as

an aspect of early AD. Molecular analysis indicates extensive homology of canine Aβ, its precursor protein (APP), and enzymes involved in APP processing with their human counterparts (100 %, 98 %, and 92–100 % similarity, respectively) (Johnstone *et al.,* 1991). Pathogenetic analysis of the brains of aged dogs has shown Aβ deposits and amyloid angiopathy. In addition, increased Aβ deposition leads to decreased cognitive function (Head *et al*., 2002; Skoumalova *et al*., 2003; Pugliese *et al*., 2006; Rofina *et al*., 2006; Siwak-Tapp *et al*., 2008). In addition, neurodegeneration and oxidative damage have been observed in the brains of aged dogs (Borras *et al*., 1999; Colle *et al*., 2000; Rofina *et al*., 2006). Canine neurology with behavioral medicine is a well-developed field among veterinary sciences, and numerous longitudinal records are available on the same (Overall, 1997). Several effective strategies from cholinergic agonists to antioxidant and mitochondrial enzymatic cofactors have been applied in dogs to prevent the preclinical development of AD (Milgram *et al*., 2002; Araujo *et al*., 2005; Landsberg, 2005; Siwak-Tapp *et al*., 2005; Head, 2007). Thus, these previous observations suggest that dogs are a suitable spontaneous model for studying the mechanisms underlying AD (Sarasa *et al.,* 2009).

3.3. Characteristics of AD in dogs

Extensive research on the canine model of AD has been conducted using beagle dogs. However, companion dogs may be more beneficial because these

dogs share their living space with humans and are exposed to similar environmental stress throughout their life as they age (Cotman *et al.*, 2008). In elderly pet dogs, cognitive dysfunction syndrome is characterized by behavioral and cognitive deficits. It is assessed using the following four main behavioral categories (Colle *et al*., 2000; Pugliese *et al*., 2006):

1) Disorientation (on daily walks or at home)
2) Interaction with owners (lost of interest in greeting or playing lost of response to incitation)
3) Sleep/awake cycle (wander and/or bark during night)
4) Failure to remember trained habits

A survey of owners indicated that almost 30 % dogs aged ≥ 10 years showed impairments in more than one of these categories, with 10 % of these dogs showing impairments in more than two categories (these dogs were considered as having severe cognitive impairment). Among dogs aged > 15 years, almost 68 % showed impairments in more than one category, with 35 % of these dogs showing impairments in more than two categories (Bain *et al.,* 2001; Neilson *et al.*, 2001).

3.4. Amyloid plaques formation in brain

Two main pathways have been recognized in AD. The two major proteins APP and microtubule-associated tau protein have been implicated in the

formation of the two characteristic lesions—extracellular plaques (Glenner *et al.*, 1983) and intracellular NFTs (Goedert *et al.*, 1992). These amyloid plaques and NFTs are the major causes of AD that alter neuronal morphology, which eventually leads to neuronal death (Gómez-Isla *et al*., 1997; Buée *et al*., 2000; Ballatore *et al*., 2007; Meyer-Luehmann *et al*., 2008).

A schematic diagram of APP processing in the amyloidogenic pathway is presented in Figure 2. Amyloid plaques are formed by the proteolysis of APP as follows. First, the ectodomain of APP is released by the activities of either α- or β-secretase (Vingtdeux *et al.*, 2007). In the non-amyloidogenic pathway, the α-secretase cleaves APP into a soluble APP fragment α (sAPPα, a soluble N-terminal fragment) and a C-terminal fragment α (CTFα) containing 83 amino acids, resulting in the formation of the precursor of Aβ peptide. The β-secretase cleaves APP into a soluble fragment sAPPβ and a CTF β (a membrane-bound CTF) containing 99 amino acids. Subsequently, γ-secretase cleaves all the CTFs (CTFα and CTFβ) in the juxtamembrane region. Cleavage of APP ectodomain is mandatory for the intramembrane γ-secretase proteolysis of APPCTFs. Cleavage by γ-secretase at ε site releases APP intracellular domain (AICD) from both CTFα and CTFβ. The cleavage of CTFβ by γ-secretase produces the AICD and the soluble Aβ. Plaques are aggregations of a protein called amyloid, which is an insoluble fibrous protein aggregate, organized in β-sheet strands, and deposited in the outer part of the brain (De Strooper, 2010; Jucker *et al.*, 2011). It accumulate steadily into microscopic deposits via a multistep mechanism in

which Aβ peptides aggregate and fold into oligomers with a regular β-sheet structure, leading to the formation of fibrillar aggregates.

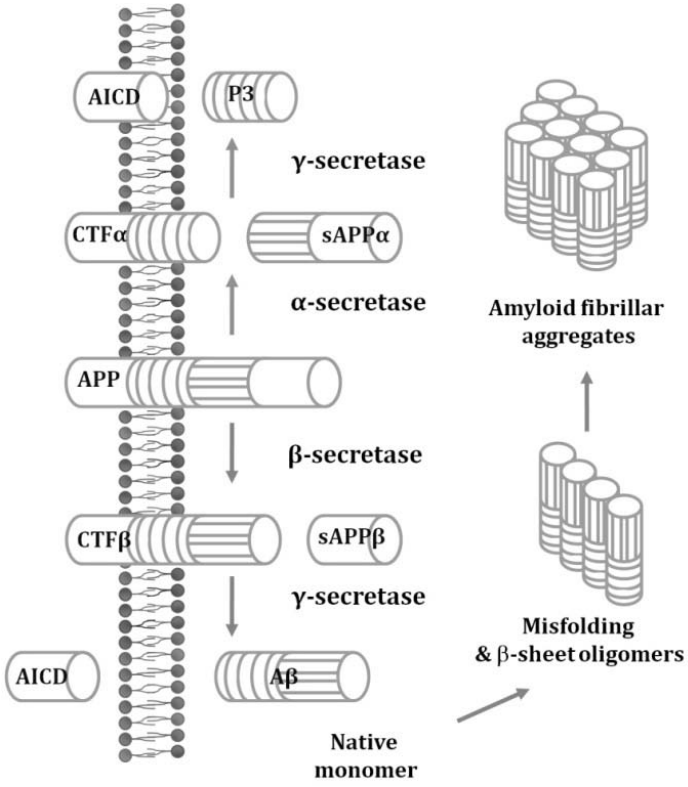

Figure 2. Amyloidogenic pathways of APP

Formation of fibrillar amyloid plaques due to the excessive cleavage of APP by secretase enzymes that results in the production of Aβ peptide.

Amyloid plaque formation is the hallmark of AD. However, mouse models of AD available at present do not form amyloid plaques in their brains in the same manner as those in the brains of human patients with AD. Furthermore,

significant brain cell death, which is observed in the brains of human patients with AD, is not observed in the brains of the current mouse models.

3.5. Genes implicated in AD

The apolipoprotein (APOE) gene has been considered as a predisposing factor for late-onset AD (Saunders *et al.,* 1993). Vascular risk factors such as hypertension and hypercholesterolemia are believed to facilitate the production of Aβ, which is aggravated by the inheritance of the E4 allele of the APOE gene. Therefore, this allele influence on increases the risk of developing both vascular dysfunction and, consequently resulting in AD (Cacabelos *et al.*, 2003).

In addition to APP, mutant forms of PSEN1 (encoding presenilin 1) or PSEN2 (encoding presenilin 2) have been implicated in the pathogenesis of early-onset AD (Tanzi *et al*., 2005). Presenilin1 is a part of the γ-secretase complex that plays a pivotal role in the proteolysis of APP (Li *et al*., 2000). Mutant PSEN1encodes an abnormal presenilin1 that hampers the function the γ-secretase complex, thus altering the processing of APP and leading to the overproduction of the Aβ peptide. Presenilin2 cleaves the APP into soluble (sAPP). Mutation in the PSEN2 increases the processing of APP, leading to the overproduction of Aβ peptide.

APOE is the predisposing factor for late-onset AD (Saunders *et al.*, 1993). Vascular risk factors such as hypertension and hypercholesterolemia are believed to facilitate the production of Aβ peptide, which is aggravated by the inheritance of the E4 allele of APOE. Thus, the presence of this allele increases the risk of

both vascular dysfunction and consequently AD (Cacabelos *et al.*, 2003).

3.6. Functional validation of AD symptoms

Assessment of spatial learning and memory function is well characterized in mouse models of AD (Figure 3). Typical methods such as Morris water maze, Y-maze, radial arm maze, and novel object recognition test are generally used to evaluate memory and hippocampal functions. The Morris water maze is widely used to measure spatial learning and cognitive function. The mouse is placed in a large circular pool of water and is made to find invisible cues that allow it to escape the water (Sharma *et al.*, 2010). The Y-maze or T-maze provides spatial working memory. One of the two paths of the maze is closed, and the mouse made to explore the other two paths for minutes. The closed path is then opened, and the mouse is allowed to explore the maze. The radial arm maze measures short-term spatial memory in mice (Crusio *et al.*, 1987). During training, memory is measured by placing food pellets in each arm. The mouse is made to explore each arm only once to successfully complete the maze. The novel object recognition task involves an open arena with two different objects. During habitation, mice are allowed to explore the arena and are exposed to two identical objects placed at an equal distance. On the next day, the mice are allowed to explore the arena with one familiar object and one novel object to measure long-term recognition memory.

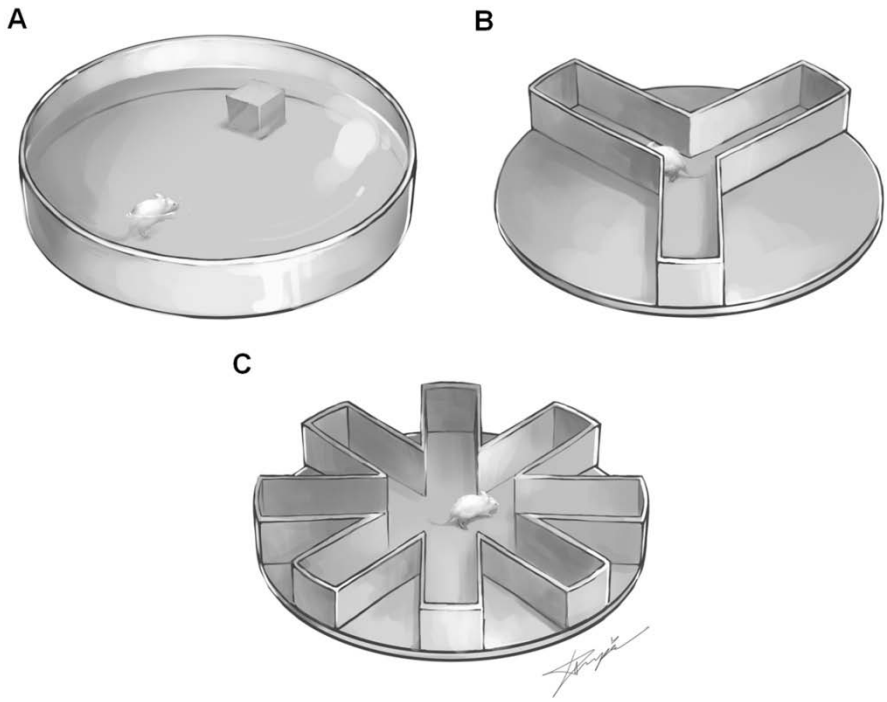

Figure 3. A representative diagram of the maze tests for evaluating memory function in mice

A. The Morris water maze
B. The Y-maze
C. The radial arm maze

However, evaluation of spatial learning and memory function in canine models of AD is not well established. Over the last 20 years, interest in canine cognition has tremendously increased in order to understand interspecies

communication in dogs (Bensky, 2013). However, limited effort has been put in organizing and summarizing a more comprehensive protocol to study AD in dogs. Therefore, a useful protocol to evaluate memory function in dogs is needed.

4. Nuclear transfer in dogs

4.1. Genetic advantages of dogs

Throughout the history, a domestic dog has been perceived as man's friend (Savolainen *et al*., 2002; Lindblad-Toh *et al*., 2005; Morey, 2006). Domestic dogs have evolved through a mutually beneficial relationship with humans by sharing living space and food resources. Therefore, several diseases are common between both canine and human populations, and the clinical manifestations of these diseases are often similar (Lindblad-Toh *et al.*, 2005). Kirkness *et al*. (2003) reported that diseases and dysfunctions associated with more than 400 different genes are common between dogs and humans. This indicates that canines are extremely important animal research models for studying human diseases. However, many of the similarities with human disorders are restricted to a particular breed or a group of breeds, with underlying genetic variations in loci affecting phenotypic traits.

4.2. Reproductive parameter were influence by body conformation

The domestic dog (*Canis familiaris*) shows the most remarkable morphological variations among mammalian species. Dogs vary in size from toy dog breeds to giant dog breeds, with variant conformation. Behavioral traits and physiological differences are also substantial between these breeds (Hart, 1995). Over decades, intensive artificial selection has influenced the shape, color, and behavior of dogs (Clutton-Brock, 1999). In particular, morphological variations between breeds may influence male and female reproductive traits. Female reproductive parameters such as litter size, and gestation period also differ in dogs with variant body conformation largely due to physiological limitations. Furthermore, the inception of puberty is associated with the mature weight. The mean age at puberty inception is between 6 and 10 months for female dogs of many smaller breeds but 2 years for female dogs of some larger breeds (Johnston *et al.,* 2001). In male dogs, body weight may potentially influence sperm production because it is strongly associated with gonad weight (Woodall *et al*., 1988; Ortega-Pacheco, 2006).

4.3. Canine cloning

Despite, in canine, insufficient results from *in vitro* maturation and culture system and limitation of application of ART, cloned puppy have been reported using *in vivo*-derived oocyte. Innovative technique for ovum flushed-out from

oviduct allows the generation of cloned dogs to provide an essential source to dog cloning (Lee *et al*., 2005). A number of recent studies using *in vivo* oocyte collecting protocols have been reported for successful generation of cloned small-breed (Jang *et al*., 2008), medium-sized (Hossein *et al*., 2009), and large-breed dogs (Kim *et al*., 2009), as well as production of one cloned dog from somatic cells that had been cryopreserved for a long period of time (Hossein *et al*., 2009). Furthermore, coyote nuclei successfully were reprogrammed to full-term pregnancy into dog oocyte, which demonstrated alternative ways of rescuing the endangered canine species (Hwang *et al.*, 2013). Recently, coupling with gene manipulation techniques, transgenic dogs were produced by nuclear transfer (NT) of genetically manipulated somatic cells (Hong *et al.*, 2009).

4.4. Bottlenecks of canine ART

Oocyte is a basic material for ART and developmental biology research, of which may ensure the developmental competence and further fertility for *in vitro* embryo production such as IVF and SCNT (Edwards, 1965; Schroeder *et al*., 1984; Galli *et al*., 1991; Schramm *et al*., 1996). In general, reproductive tissues from the carcasses at a slaughterhouse and application of superovulation are a useful source for sufficient number of oocyte in domestic animals. Peculiar canine reproductive physiology hampers the application of artificial reproductive technique (ART) well established from other domestic animals (Reynaud *et al*.,

2004; Luvoni *et al*., 2005; Reynaud *et al*., 2009). Although a number of attempts to improved meiotic competence of canine oocyte have been conducted by mimic cultivation of the *in vivo* follicular and oviductal biochemical conditions (Hewitt *et al*., 1998), several cultures media (Rota *et al*., 2004) and supplementations such as hormones (Hewitt *et al*., 1999; Hatoya *et al*., 2009), proteins (Rodrigues *et al*., 2003), energy substrates (Songsasen *et al*., 2002; Sturmey *et al*., 2009), antioxidants (Hossein *et al*., 2007) and other compounds, these efficiency is still very limited (Luvoni *et al*., 2003).

Despite the aforementioned achievements in canine cloning and its genetic significance for human disease, the exact chronology of preimplantation canine embryo is not elusive due to peculiarity of canine reproduction. At the ovulation, oocytes in the first meiotic prophase at GV stage are expelled from their follicle into the ovarian bursa, becomes competent to support normal development (Reynaud *et al*., 2005). Long incubation periods, prior to *in vitro* culture, presumably affect embryo competence. Moreover, after maturation *in vitro*, embryos are particularly vulnerable to suboptimal *in vitro* conditions.

It was clear that timing of oocyte retaining maturational competence after ovulation was heterogeneous in this species, since MII oocytes were observed starting from 54 h up to 130 h in reference to ovulation (Reynaud *et al*., 2005). Therefore, initiation of cleavage following fertilization with sperm varies between the embryos of the same cohort. In addition, the previous studies on embryonic developmental timing were conducted on an imprecise onset of the

estrus or the acceptance of the male. This difficulty to determination of ovulation may be implicated in long lasts LH peak for 24-72 h (De Gier *et al*., 2006), leading to the asynchrony of follicle ovulation (Boyd *et al*., 1992).

4.5. Canine oocyte maturation

Although a number of studies were conducted to determine exact time to nuclear maturation of canine oocyte *in vivo* and *in vitro,* the incubation period to meiotic competence are still controversial and unsatisfactory. Canine oocytes require protracted time to complete nuclear maturation *in vivo* and *in vitro* (48-72 h post-ovulation) compared to other mammalian species (Concannon *et al*., 1989). Lately, it has been shown that oocytes collected *in vivo* vary over a wide range from the germinal vesicle (GV) stage to early preimplantation stage with staining DNA/tubulin and confocal microscopy of oocytes collected *in vivo*. GV stage was observed up to 44 h postovulation. metaphase I (MI) stage is observed around 45 h after postovulation and the first metaphase II (MII) stage occurs from 50 and 130 h after ovulation. Preimplantation embryo up to 8-cell has been observed in oviduct with the innermost layer of corona radiata (Reynaud *et al*., 2005). *In vitro* culture period for canine oocyte require an extensive time and its subsequent maturation competence varies according the estrous cycle and reproductive status of donor in difference other species. Culture period of canine oocyte *in vitro* varies according the donor's estrous cycle phase or reproductive status. Oocyte collected from ovarian follicles start to proceed beyond

the MII stage by as early as 24 h of culture (Nickson *et al*., 1993; Yamada *et al*., 1993; Saint-Dizier *et al*., 2004). The biphasic culture system yield a higher proportion of oocytes resumed meiosis after 72 h, however oocyte degeneration significantly increased over prolonged periods of culture time (Luvoni *et al*., 2005).

4.6. Oviductal environment during maturation

The accumulated fluid in the oviductal lumen varies with the estrous cycle, which were affected by the relative contribution of transudation from the capillary bed with epithelial secretion. However, there have been no reports of oviductal fluid in canine due to technical and anatomical limitations associated with narrow and tubular structure. In other large animals, generally two different methods have been employed to collect oviductal luminal fluid. Dissected oviduct from the reproductive tract (salpingectomy) was flushed from the uterotubal junction to the ampulla by ascendant aspiration with a pipette (Hunter, 2012). The other method, collection from the accumulated luminal fluid with ligatures cranial to the ampulla was used (Carrasco *et al*., 2008). Previously, recent studies have demonstrated that follicular fluid steroid levels increases in accordance with the increases of serum steroid level and the follicular growth during preovulatory period using salpingectomy methods (Fahiminiya *et al*., 2010).

Ovarian steroid hormone directly affects the oviduct such as its micro-environmental structures and physiological composition of oviductal fluids (Hunter,

2012). In particular, hormonal changes resulting from preovulatory luteinization in canine might affect on the reception and maturation of gametes and thereafter for fertilization and early embryonic development. To mimic high progesterone *in vivo* milieu, results from progesterone supplementation on maturation medium are not sufficient explain of effects canine oocyte maturation due to the poor developmental competence, although it is not clear whether these hormones play a role *in vitro*. Nevertheless, the most commonly accepted physiological hormone that accelerates and induce MPF (maturation promoting factor) activation GVBD in lower vertebrates is progesterone (Hammes, 2004). As the acquisition of meiotic competence in canine was take place in the oviduct, further investigation were needed on the elucidation of molecular dynamics between the oviduct and the cumulus or, the oocyte.

5. Canine embryo development

5.1. Canine embryo development during preimplantation period

Basic information about reproductive physiology like gametogenesis, early embryonic development, implantation, pregnancy and parturition is helpful to improve ART. Canine artificial reproductive techniques lag behind compared to other species because a reliable protocol of providing oocyte from *in vitro* maturation and *in vivo* induction still not determined. Therefore, to date, all cloned offspring have been produced from *in-vivo* oocyte retrieved from bitches

showing natural cycling (Hossein *et al.*, 2009). Despite this notable achievement in canine ART, early developments of cloned canine embryos were still elusive.

5.2. *In vitro* development of canine embryos

Although numerous effort have been made to improve *in vitro* maturation and *in vitro* culture system in canid species, developmental competence of canine embryos during early stage still remain low. Of particular, using *in vitro*-matured oocyte, fertilization with fresh semen has shown poor developmental capability on subsequent *in vitro* cultivation. Factors affecting meiotic competence *in vitro* such as estrus cycle of oocyte donor, coculture of epithelial cell and, oxygen tension for culture may be responsible for this. Otoi *et al*. (2004) achieved that lesser than 40 % cleavage rate, of which *in vitro* matured with bovine oviductal epithelium and subsequent fertilized with fresh semen, lead to 1 morula for 8 days culture. Rodrigues *et al*. (2013) evaluated the developmental competence of fertilized embryos originated from matured oocyte in different estrus cycles, in which the percentage of oocytes cleaving into early stage embryos was below 10 %. However there were no difference among oocytes recovered from bitches at the follicular, anestrus, and luteal stages (Rodrigues *et al*., 2004). Cleavage rate of *in vitro*-matured following insemination was 7.7 % at an 5 % oxygen tension, whereas 5.4 % were morphologically normal at an oxygen tension of 20 % (Rodrigues *et al*., 2013). The poor fertilization and subsequent development of *in*

vitro-originated oocyte and embryos is due, in part, to prerequisite the meiotic capability and the reliable culture condition, which is not referral developmental ability to cloned canine embryos. Lately, *in vivo* development of canine embryos by surgical excision of reproductive tract after insemination has been revealed higher rates of cleavage rate and blastocyst rates than those reported for *in vitro* development. After insemination, approximately 90 % of embryo cleaved and more than 50 % of those developed to blastocyst when recovering at Day 12 (Luz *et al*., 2011; Commin *et al*., 2012).

5.3. *In vivo* development of canine embryos

Using *in vivo* matured oocyte collected from cycling dog and subsequent 7 days *in vitro* culture after embryo manipulation that the developmental competence of parthenotes and cloned embryos were limited to morula stage and their developmental rate also low (Jang *et al*., 2008).

In vitro culture system of canine embryo is still not capable of supporting development to the blastocyst stage despite using highly competent *in vivo* oocytes, which is impossible to determine the development. Reynaud and his colleagues (2005) observed the extended period of the chronology of embryo development, which was limited to the early preimplantation period up to 8 cell as well as, shown wide variation each developmental stage. Tsutsui *et al*. (2001) demonstrated that blastocyst was firstly observed at 9 days after ovulation and

since then it was observed up to 10 days after ovulation. In contrast, Commin *et al*. (2012) reported that the blastocysts were observed at protracted period based ovulation by 2 more days, showing the highest blastocyst formation at Day 12 postovulation.

5.4. Fragmentation of canine embryos

Fragmentation and unequal size of blastomere by karyorrhexis are observed in unarrested embryos produced both *in vivo* and *in vitro*, which has been responsible for the compromised developmental competence of embryos (Gjørret *et al*., 2003). These degree and patterns of cell fragmentation may affect increase the apoptotic incidence also decrease implantation rates, increase fetal absorption and, fetal viability (Gjørret *et al*., 2003). These nuclear changes has been observed more than half percentages of all *in vitro* produced blastocyst from mice (Handyside *et al*., 1986), human (Hardy *et al*., 1989), cattle (Byrne *et al*., 1999), and porcine. In domestic dog, lesser than 10 % of fragmented embryos were presented in recovered *in vivo* at the preimplantation period were observed (Tsutsui *et al.*, 2001; Head, 2007). Of particular, in *in vitro*-fertilized embryos using *in vitro*-matured oocyte lesser than 1 % of fragmented embryo were observed in cultured *in vitro* (Rodrigues *et al*., 2004). Early arrest resulting from insufficient maturation condition may explain lesser incidence of fragmentation *in vitro*.

5.5. Developmental arrest in canine embryos

The relatively high rate of development arrest was observed between 4- and 8-stage. During early preimplantation period, a precise and accurate interaction between nucleus and cytoplasm determines the success development into a complete and normal fetus. Coupling with this reprogramming, fundamental event implicated in the timely utilization of maternal inherited proteins and mRNA must occur. Activation of embryonic nuclei critically depends on the recipient cytoplasm including maternally inherited proteins and mRNAs (Minami *et al*., 2007). Developmental arrest in early embryo is due to improper reprogramming of nuclei as donor following micromanipulation process such as IVF, SCNT or intracytoplasmic sperm injection (ICSI) and subsequent culture on suboptimal *in-vitro* condition. The commencement of embryonic transcription at the maternal-embryonic transition varies with species, starting from 1- to 2-cell in mice (Bolton *et al*., 1984), the 8- to 16-cell stage in cattle (Barnes *et al*., 1991), the 4- to 8-cell stage in rhesus monkeys (Schramm *et al*., 1999) and the 4- cell stage in pig (Jeong *et al*., 2006). In canine embryos, embryonic genome activation seem to occurs by the 6- to 8-cell stage (Farstad, 2000). Using *in vivo* retrieved oocyte, when parthenogenetic embryo and SCNT embryos subsequently cultured and subsequent culture, eight cell arrest were observed (Jang *et al*., 2008), are consistent with developmental arrest in the present study, leading to 8-cell arrest occurs at the time of genome activation.

5.6. *In vivo* localization of canine embryo

In accordance previous reports, embryo migration from oviduct to uterus occurred between 10 and 11 days after ovulation (Head, 2007). Interestingly, all most of arrested embryos were remain oviduct until preimplantation period. Embryo that has the superior capability to develop that were transported toward the uterus faster than their inferior counterparts in the oviduct (Ortiz *et al*., 1986). The developing embryos and the cumulus matrix and cumulus cells affected tubal migration *in vivo* and *in vitro* (Wetscher *et al*., 2005; Kölle *et al*., 2009). The dissociation of cumulus cell chemically or mechanically from the oocyte is essential procedure to visualize nuclei for nuclear transfer. Moreover, the reconstructed embryos by nuclear transfer were lower developmental competence compare with their *in vivo* counterparts, which result in constraint migration of embryo into uterine cavity. All together, developmentally compromised embryo may lead to lack of signal to ciliary beating of the epithelial cells in oviduct.

6. Embryo collection

Embryo collection techniques have been developed in small ruminant, by which collection of *in-vivo* fertilized embryos has progressed to multiply and preserve the superior genetic stock (Ishwar *et al*., 1996). Surgical collection using balloon tip catheter from intact uterus is commonly applied due to the

higher yield rate rather than transcervical embryo collection and laparoscopic approach despite of several disadvantages such as those include the stress of anesthesia and surgery, postoperative adhesions limiting the number of possible interventions and high expense. Commin *et al*. (2012) have reported recovery of AI embryos from the reproductive tract *ex vivo* and overall recovery rate was 61.3%. Recovery of embryo by uterine flushing during laparotomy gives a poor recovery rate (42.3 %) compared to the excision method (97.1 %) (Tsutsui *et al*., 2001).

7. Genomic imprinting

In mammal, imprinted genes show parental-specific mono-allelic expression, and their proper expression is indispensible to normal development, fetal growth, nutrient metabolism and adult behavior. Most of the imprinted genes are observed in clusters in the genome from hundreds to thousands of kilo bases. Imprinted region constituting paternally and maternally expressed imprinted genes correspond to non-coding RNAs and non-imprinted genes (Thorvaldsen *et al*., 2007; Peters *et al*., 2008; Royo *et al*., 2008). The clusters also contain CpG-rich regions that are DNA-methylated only on one of the two parental chromosomes (differentially methylated regions, DMRs).

A number of studies suggest that the manipulation *in vitro* of embryos may impose inherent risks to normal development which is associated with aberrant

epigenetic reprogramming in preimplantation stage embryos, resulting in specific phenotypic abnormalities during fetal and postnatal development (Niemann *et al*., 2008). These pathphysiological characteristics are collectively referred to as the large offspring syndrome (LOS). Affected animals exhibit various phenotypes, including large BW, placental abnormality and histological defects in most organs (Hill *et al*., 1999; Barnes, 2000; Chavatte-Palmer *et al*., 2000; De Sousa *et al*., 2001; Hammer *et al*., 2001; Renard *et al*., 2002). Improper reprogramming of imprinting gene during early developmental stage is recognized as main cause of LOS (Younga *et al*., 2000; Humpherys *et al*., 2001; Chung *et al*., 2003).

Most imprinted genes comprise growth factors such as IGF or as regulators of gene expression controlling growth (Eggermann *et al*., 2008). Paternal imprinting genes generally involved in enhance growth. On the other hand, maternal imprinting genes associated with suppress growth. Disorders implicated with imprinting gene occur by either genetic and epigenetic mutations or defects including disruption of DNA methylation within the imprinting controlling regions of these genes. Imprinting gene play a crucial part in development and its regulation with hypomethylation or hypermethylation of CpG island can lead to certain defined disease states such as IGF2 in Beckwith-Wiedemann syndrome and the GRB10 gene in Silver-Russell syndrome (Maher *et al.*, 2003).

However, evaluation of genetic quality and morphological parameter in dog derived from nuclear transfer were not well documented. Previous results revealed that the myostatin is one of the crucial gene contribute to developmental

abnormalities in cloned dog and its reduction might be related with the muscle hypertrophy in the tongue, forelimb, and the viscera (Hong *et al.*, 2011). Therefore, further in-depth study of pertinent nuclear reprogramming in the early embryos can improve cloning efficiency in dog cloning and reduce incidence of abnormalities in newborn.

Research purpose

Undoubtedly, dogs provide extremely valuable resources for studying the genetic disease and for developing animal models that reflect human diseases because of their pathophysiological similarities with human. To date, spontaneous or experimentally induced diseases in dogs have been studied extensively. Therefore, transgenic dog models that express the major genetic risk factors for human diseases pave the way for developing therapeutic opportunities and studying signal pathway.

The purpose of the first chapter of the study entitled “Optimization of procedures for cloning by somatic cell nuclear transfer in dog” was to assess whether the breeds providing the donor nuclei for nuclear transfer affects reproductive parameters in transferring reconstructed embryos from each breed into the surrogates. Moreover, the factors that may affect canine cloning efficiency such as the meiotic status of the recipient oocyte and the passages of fibroblast as nuclear donor have been investigated.

The objective of the latter chapters of the study entitled “Establishment of transgenic cloned dogs for human diseases via somatic cell nuclear transfer” and “Canine model of Alzheimer’s disease over-expressing a mutated human amyloid precursor” was to produce transgenic dogs carrying the exogenous genes implicated in a human genetic disease using the SCNT technique. PEPCK gene that participates in the induction of gluconeogenesis for T2DM models, and APP gene that contributes to the induction of amyloidogenesis for AD models were isolated and transfected into the canine fetal fibroblasts using the liposomal carrier. The

transfected fibroblasts as donor nuclei were injected into collected dog oocytes *in vivo*. After fusion and activation, subsequently reconstructed embryos were transferred into the spontaneous cycling surrogates. The presence and expression of the inserted gene were examined with PCR and RT-PCR. Furthermore, phenotypes associated with the integrated genes from the resultant transgenic dogs were studied.

Various factors such as the breed of nuclear donor, the maturational status of an oocyte, and the donor passages, that can affect the cloning efficiency were evaluated following embryo transfer into the surrogates. Furthermore, transgenic dogs overexpressing exogenous PEPCK or APP were successfully produced in order to establish the model dog of T2DM or AD. The present study was a leap forward in the canine model for developing therapeutic opportunities and studying signal pathway.

Production of transgenic dogs carrying human phosphoenolpyruvate carboxykinase and amyloid precursor protein gene by nuclear transfer

Chapter I.

Optimization of procedures for cloning by somatic cell nuclear transfer in dog

1. Introduction

Dogs have been raised for human purposes for over a millennia (Savolainen *et al*., 2002; Lindblad-Toh *et al*., 2005; Morey, 2006). Artificial breeding selection has been imposed on dogs and has lead to the evolution of modern domestic dogs (Akey *et al*., 2010). The diversity of their sizes ranges from toy breeds to large breeds (Wilcox *et al*., 1995). The body weight of an ultra-large sized dog at full maturity is ninety times heavier than those of toy breed dog (Wilcox *et al*., 1995). These variations in body size indicate that the differences in female reproductive characteristics would also be substantial for dogs. It is believed that there is a positive correlation between the size of a breed with their mean litter size (Robinson, 1973; Gill, 2001; Thomassen *et al*., 2006; Borge *et al*., 2011). Moreover, there also appears to be a correlation between energy expenditure and increased life expectancy (Speakman *et al*., 2003). It can be postulated that basal energy expenditure according to body composition might affect the viability of cells and their growth, thereby influencing the donor cell transferring and reprogramming into oocytes during nuclear transfer.

ART in domestic animals for the purposes of improving fertility has made impressive progress over the past decade, however basic research and the commercialization of reproductive biotechnologies in canids has lagged behind despite relatively early-on recognition of the importance of canine artificial insemination (Bartlett, 1962). The main reason for this slow progress in canine ART was the lack of commercial interest originating from fact that there are high

fertility rate in the wild (Thomassen *et al*., 2009). Until recently, there had been relatively limited interest in the conservation of wild canids. Although reconstructed interspecies embryos could successfully undergo normal development to term following embryo transfer into a surrogate dogs, interspecies cloning (Otoi *et al*., 2004; Commin *et al*., 2012; Rodrigues *et al*., 2013).

The consequences of *in vitro* experiments involving the maturation and culture of oocytes pose limitations for the application of ART in canines. Studying the exact mechanisms of acquisition of meiotic competence is still elusive due to the peculiarity of canine reproduction. In the ovulation stage, oocytes in the first meiotic prophase of the GV stage are expelled from their follicles into the ovarian bursa instead of the second meiotic prophase (Reynaud *et al*., 2005). It was clear that the timing of oocytes retaining maturational competence after ovulation was heterogeneous in canines, since MII oocytes were observed starting from 54 h up to 130 h after ovulation (Reynaud *et al*., 2005). The current ovum flushing technique that is performed at optimal time provides the oocyte with various stages of maturation. Therefore, the reconstructed embryos from oocytes with lower meiotic competence may affect their *in vivo* development after embryo transfer.

Primary cells are capable of undergoing a limited number of passages in cultures before becoming aged (Hayflick *et al*., 1961; Pignolo *et al*., 1992). Cell senescence is associated with the number of divisions a cell has undergone. The life span of a cell *in vitro* decreases as the age of the donor increases (Cristofalo

et al., 1998). Although, during nuclear transfer, somatic cells gain the ability to reprogram to reach a pluripotent state by fusing with oocytes, the viability of donor nuclei is considered to be directly connected to the developmental competence of reconstructed embryos and the further development to term. Kubota *et al*. (2000) demonstrated that the developmental competence of reconstructed embryos was similar for cells from both low-passage and high-passage. Conversely, Roh *et al*. (2000) reported that the reconstructed embryos originating from low-passage had higher developmental ability to blastocyst than those from high-passage. In particular, a long-term culture of primary cells under antibiotic selection has become an indispensable part of the production of transgenic animals.

There are no reliable culture systems for canine preimplantation embryos. Before the reconstructed embryo is transferred into the surrogate, it is hard to predict their developmental competence to full term pregnancy. The factors that determine the pregnancy output are evaluated in accordance with the genotypic factor such as breed size and the passage of donor nuclei, as well as cytoplasmic factors such as meiotic status of recipient oocytes.

2. Materials and methods

2.1. Animals

From January 2009 to December 2011, 52 pregnancies in 454 bitches with transferred cloned embryos were included in this study with adult somatic cells from: Tibetan mastiff (n=2), Great pyrenees (n=1), Golden retriever (n=2), Labrador retriever (n=2), Grey hound (n=1), German shepherd (n=2), Beagle (n=2), Jindo dog (n=2), Boston terrier (n=1), and Pomeranian (n=1). Donor breeds were categorized according to body weight at the time of biopsy as the following: small (≤9 kg), medium (>9 to 20 kg), large (>20 to 40 kg), and ultra-large (>40 kg).

The female mixed breed dogs for surrogates, between the ages of 1 and 7 (body weight 20-25 kg), were housed singly in an indoor kennel. To avoid biases from the maternal implication, all oocyte donors and surrogates were selected from the homogeneous population, showing normal estrus cycle and providing approximately 8-11 oocytes per each cycles (Hossein *et al.*, 2008). They were fed standard commercial dog food once a day, and given water *ad libitum* in accordance with the animal study guidelines of Sooam Biotech Research Foundation's Accreditation for Laboratory Animal Care.

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise stated.

2.3. Preparation of donor cells

Adult fibroblasts were obtained by biopsies after owners' consent. Tissue samples measured approximately 1 × 3 cm and were collected under light tranquilization by intravenous injection of 5 mg / kg tiletamine hydrochloride and zolazepam hydrochloride (Zoletil 50; Virbac Korea Co. Ltd., Seoul, Korea) and local anesthesia. Subcutaneous tissues were cut into small (approximately 1 mm^2) sections and were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Rockville, MD) containing 10 % fetal bovine serum (FBS; Life Technologies) at 37 °C in an atmosphere of 5 % CO_2 and air to obtain fibroblasts. Explants were maintained in the culture until they approached 90 % confluence. Cells were then trypsinized and reconstituted to obtain concentrations of approximately 1×10^6 cells per mL; lastly, the cells were cryopreserved in cryovials containing DMEM+10 % dimethyl sulfoxide (DMSO;Sigma).

2.4. Laparotomy and collection of oocytes

The estrous of bitches was followed weekly by showing vaginal bleeding to detect the onset of heat period, during which a blood sample (2 ml) was collected by cephalic venipuncture every day, and serum progesterone levels were determined by electro chemiluminescence immunoassay (ECLIA; Cobas e411, Roche Diagnostics, Mannheim, Germany). The oocyte retrieval and cloned embryos transfer were performed under general surgical procedures. Briefly, the oviducts of oocyte donor dogs were flushed upward with TCM 199 under surgical procedures.

2.5. Determination of meiotic stage

After retrieval, collected oocytes were stripped from cumulus cells by repeated pipetting in 0.1 % (w/v) hyaluronidase in TCM-199 supplemented with 10 % (w/v) FBS. The oocytes had been previously stained with 5 mg / mL bisbenzimide (Hoechst 33342) under an inverted microscope equipped with epifluorescence (TE2000-E; Nikon Corporation, Japan). Oocytes were classified into one of the following stage (Figure 4, 5a):

1) Immature or maturing oocyte (IM: cumulus very closely attached to oocytes, nuclear stage is metaphase I and telophase I),
2) Matured oocyte (MA: MII oocytes with several layers of cumulus cells and homogeneous cytoplasm)
3) Aged oocyte (PM: unidentified nuclear status, MII oocytes with less than

50 % of cytoplasm and loosely attached cumulus cells, oocytes with broken zona or broad perivitelline space).

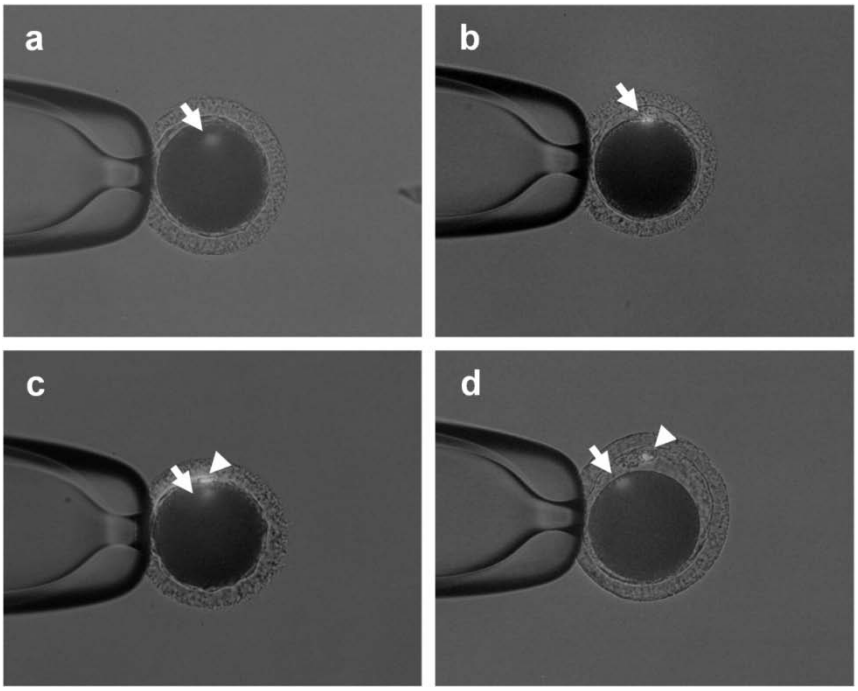

Figure 4. Representative pictures of maturational status of canine oocytes

a-d) Oocytes at different stages in the normal progression of meiotic maturation; arrow: nuclei; arrowhead: polar body

a. Immature or maturing oocyte (MI)

b. Maturing oocyte (TI)

c. Matured oocyte (MII)

d. Post-matured oocyte (aged)

2.6. Nuclear transfer

The oocytes were enucleated by squeezing out a tiny amount of cytoplasm including the first polar body and the nucleus using a glass pipette. The cells were transferred into the perivitelline space of enucleated oocytes (Figure 5b). Using a fine pipette, a trypsinized fibroblast was placed into the perivitelline space of an enucleated oocyte (Figure 5c). The couplets were equilibrated with 0.26 M mannitol solution containing 0.5 mM of HEPES, 0.1 mM of $CaCl_2$ and $MgSO_4$ for 4 min and then transferred to a chamber containing two electrodes overlaid with mannitol solution (Figure 6). The couplets were fused with two DC pulses of 1.75-1.85 kV / cm for 15 μs using a BTX Electro-Cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA) (Figure 7). Only the fused embryos were selected and equilibrated for 2 h in modified synthetic oviductal fluid (mSOF). The formula for mSOF was basically the same as the original formulation (Tervit *et al.*, 1972) except for the 1.5 mM concentration of glucose and the addition of 2 % MEM essential (Invitrogen, Carlsbad, CA) and 1 % nonessential amino acids (Invitrogen, Carlsbad, CA), 8 mg/ml of BSA and 1 % (v/v) mixture solution of insulin, transferrin and selenium (Invitrogen, Carlsbad, CA).

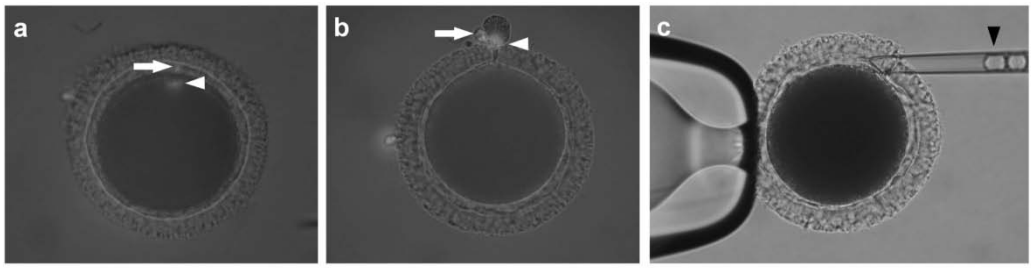

Figure 5. Canine nuclear transfer

a. Denuded oocyte collected from *in vivo*: stained MII chromosome (arrowhead) and the first polar body (arrow)

b. Enucleated oocyte image under light and fluorescence: stained MII chromosome located in blebs-like cytoplasm (arrowhead), the first polar body (arrow).

c. Injection of somatic cell (arrowhead) into the perivitelline space.

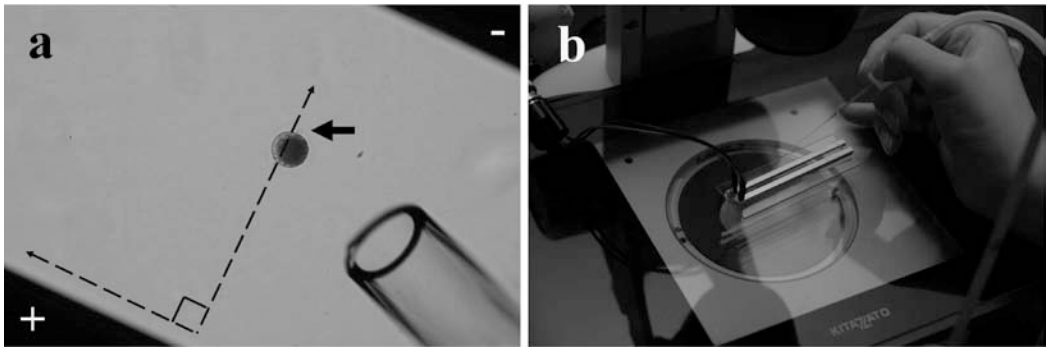

Figure 6. Electrical activation of canine oocyte

a. One oocyte injected with donor nuclei was aligned between the electrodes and the cleavage plane of the first polar body was oriented perpendicularly to the line between both electrodes.

b. Oocyte were identified by the presence of the first polar body under a stereomicroscope and delivered electrical pulse with a BTX Electro-cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA).

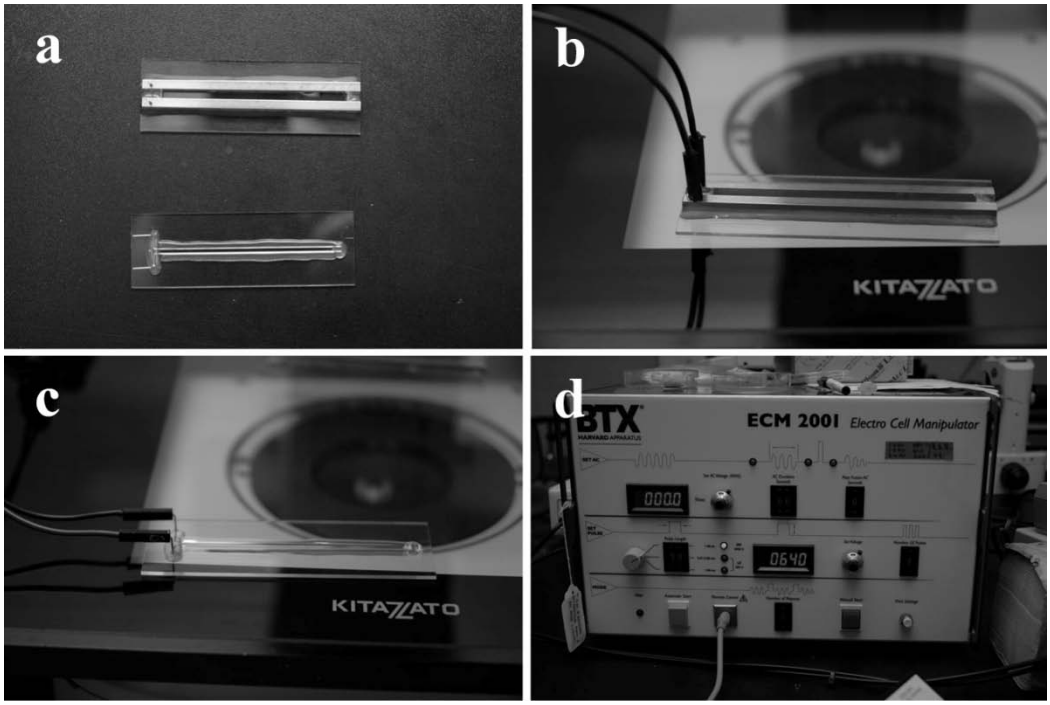

Figure 7. Instrument for electrical activation

a. 3.2-mm gap chamber (upper) and 10mm gap chamber (lower)

b. 10-mm gap chamber connected with the electrode leads

c. 3.2-mm gap chamber connected with the electrode leads

d. BTX Electro-cell Manipulator 2001 (BTX, Inc., San Diego, CA, USA)

2.7. Embryo transfer

Recipient animals were anesthetized as described earlier in oocyte retrieval and placed in ventral recumbency. The ovary containing more number of corpora luteum was approached by performing ventral laparotomy. The fat layer covering the ovary was gently grasped using forceps and suspended with a suture to exteriorize the fimbriated end of the oviduct. The embryos were loaded into a Tomcat catheter (Sovereign, Sherwood, USA), with minimum medium volume, and were gently transferred into the distal position of the oviduct without insufflating air. Pregnancy was confirmed by transabdominal ultrasonography on days 25–30 after embryo transfer.

2.8. Statistical analysis

Data are described as mean±SE. Statistical analysis was undertaken using SAS statistical software (SAS Corporation, Cary, NC, USA). To determine whether the breed size, the meiotic status of the recipient oocyte and the passages of donor nuclei can affect the pregnancy outcome, the cloning efficiency and litter size, a regression analysis (Proc GLM) was employed. A Duncan test was applied to evaluate the following value: the mean number of embryos transferred, the mean number of litter size, the mean gestation length, and the mean BW. In the study of the meiotic status of the recipient oocytes and the passage of donor nuclei on pregnancy outcome, data derived from transfer

into multiple surrogate mothers without distinction of its origin were eliminated. Statistical significance was set at $P<0.05$.

3. Results

3.1. Effects of the breed size of somatic cell donor on pregnancy outcome

The effects of the breed size of somatic cell donor on pregnancy outcome are summarized in Table 1. The numbers of recipient that received reconstructed embryos for ultra large-sized breed, large-sized breed, medium-sized breed, and small-sized breed was 115, 154, 100, and 85, respectively. The number of embryos transferred into ultra large-sized breed, large-sized breed, medium-sized breed, and small-sized breed were 1,641, 2,142, 1,579, and 1,122, respectively. The model effect of donor cells indicated as p-value were 0.001, 0.277, 0.215, 0.001 and 0.053 in the mean number of embryos transferred, the number of the recipients diagnosed as clinical pregnancy, delivered, the number of live pups delivered, and mean litter size, respectively. The clinical pregnancy rates on Day 30 of the embryo transfer were 20.9 %, 13.6 %, 14.0 %, and 12.9 % for the donor cells originated from ultra-large, large, medium and small breeds, respectively. Full term pregnancy rates were 16.5 %, 11.0 %, 10.0 % and 7.1 % for the donor cells originated from ultra-large, large, medium and small breeds, respectively. However, the differences were not significant in clinical pregnancies and full-term pregnancies among the donors originated from different sized breeds. The highest pregnancy performances in

accordance to donor cell origins were observed in the ultra-large group, and the lowest pregnancy performances were examined in the small groups. Litter size varied from 1 to 5 for different breeds. The mean litter size was 2.1, 1.2, 1.5 and 2.0 for ultra-large, large, medium and small breeds, respectively.

3.2. Effects of the breed size of somatic cell donor on pregnancy outcome in ultra large-sized breed

The effects of the breeds on pregnancy outcome in ultra large-sized dog are summarized in Table 2. A total of 1,336 and 305 reconstructed embryos were transferred into 94 and 31 recipients for Tibetan mastiff and Great pyrenees. The model effect of donor cells indicated as p-value were 0.553, 0.486, 0.039, 0.006 and 0.865 in the mean number of embryos transferred, the number of the recipients diagnosed as clinical pregnancy, delivered, the number of live pups delivered, and mean litter size, respectively. The clinical pregnancy rates on Day 30 of the embryo transfer were 20.2 % and 23.8 % and full term pregnancy rates were 18.1 % and 9.5 % for Tibetan mastiff and Great pyrenees. However, the differences were not significant in clinical pregnancies, full-term pregnancies, the cloning efficiency and litter size among the donors originated from different breeds in the group.

Table 1. Effects of the breed size of somatic cell donor on pregnancy outcome

Donor cells[a] collected from	No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
	Embryo recipients	Embryos transferred		Diagnosed as clinical pregnancy	Delivered		
UL	115	1,641	14.3±0.5[d]	24 (20.9)	19 (16.5)	39 (2.4)[d]	2.1±0.3[*]
L	154	2,142	13.9±0.3[d]	21 (13.6)	17 (11.0)	21 (1.0)[e]	1.2±0.1
M	100	1,579	15.8±0.5[e]	14 (14.0)	10 (10.0)	15 (0.9)[e]	1.5±0.2
S	85	1,122	13.2±0.5[d]	11 (12.9)	6 (7.1)	12 (1.1)[e]	2.0±0.4[*]

[a]UL: ultra-large sized breed of Tibetan mastiff and Great pyrenees; L: large-sized breed of Golden retriever, Labrador retriever, Grey hound and German shepherd; M: medium-sized breeds of Beagle and Jindo dog; S: small sized breeds of Boston terrier and Pomeranian were provided for somatic cell donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

[de]Different superscripts within the same column indicate significant difference, $P<0.05$.

[*]Different superscripts within the same column indicate significant difference, $P<0.06$.

Table 2. Effects of the breeds of somatic cell donor on pregnancy outcome in ultra large-sized breed

Donor cells[a] collected from		No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
		Embryo recipients	Embryos transferred		Diagnosed as clinical pregnancy	Delivered		
UL	TM	94	1,336	14.2±0.5	19 (20.2)	17 (18.1)	35 (2.6)	2.1±0.3
	GP	21	305	14.5±1.2	5 (23.8)	2 (9.5)	4 (1.3)	2.0±1.0

[a]UL: ultra large-sized breed; Ultra-large sized breed of Tibetan mastiff (TM) and Great pyrenees (GP) were provided for somatic cell donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

3.3. Effects of the breed size of somatic cell donor on pregnancy outcome in large-sized breed

The effects of the breeds on pregnancy outcome in large-sized dog are summarized in Table 3. A total of 343, 104, 190 and 1,505 reconstructed embryos were transferred into 23, 8, 11 and 112 recipients for Golden retriever, Labrador retriever, Grey hound and German shepherd, respectively. The model effect of donor cells indicated as p-value were 0.005, 0.485, 0.688, 0.624 and ND in the mean number of embryos transferred, the number of the recipients diagnosed as clinical pregnancy, delivered, the number of live pups delivered, and mean litter size, respectively. The clinical pregnancy rates on Day 30 of the embryo transfer were 17.4 %, 25.0 %, 18.2 %, and 11.6 % and full term pregnancy rates were 13.0 %, 12.5 %, 18.2 % and 9.8 % for Golden retriever, Labrador retriever, Grey hound and German shepherd in large-sized group, respectively. However, the differences were not significant in clinical pregnancies, full-term pregnancies, the cloning efficiency and litter size among the donors originated from different breeds in the group.

Table 3. Effects of the breeds of somatic cell donor on pregnancy outcome in large-sized breed

Donor cells[a] collected from		No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
		Embryo recipients	Embryos transferred		Diagnosed as clinical pregnancy	Delivered		
L	GR	23	343	14.9±1.0[de]	4 (17.4)	3 (13.0)	4 (1.2)	1.3±0.3
	LR	8	104	13.0±1.6[d]	2 (25.0)	1 (12.5)	1 (1.0)	1.0
	GH	11	190	17.3±1.6[e]	2 (18.2)	2 (18.2)	3 (1.6)	1.0±0.0
	GS	112	1,505	13.4±0.3[d]	13 (11.6)	11 (9.8)	14 (0.9)	1.3±0.1

[a]L: large-sized breed; large-sized breed of Golden retriever (GR), Labrador retriever (LR), Grey hound (GH) and German shepherd (GS) were provided for somatic cell donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

3.4. Effects of the breed size of somatic cell donor on pregnancy outcome in medium-sized breed

The effects of the breeds on pregnancy outcome in medium-sized dog are summarized in Table 4. A total of 664 and 915 reconstructed embryos were transferred into 36 and 64 recipients for Beagle and Jindo dog. The model effect of donor cells indicated as p-value were 0.122, 0.399, 0.958, 0.169 and 0.274 in the mean number of embryos transferred, the number of the recipients diagnosed as clinical pregnancy, delivered, the number of live pups delivered, and mean litter size, respectively. The clinical pregnancy rates on Day 30 of the embryo transfer were 11.1 % and 14.1 % and full term pregnancy rates were 11.1 % and 10.9 % for Beagle and Jindo dog. However, the differences were not significant in clinical pregnancies, full-term pregnancies, the cloning efficiency and litter size among the donors originated from different breeds in the group.

Table 4. Effects of the breeds of somatic cell donor on pregnancy outcome in medium-sized breed

Donor cells[a] collected from		No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
		Embryo recipients	Embryos transferred		Diagnosed as clinical pregnancy	Delivered		
M	BG	36	664	18.4±0.9[d]	4 (11.1)	4 (11.1)	5 (0.8)	1.3±0.3
	JD	64	915	14.3±0.5[e]	9 (14.1)	7 (10.9)	10 (1.1)	1.4±0.2

[a]M: Medium-sized breed; S: small-sized breed; medium-sized breeds of Beagle (BG) and Jindo dog (JD) were provided for somatic cell donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

[de]Different superscripts within the same column indicate significant difference, $P<0.05$.

3.5. Effects of the breed size of somatic cell donor on pregnancy outcome in medium-sized breed

The effects of the breeds on pregnancy outcome in medium-sized dog are summarized in Table 5. A total of 664 and 915 reconstructed embryos were transferred into 50 and 35 recipients for Boston terrier and Pomeranian. The model effect of donor cells indicated as p-value were 0.003, 0.041, 0.422, 0.164 and 0.164 in the mean number of embryos transferred, the number of the recipients diagnosed as clinical pregnancy, delivered, the number of live pups delivered, and mean litter size, respectively. The clinical pregnancy rates on Day 30 of the embryo transfer were 16.0 % and 8.6 % and full term pregnancy rates were 8.0 % and 5.7 % for Boston terrier and Pomeranian. However, the differences were not significant in clinical pregnancies, full-term pregnancies, the cloning efficiency and litter size among the donors originated from different breeds in the group.

Table 5. Effects of the breeds of somatic cell donor on pregnancy outcome in small-sized breed

Donor cells[a] collected from		No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
		Embryo recipients	Embryos transferred		Diagnosed as clinical pregnancy	Delivered		
S	BT	50	637	12.7±0.5	8 (16.0)	4 (8.0)	8 (1.3)	2.0±0.7
	PM	35	485	13.9±0.9	3 (8.6)	2 (5.7)	4 (0.8)	2.0±1.0

[a]S: small sized breeds of Boston terrier (BT) and Pomeranian (PM) were provided for somatic cell donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

3.6. Effects of donor cell on pregnancy maintenance of cloned puppies

The pregnancy maintenance in different breeds are summarized in Table 6. The model effect of donor cells indicated as p-value were 0.657, 0.586 and 0.586 in the mean number of the recipients aborted, and the number of the recipients detected early abortion prenatal mortality and stillbirth, respectively. There were no significant differences for abortion rates among different donor cell lines. The highest abortion rate was observed in the small breed and the lowest was examined in the large breed. The abortion rates at the first trimester according to the number of the recipients aborted were 60.0 %, 25.0 %, 25.0 % and 20.0 % for the donor cells originated from ultra-large, large, medium and small breeds, respectively. The abortion rates during second trimester and later 40.0 %, 75.0 %, 75.0 % and 80.0 % for the donor cells originated from ultra-large, large, medium and small breeds, respectively.

Table 6. Effect of donor cell on pregnancy maintenance of cloned puppies

Size of donor cell breeds[a]	No. (%)[b] of		No. (%)[c] of aborted recipients	
	Embryo recipients	Recipients aborted	At the first trimester	Later than the second trimester
UL	115	5 (4.3)	3 (60.0)	2 (40.0)
L	154	4 (2.6)	1 (25.0)	3 (75.0)
M	100	4 (4.0)	1 (25.0)	3 (75.0)
S	85	5 (5.9)	1 (20.0)	4 (80.0)

[a]UL: ultra-large sized breed of Tibetan mastiff and Great pyrenees; L: large-sized breed of Golden retriever, Labrador retriever, Grey hound and German shepherd; M: medium-sized breeds of Beagle and Jindo dog; S: small sized breeds of Boston terrier and Pomeranian were provided for somatic cell donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of the recipients aborted

3.7. Comparison of neonatal parameters in different breeds

The mean gestation period, litter size and body weight of cloned puppies at birth are presented in Figure 8. The model effect of donor cells indicated as p-value was both 0.0001 in the gestation length and BW. The gestation period was 59.8±0.1 d, 60.7±0.3 d, 60.3±0.3 d, and 58.8±0.3 d for ultra-large, large, medium and small breeds, respectively. A significantly shorter gestation period was observed in the small breed compared to others. The mean body weights at birth were 575.4±18.8 g, 551.4±28.1 g, 412.7±15.3 g, and 209.2±10.5 g for ultra-large, large, medium and small breeds, respectively.

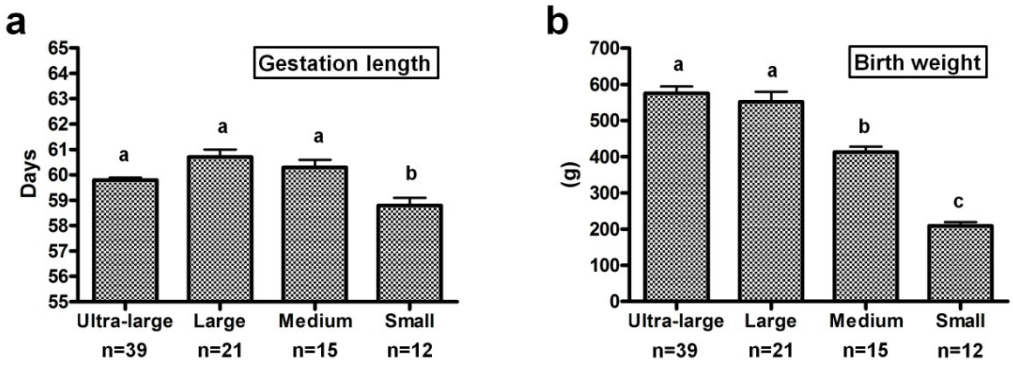

Figure 8. Effect of donor cell on gestation length and litter size

[abc]Superscripts in different rows in the same column differ significantly ($P<0.05$).

3.8. Effects of the meiotic status of recipient oocytes

To observe whether the meiotic status of recipient oocyte for nuclear transfer could influence *in vivo* development, the clinical pregnancy, the full term pregnancy and the cloning efficiency were examined following embryo transfer (Table 7). The model effect of donor cells indicated as p-value were was 0.0001, 0.313, 0.545, 0.074 and 0.495 in the mean number of embryos transferred, number of the recipients diagnosed as clinical pregnancy and delivered, number of live pups delivered, and the number of live pups delivered and mean litter size, respectively. The clinical pregnancy rate (Day 30) of gestation was 0.0 %, 17.8 %, 8.6 %, 11.4 %, 17.4 %, 0.0 % and 0.0 % for IM, MA, PM, IM+MA, MA+PA, IM+PM and IM+MA+PA group. The full term pregnancy rate of gestation was 0.0 %, 14.0 %, 5.7 %, 9.7 %, 11.6 %, 0.0 % and 0.0 % for IM, MA, PM, IM+MA, MA+PA, IM+PM and IM+MA+PA group. There was no significant difference in the pregnancy outcomes among groups. However, the highest clinical pregnancy rate and the full term pregnancy rate were observed in MA group. On the contrary, no pregnancy outcome data were observed in groups including immature oocyte, i.e. IM, IM+PM, IM+MA+PA group.

Table 7. Effects of the meiotic status of recipient oocytes subjected SCNT on pregnancy outcome

Meiotic status[a]			No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
			Embryo recipients	Embryos transferred[†]		Diagnosed as clinical pregnancy	Delivered		
IM			6	71	11.8±1.7[e]	0	0	0	0
	MA		157	2,127	13.5±0.4[e]	28 (17.8)	22 (14.0)	37 (1.7)	1.3±0.1
		PM	35	402	11.5±0.7[e]	3 (8.6)	2 (5.7)	3 (0.7)	1.5±0.5
IM	MA		44	687	15.6±0.6[ef]	5 (11.4)	4 (9.1)	5 (0.7)	1.0±0.0
	MA	PM	86	1,399	16.3±0.5[e]	15 (17.4)	10 (11.6)	15 (1.1)	1.5±0.2
IM		PM	4	48	12.0±1.7[e]	0	0	0	0
IM	MA	PM	13	227	17.5±1.2[f]	0	0	0	0

[a]Meiotic status of oocyte subjected to nuclear transfer, IM=immature or maturing (PI-TI); MA=mature (MII); PM=post-maturation (aged).

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

[†]Data derived from transfer into multiple surrogate mothers without distinction of its origin were eliminated.

3.9. Effects of the passages of donor nuclei

To determine the optimal passage of the donor nuclei for nuclear transfer, the clinical pregnancy, the full term pregnancy and the cloning efficiency were examined following embryo transfer (Table 8). The model effect of donor cells indicated as p-value were 0.589, 0.613, 0.859, 0.942 and 0.727 in the mean number of embryos transferred, number of the recipients diagnosed as clinical pregnancy and delivered, number of live pups delivered, and the number of live pups delivered and mean litter size, respectively. The clinical pregnancy rate (Day 30) of gestation was 15.0 %, 14.6 %, 18.0 % and 11.3 % for below passages 3, passages 4, passages 5 and more than passages 6 group. The full term pregnancy rate of gestation was 10.6 %, 10.8 %, 14.3 % and 8.5 % for below passages 3, passages 4, passages 5 and more than passages 6 group. There was no significant difference in the pregnancy outcomes among the groups. However, relatively higher clinical pregnancy rate and the full term pregnancy were observed in passages 5 group.

Table 8. Effects of the passages of donor nuclei on pregnancy outcome

Passage of nuclear donor[a]	No. of		No. of embryos transferred (Mean±SE)	No. (%)[b] of the recipients		No. (%)[c] of live pups delivered	Litter size (Mean±SE)
	Embryo recipients	Embryos transferred[†]		Diagnosed as clinical pregnancy	Delivered		
<3	113	1,555	13.8±0.4	17 (15.0)	12 (10.6)	15 (1.0)	1.4±0.2
4	130	1,887	14.5±0.4	19 (14.6)	14 (10.8)	18 (1.0)	1.4±0.2
5	133	1,925	14.4±0.5	24 (18.0)	19 (14.3)	21 (1.1)	1.2±0.1
>6	71	1,037	14.4±0.6	8 (11.3)	6 (8.5)	9 (0.9)	1.5±0.2

[a]Passage of nuclear donor.

[b]Percentage of the number of embryo recipients.

[c]Percentage of the number of embryos transferred.

[†]Data derived from transfer into multiple surrogate mothers without distinction of its origin were eliminated.

4. Discussion

Numerous factors including nucleation methods (Vajta *et al*., 2001; Oback *et al*., 2003), activation and fusion condition (Wells *et al*., 1997; Galli *et al*., 2002; Akagi *et al*., 2003), *in vitro* culture conditions (Kubota *et al*., 2000; Ogura *et al*., 2000; Powell *et al*., 2004) and gene modifications (Cibelli *et al*., 1998; Arat *et al*., 2002), cell origin (Kato *et al*., 2000), degree of differentiation (Heyman *et al*., 2002; Hochedlinger *et al*., 2003) and donor age (Hill *et al*., 2000) have effects on the efficiency of the production of viable cloned offspring. In this study, somatic cells from 10 different breeds were employed as the nuclear donor cell and *in vivo*-collected oocytes were used as recipient cytoplasts for nuclear transfer. The resulting pregnancy output was statistically analysed in accordance with the meiotic status of the recipient oocyte and the passages of donor nuclear.

Since the first report of a viable pup using SCNT in 2004, various breeds of dog have been successfully produced such as the Beagle (Hossein *et al*., 2009), Afghan hound (Jang *et al*., 2007), Sapsaree (Jang *et al*., 2009), Labrador retriever (Oh *et al*., 2009), Toy poodle (Jang *et al*., 2008), Pekingese (Park *et al*., 2011) and Golden retriever (Kim *et al*., 2009). Only a small percentage of the reconstructed embryos transferred into surrogates developed into a full term pregnancy. When the first cloned puppy was reported, Lee *et al*., (2005) the cloning efficiency (the number of puppies born per the number of embryos

transferred) was around 0.2 %. A number of studies have been conducted to improve the cloning efficiency into areas such as the optimal fusion and activation conditions (Kim *et al*., 2009; Oh *et al*., 2009), the hormonal condition of surrogates (Hossein *et al*., 2008), the optimal number of transferred embryos (Hossein *et al*., 2009) and the cell types of the donor (Oh *et al*., 2011). Although significant advances have been made in the production of a range of diverse breeds of cloned dogs, efficiency is still low below 5 %. This result demonstrated that the genotype of dog breed can affect the donor nuclei influencing the cloning efficiency. The highest cloning efficiency was observed in the ultra-large breeds; however there were no differences within the group.

The gestation length in mammals is calculated based on sexual intercourse during the fertile period such as the onset of LH peak. Oocytes that ovulated from the full-grown follicle shortly after LH surge should be arrested at the second meiotic stage and are capable of being fertilized with sperm. However, it is known that the LH surge in dogs occurs 2 days before ovulation, and ovulated oocytes required 2-5 days for the completion to maturation following ovulation (Luvoni *et al*., 2001). Therefore, the gestation length in bitches ranges widely from 57 to 72 days according to the breed and litter size (Holst *et al*., 1974; Okkens *et al*., 1993; Okkens *et al*., 2001). In this study, the donor genotype affects its gestation length when the reconstructed embryos were transfer into

surrogates. Furthermore, except for the small breeds, all other breeds showed consistency of gestation length at approximately 60 days from the day of embryo implantation. This may be explained by transferring of activated embryos via electrical and chemical means.

The BWs in dogs vary by breed, ranging from 100 to 200 g for toy breeds, to 250 to 350 g for medium breeds, 400 to 500 g for large breeds and up to 700 g for giant breeds (Peterson *et al*., 2010). In this study, genetic background affected the cloned pups' weights at birth. However, decreased average BW was observed in the ultra-large group and increased BW was observed in the other breeds compared to the reference range. Lower BW in the ultra-large group may be explained by the relatively limited uterine capacity of physical surrogates to their litter. On the other hand, slightly increased BW in other groups may result from imprinting gene alterations throughout the cultivation period.

One of the prerequisites for mammalian reproduction is an effective reciprocal interaction between the conceptus and the receptive uterus (Paria *et al*., 2001). In normal pregnancies, initiation and maintenance of pregnancy following fertilization requires sufficient signals from the fetus and the extraembryonic membrane (Spencer *et al*., 2004). In the case of SCNT, the aberrant reprogramming of cloned embryos induces insufficient maternal recognition of pregnancy and abnormal placentation following transfer to recipients (Palmieri *et*

al., 2008). As consequence of the aberrant placentation, high incidences of pregnancy loss usually occur in somatic cell cloning (Spencer *et al*., 2004). In previous studies, the abortion rate in canine cloning was shown to be relatively low compared to other domestic animals due to the use of *in-vivo* matured oocyte for nuclear transfer (Hossein *et al*., 2009; Kim *et al*., 2009). In the present study, pregnancy loss during early and late gestation was observed in all groups, but there were no significant difference between the breeds. Further study is required to investigate whether abortion can be attributed to placentation in canine cloning.

As a source of recipient oocytes for the nuclear transfer, the competence of meiotic maturation is one of the most significant factors in supporting implantation and subsequent pregnancy following *in vitro* manipulation. MII-arrested oocytes that are not fertilized within the period for optimal fertilization undergo an aging process with deterioration in quality (Miao *et al*., 2009). Fertilizations with aged oocytes have compromised developmental potential arising from lower implantation rates, polyspermy, aneuploid, a reduction of MPF and MAPK (mitogen-activated protein kinases) (Kikuchi *et al*., 2002) and the perinatal complications including a shortened gestation period (Tarín *et al*., 2000), lower litter size and higher incidence of fetal mortality (Tarín *et al*., 2002). However, in humans ART, the fertilization of aged oocytes with sperm by IVF

results in a full-term pregnancy, which indicates that the aged oocytes could support a full-term birth without any loss of developmental potential (Lei *et al*., 2008).

On the contrary, immature oocytes obtained from infertility clinics were used for research purposes instead of being subjected to compatible cytoplasts for the ART techniques due to their limited abilities to develop into blastocysts and a subsequent pregnancy (Heindryckx *et al*., 2007). Studies for domestic animals have demonstrated that a GV-matured oocyte is less capable of supporting the preimplantation development compared to their *in vivo* counterparts, which can be explained by the asynchronization of the nuclear and the cytoplasmic maturation (Wells *et al*., 1997), as well as the high incidence abnormalities in meiotic spindles, chromosome, and nucleus (Lanzendorf, 2006). Consistent with the previous studies, the reconstructed embryos from immature oocytes used as recipient cytoplasts had neither attached nor implanted, on the other hand, those from meiotic aged had maintained a full term pregnancy.

The passages of donor cells as donor nuclei for nuclear transfer are important factors in determining the developmental competence of the preimplantation embryos. Over consecutive passages, cell viability was decreased with the wide extent of morphological changes and cellular changes (Sabin *et al*., 2011). Therefore, the cloning efficiency can be affected by the donor cells for the

cultures progressing towards senescence. During the preimplantation period, the reconstructed embryos derived from the donor cells that had less than 16 passages showed increased developmental competence compared to those with more than 17 passages (Roh *et al*., 2000). In bovines, long-term cultured cells used as donor cells (up to 15 passages) can be reprogrammed to support a full term pregnancy without compromising cloning competence (Kubota *et al*., 2000). The effects of donor cell passages on the cloning efficiency in dog cloning were investigated. No significant differences were observed in the cell passages. Cell passages employed in the present study were categorized as the early passages in comparison to the previous studies. Therefore, cells from passages higher than 16 should be used as the donor cells in order to investigate the effects of donor cell passages on cloning efficiency.

The purpose of the this study was to investigate the factors that can affect the pregnancy outcomes in canine cloning including the genotypes of donor cell, the maturational stage of recipient oocyte and the degrees of cell senescence of donors. In conclusion, the genotypes of donor cell influence the cloning efficiency, whereas the meiotic maturation of recipient oocytes and the passages of donor nuclei do not.

Chapter II.

Canine disease model of human type 2 diabetes mellitus by over-expressing phosphoenolpyruvate carboxykinase

1. Introduction

Since the development of SCNT and the production of a cloned sheep in 1997 (Wilmut *et al.*, 1997), a number of scientist have successfully somatic cells from several species to produce cloned offspring using similar method (Cibelli *et al*., 1998; Wakayama *et al*., 1998; Baguisi *et al*., 1999; Polejaeva *et al*., 2000; Chesne *et al*., 2002; Zhou *et al*., 2003; Lee *et al*., 2005; Li *et al*., 2006). Although the canine reproductive technique have not been properly developed, the technique of cloned dogs has dramatically advanced since the first dog was cloned (Lee *et al.*, 2005). A number of recent studies reported successful generation of cloned small-breed (Jang *et al.*, 2008), medium-sized (Hossein *et al.*, 2009), and large-breed dogs (Kim *et al.*, 2009), as well as production of one cloned dog from somatic cells that had been cryopreserved for a long period of time using *in vivo* oocyte collecting protocols (Hossein *et al.*, 2009).

The gene modification technique in mice is well characterized when compared with other species and these mice have been widely used as a model of human diseases (Capecchi, 2005). Gene manipulation of germ line and treatment of specific drug often cause diseases that closely resemble the human disorder with respect to phenotype and underlying pathological mechanism (Palmiter *et al*., 1986; Stacey *et al*., 1988; Gordon, 1989). However, the mouse models could not fully reflect the human disease process. Thus, alternative animals are needed

to come over the limitation of rodent models. One of the best animals is dogs which provide valuable models of human disease that can be used to validate of gene level approaches which are enhanced by a specific understanding of the genome with different dog breeds (Karlsson *et al.,* 2008). In addition, dogs have similar characteristics of cohabitation with human, comparable organ size and receiving exceptional medical care comparing small laboratory animals (Ostrander *et al.,* 2000). With these advantages, combining techniques for dog cloning and genetic manipulation, will pave the way for gene function studies involving the disruption or introduction of specific genes, targeting of genes, and the molecular characterization of human hereditary disease in mammalian species.

In the present study, transgenic dogs overexpressing PEPCK were produced, which is a key gluconeogenic enzyme in the liver and kidneys with an established role in the metabolism of intermediates (Hanson *et al.,* 1997). PEPCK-overexpressing mice successfully shares the key characteristics with those of human T2DM (Valera *et al.,* 1994). Based on the previous mouse models, firstly, the promoter activities of canine PEPCK were assessed and targeting vectors to produce the disease model of T2DM were generated. In addition, the nuclear source of SCNT to increase the cloning efficiency of transgenic dogs was optimized. Finally, the PEPCK overexpression in the transgenic dogs was characterized.

2. Materials and methods

2.1. Care of animals

Female mixed breed dogs (age, 1−7 years; weight, 20–25 kg) were housed singly in an indoor kennel. They were fed a standard commercial dog diet once a day and were given water *ad libitum* in accordance with the animal study guidelines of the Sooam Biotech Research Foundation for Accreditation for Laboratory Animal Care.

2.2. Establishment and culturing

Unless otherwise indicated, all cells were grown at 37 °C, all cell culture materials were obtained from Invitrogen, and all other chemicals were obtained from Sigma-Aldrich (Louis, MO, USA). Adult fibroblasts were isolated from the abdominal skin of a 2-year-old beagle dog. Normal or cloned fetal fibroblasts were isolated from a fetus on Day 25 of gestation after mating or embryo transfer. Fetuses were retrieved aseptically by laparotomy. The isolated tissues were minced in a culture dish (35 or 100 mm; Becton Dickinson, Lincoln Park, NJ) by using a surgical blade and were dissociated in DMEM supplemented with 0.1 % (w/v) trypsin/1mM EDTA for 1–2 h. Trypsinized cells were subsequently cultured for 6–8 days in DMEM supplemented with 10 % (v/v) FBS, 1mM

sodium pyruvate, 1 % (v/v) NEAAs, and 10 μg/mL penicillin/streptomycin solution. When the cells reached complete confluence, they were trypsinized and frozen in DMEM supplemented with 40 % FBS and 10 % DMSO.

2.3. Genomic DNA extraction and PCR

Genomic DNA was isolated from the umbilical cells of transgenic and cloned puppies at the birth by using G-DEX™ II Genomic DNA Extraction Kit (iNtRON Biotechnology, Inc., Suwon, Korea). For the polymerase chain reaction (PCR), 1 μg of genomic DNA was amplified in 20 μL reaction mixture containing 1UEx-Taq polymerase (TaKaRa, Otsu, Shiga, Japan), 2 mM dNTPs (TaKaRa), and 10 pmol specific primers. The details of all the primers are described in Table 9. The PCR conditions were as follows: Denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 or 3 min. Next 8 μL of the PCR products were fractionated by electrophoresis on 0.7 % agarose gel, stained with ethidium bromide, and visualized under UV illumination. The films were scanned and analyzed using Quantity One Program (Gel Doc EQ, Bio-Rad).

Table 9. Primer list

Name	Restriction enzyme	Direction	Sequences (5'→3')
Dog PEPCK promoter (-3,180)	NheI	Forward	GCT AGC CAT GGC TTC CTT TCC ACT
Dog PEPCK promoter (-2,349)	NheI	Forward	GCT AGC GAC AGT TGC AAA GGA ATA A
Dog PEPCK promoter (-1,018)	NheI	Forward	GCT AGCAAG GGC ATT GAG AAG TGT
Dog PEPCK promoter (-746)	NheI	Forward	GCT AGC TCC TAT AGG CCT TGG CTG
Dog PEPCK promoter (+1)	NcoI	Reverse	CCA TGG CCA AGG CTT CCT GAA ACA
PEPCK cDNA	Nco I	Forward	CCA TGG CGA GGT CAT CCC AAA ACA AG
PEPCK cDNA	Xba I	Reverse	TCT AGA GGG TCT GAT CAC ATC TGG CT
EGFP cDNA	EcoR V	Forward	GAT ATC CAC AAC CAT GGT GAG CAA GGG CGA
EGFP cDNA	BamH I	Reverse	GGA TCC TTA CTT GTA CAG CTC GTC CAT GCC
Confirming primer a		Forward	CAT GAA GCA GCA CGA CTT CT
Confirming primer b		Reverse	CCT AGG AAT GCT CGT CAA GA
Confirming primer c		Forward	TCC TAT AGG CCT TGG CTG
Confirming primer d		Reverse	GGG TCT GAT CAC ATC TGG CT
PEPCK		Forward	GAC ATC GCC TGG ATG AAG TT
PEPCK		Reverse	CCA GTG CCT GGT CAA TAC CT
GAPDH		Forward	AGA ACA TCA TCC CTG CTT C
GAPDH		Reverse	TTG AAG TCA CAG GAG ACC AC

2.4. Establishment of fetal fibroblast cell line

Fetal fibroblasts were obtained from a fetus on Day 25 after natural breeding. Fetuses were retrieved aseptically by laparotomy. A piece of the fetal skin tissue was cut into small pieces, and these small explants were cultured in 35-mm tissue culture plates. The minced tissues were dissociated in DMEM supplemented with 0.1 % (w/v) trypsin/1mM EDTA (Invitrogen) for 1–2 h. Trypsinized cells were washed once by centrifugation at 300 × g for 5 min and seeded onto 100-mm plastic culture dishes. Subsequently, cells were cultured for 6–8 days in DMEM supplemented with 10 % (v/v) FBS (Invitrogen), 1mM sodium pyruvate, 1 % (v/v) NEAAs, and 10 μg / mL penicillin/streptomycin solution at 39 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. When the cells reached confluence, they were collected by trypsinization and frozen in DMEM supplemented with 40 % FBS and 10 % DMSO.

2.5. Genetic transformation of cells

The selection cassette construct contains CMV, EGFP, and Neo and the PEPCK expression cassette construct -0.7 kb of the PEPCK promoter, a cDNA encoding PEPCK and an SV40 poly (A) signal. The PEPCK gene overexpression construct was linearized through MluI digestion. A linearized fragment was purified and transfected into normal fetal fibroblast derived using a lipofectamin TM 2000 (Invitrogen, Co.) according to the manufacturer’s instructions.

2.6. Promoter study

Unless otherwise indicated, all restriction enzymes were obtained from TaKaRa. Canine PEPCK promoter (~3,180 kb nucleotides (nt) +1 nt [transcriptional start site]) was prepared by PCR amplification of a genomic DNA template (obtained from beagle fibroblasts) containing an NheI site at the 5' end and an NcoI site at the 3' end. The amplified fragments were digested using the specified restriction enzymes and ligated to promoter-less pGL3-Basic vector (Promega Co., Madison, WI, USA). The vector was transiently transfected using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. The Rous sarcoma virus (RSV)-lacZ plasmid was co-transfected into hepatoma cell line (H4IIE; ATCC, Manassas, VA, USA) along with the indicated PEPCK promoter-conjugated luciferase construct as a control for different transfection efficiencies of various luciferase constructs. Briefly, 3×10^5 cells were seeded into 6-well tissue culture plates one day before transfection. Next, 4 μg of the luciferase constructs and 0.5 μg of RSV-lacZ plasmid were co-transfected into the cells under serum-free conditions. At 4h after transfection, the media was replaced with treatment media containing 1 mM dexamethasone, and the cells were incubated for additional 48 h. Cellular lysates were prepared using 150 μL of reporter lysis buffer (Promega) and assayed for luciferase activity by using the luciferase assay system (Promega). Luminescence was measured using the Glo Max 20 / 20 Luminometer (Promega), and β-galactosidase activity was measured using β-galactosidase enzyme assay system (Promega). Relative promoter activity was calculated as luciferase normalized by β-galactosidase (%).

2.7. Targeting vector construction

PEPCK cDNA was prepared by PCR by using cDNA obtained from the liver of a beagle dog as a template. Amplified fragments were digested by NcoI and XbaI and ligated into the recombinant pGL3_PEPCK promoter (–746 nt to +1 nt) plasmid to replace the luciferase gene. The selection cassette plasmid was made using several steps. The enhanced green fluorescence protein (EGFP) was amplified by PCR by using pIRES2_EGFP (BD Biosciences Clontech, USA) as the template. The fragments were digested using EcoRV and BamHI and ligated into the pIRES-Neo vector (BD Biosciences). The selection cassette was amplified by PCR by using the recombinant pIRES_EGFP plasmid containing the neomycin resistant gene (Neo^r). The selection cassette fragments were digested using MluI and NheI and ligated into the recombinant pGL3_PEPCK promoter_PCPEK cDNA plasmid. The sequences of targeting vectors were confirmed by nucleotide sequencing (Genotech Co. Ltd., Daejeon, Korea).

2.8. Gene targeting

The structure of the targeting vector is shown in Figure 9. The selection cassette construct contains cytomegalovirus (CMV) promoter, EGFP, and Neo^r. The expression cassette consists of the dog PEPCK promoter region (–746 nt to +1 nt), dog PEPCK cDNA, and a simian virus (SV) 40 poly-A tail signal. The

targeting vector was linearized by MluI digestion and transfected into normal fetal fibroblasts by lipofectamin TM 2000 (Invitrogen).

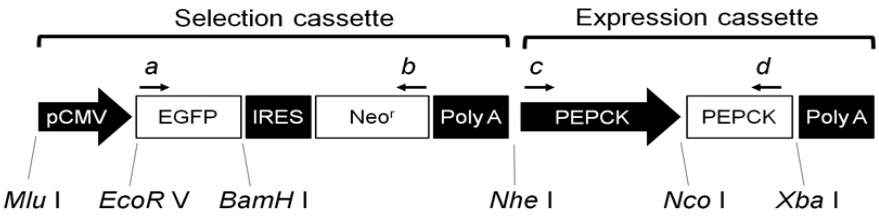

Figure 9. Schematic representation of the targeting vector

The expression cassettes contained dog PEPCK cDNA controlled by the PEPCK promoter (–746 to +1 nt). Selection cassettes consisted of enhanced green fluorescent protein (EGFP) cDNA and neomycin resistance gene (Neo^r). pCMV, cytomegalovirus promoter; IRES, internal ribosome entry site; poly A, poly A tail signal sequences. a to d indicates the locations and direction of primers used for genotyping.

2.9. Establishment of transgenic fibroblasts

Normal fetal fibroblasts (2×10^5) at passage 2 were seeded in a 35-mm culture plate 1 day before transfection. Cells were transfected with 4 μg of linearized targeting vector and then cultured under a medium containing the high dosage of antibiotics (600 μg / ml of G418, Sigma) for 20 days. The concentration of antibiotics was further reduced to 300 μg/ml for the additional

days until the formation of single colonies. The neomycin resistant colonies were picked up using cloning discs and re-seeded on 24-well plates to expand the cell number and further analysis. Following several passages, transgenic cells screened by EGFP expression and the chromosomal integration of targeting vector was further confirmed by PCR based genotyping. The colonies were expanded and frozen (6×10^5 cells per aliquot) prior to their subsequent use in SCNT.

2.10. Canine cloning

The estrous cycle of the female dogs was followed weekly by observing vulval bleeding to detect the onset of the heat period. During the heat period, a blood sample (2mL) was daily collected at the same time by perfor g cephalic venipuncture, and serum progesterone levels were assayed by Cobas E411 (Roche Diagnostics, Indianapolis, IN, USA). Oocytes were retrieved, and cloned embryos were transferred by performing general surgical procedures as described in a previous report (Hossein *et al.,* 2009). Briefly, the oviducts of oocyte donor dogs were flushed upward with TCM 199 by performing a surgical procedure. The oocytes were denuded and washed for nuclear transfer. The oocytes were then fixed with 5 μg / mL bisbenzimide (Hoechst 33342) and were mounted. The oocytes showing a nucleus and an extruded first polar body were counted and used in the routine nuclear transfer procedure.

After retrieval, MII oocytes were stripped from cumulus cells and enucleated by squeezing out the first polar body and MII plate into a small amount of surrounding cytoplasm with a glass pipette. The oocytes were previously stained with 5 μg / mL bisbenzimide. The cells were transferred into the perivitelline space of enucleated oocytes. A fine pipette was used to transfer a trypsinized fetal fibroblast with smooth cell surface into the perivitelline space of an enucleated oocyte. The obtained couplets were equilibrated with 0.26 M mannitol solution containing 0.5 mM HEPES and 0.1 mM $CaCl_2$ and $MgSO_4$ for 4 min and were then transferred to a chamber containing two electrodes overlaid with mannitol solution (Sigma). The couplets were fused using two DC pulses of 1.9–2.2 kV / cm for 30 μs by using the BTX Electro-Cell Manipulator 2001. Only the fused embryos were selected and cultured for 2 h in mSOF. The formulation of the mSOF was basically the same as the original formulation (Hossein *et al.*, 2009), except for the presence of 1.5 mM glucose and additions of 2 % MEM essential amino acids; 1 % NEAAs; 8 mg / mL BSA; and 1 % (v/v) mixture solution of insulin, transferring, and selenium.

2.11. Embryo transfer

Recipients were anesthetized as described earlier in oocyte retrieval and placed in ventral recumbency. The ovary containing more number of corpora lutea was approached by performing a ventral laparotomy. The fat layer covering

the ovary was gently grasped using forceps and was suspended with a suture to exteriorize the fimbriated end of the oviduct. The embryos were loaded into a Tom Cat catheter, with at least a medium volume, and were gently transferred into the distal two-third of the oviduct without insufflating air. Surrogate mothers were checked for pregnancy by transabdominal ultrasonography at days 25–30 after embryo transfer.

2.12. Microsatellite analysis

Parentage analysis was performed on puppies born via SCNT and on surrogate mothers to confirm the identity of the donor cells used for nuclear transfer. Genomic DNA was extracted from each newborn puppy, recipients, and donor cells. DNA microsatellite markers (PEZ1, PEZ 3, PEZ 5, PEZ 6, PEZ 8, PEZ 12, PEZ 20, FHC 2010, FHC 2054, and FHC 2079) were used to confirm the genetic identity of the cloned puppies, fetus, and fibroblast donor cells.

2.13. Quantitative real-time PCR

Total RNA was extracted from liver biopsy tissues by using Trizol reagent (Invitrogen) according to the methods outlined in the protocol. The concentration of total RNA was determined by measuring the absorbance at 260 nm. First-strand cDNA was prepared by subjecting 1 μg of total RNA to reverse

transcription by using M-MLV reverse transcriptase (Invitrogen) and random primers (9-mers; TaKaRa). Quantitative real-time PCR (RT-PCR) was performed by adding 1 μL of cDNA template to 10 μL mixture of 2 × SYBR Premix Ex Taq (TaKaRa) and 10 pmol each of specific primers. PCR was performed for 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s. Fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all the samples was set manually. The reaction cycle at which the fluorescence intensity of PCR products exceeded this fluorescence intensity threshold in the exponential phase of PCR was considered as the threshold cycle (Ct).

Expression of the target gene was quantified relative to that of an internal control gene (GAPDH) based on a comparison of Cts at constant fluorescence intensity. The amount of transcript present was inversely related to the observed Ct. For every two-fold dilution in the amount of transcript, the Ct was expected to increase by 1. Relative expression (R) was calculated using the equation $R=2^{-[\Delta CT\ sample-\Delta CT\ control]}$. To determine a normalized arbitrary value for each gene, every data point was normalized to the control gene (GAPDH) as well as to its respective control.

2.14. Western blot analysis

Liver biopsy tissues were harvested, washed two times with ice cold PBS,

and resuspended in 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (0.1mM phenylmethylsulfonyl fluoride, 5μg/mL aprotinin, 5μg/mL pepstatin A, and 1μg/mL chymostatin) and phosphatase inhibitors (5 mM Na_3VO_4 and 5 mM NaF). All the inhibitors were obtained from Sigma. Whole-cell lysate was prepared using 20 strokes of a Dounce homogenizer, followed by centrifugation at 13,000 × g for 20 min at 4 °C. Protein concentration was determined using BCA assay (Sigma). Proteins (50 μg) were separated by electrophoresis on a 12 % sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and were then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were probed using antibodies against PEPCK (dilution 1:1000; Cayman Chemical, USA) or β-actin (dilution 1:4000; Santa Cruz Biotechnology, CA, USA.). Horseradish peroxidase-conjugated anti-rabbit IgG (dilution1 : 2,000; Santa Cruz Biotechnology) was used to detect the signals. Results were visualized by exposing the treated membrane to a radiographic film. Band intensities were quantified using Gel Doc EQ system.

2.15. Statistical analysis

Data analysis was conducted using SPSS for windows (version 15, SPSS Inc., Chicago, Illinois) and graph was prepared with GraphPad Prism (version 4.0) software. For statistical evaluation, the data from the pregnancy rate were compared using the ANOVA and the results from western blot were compared

using the t-test with post hoc analysis $P<0.05$ was considered significant.

3. Results

3.1. Canine PEPCK promoter activity and targeting vector construction

To determine the promoter region of the canine PEPCK gene which would generate the maximum activity for tissue-specific expression, a series of promoter variants containing different deletions of the upstream human insulin promoter region from –3,180 nt to +1 nt using beagle genomic DNA were generated. The fragments were linked to the luciferase gene in a promoter-less pGL3-Basic vector. These PEPCK promoter constructs were finally introduced into a hepatoma cell line, H4IIE, the relative promoter activities were measured in the presence of dexamethasone. pGL3-2Kb (–2,349 nt to +1 nt) and pGL3-0.7Kb (–746 nt to +1 nt) induced 110- and 130- fold increases in luciferase activity, respectively. However, less increase in luciferase activity was observed in pGL3-3Kb (–3.180 nt to +1 nt) and pGL3-1Kb (–1.018nt to +1nt). Thus, two maximum promoter activity regions, pGL3-2Kb and pGL3-0.7Kb has the strongest luciferase activity.

It has been further investigated whether these selected promoters contribute to PEPCK transcription and translation. PEPCK cDNA was replaced with Luc genes of pGL3-2Kb and pGL3-0.7Kb to generate pGL3-2Kb-PEPCK and pGL3-0.7Kb-PEPCK, and introduced into H4IIE cells. The protein levels of PEPCK expression were higher in pGL3-0.7Kb-PEPCK than pGL3-2Kb-PEPCK empty

vector as a negative control with/without dexamethasone. Thus, pGL3-0.7Kb-PEPCK was selected as an expression cassette for the targeting vector containing dual selection marker genes, EGFP and Neo^r.

3.2. Establishment of transgenic fetal fibroblasts expressing PEPCK

Normal fetal fibroblasts were selected nuclear donor cells and introduced the targeting vector. After screening with antibiotics, the positive clones were confirmed with PCR based genotyping. The primers, a and b, presented the integration of selection cassette (Figure 10A). The chromosomal insertion of the expression cassette was confirmed by the primer c and d (Figure 10B). Endogenous PEPCK gene generated 5.2 Kb band containing intron regions whereas the inserted expression cassette produced 2.7 Kb. The finally confirmed cells expressing EGFP and resistant against neomycin were subjected to nuclear transfer.

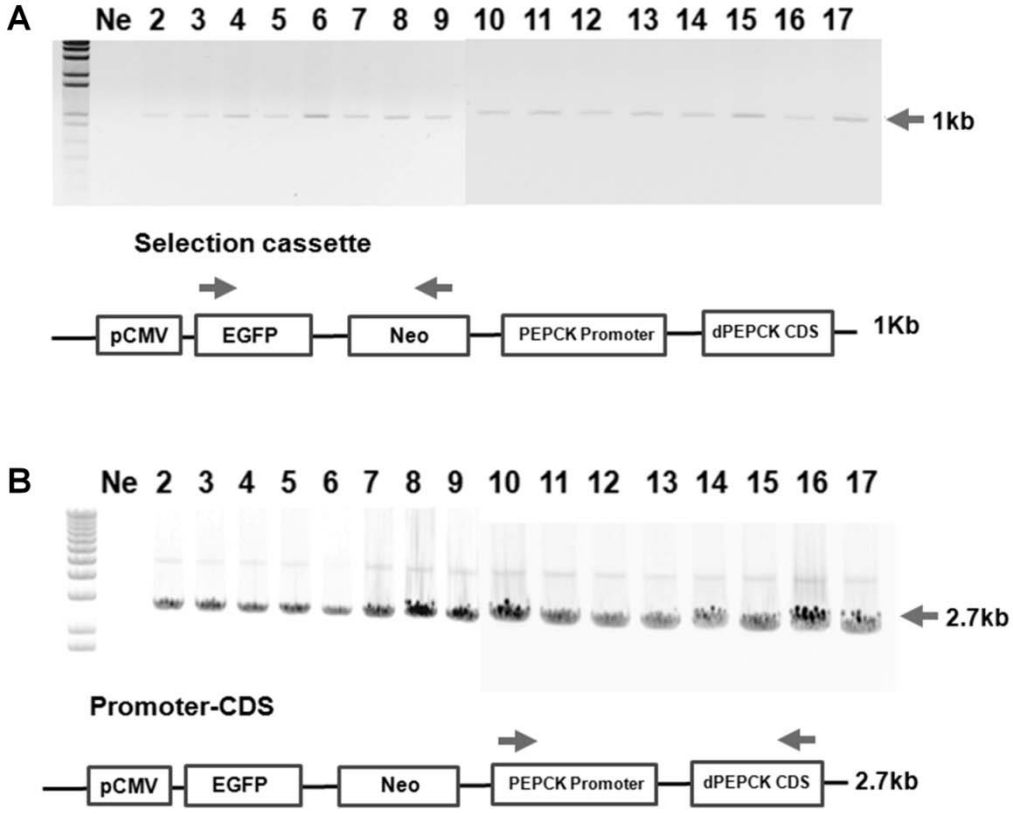

Figure 10. Confirmation of transgenic fibroblasts

Genomic DNAs were isolated from G-418 resisted transgenic fibroblasts (clone) and subject to PCR-based genotyping. The chromosomal integration of the selection cassette (A) and the expression cassette (B) were confirmed. The location and direction of primer a to d were found in Figure 9. M, molecular weights; Ne, PCR reaction without genomic DNA.

3.3. Results of embryo transfer

The results of embryo transfer are summarized in Table 10. Totally 75 oocytes were from 7 egg donors (10.7 oocytes / egg donor) and subjected to nuclear transfer using the transgenic cells. Successfully fused 47 embryos (72.3 %) were transferred into 5 recipients, and two surrogates (S2 and S3) were delivered 3 puppies (Table 11). One (DM-1) of puppies from S2 were healthy but the other (DM-2) was died within 24 h which was the same symptoms with previously reported (Hong *et al.*, 2009). Although DM-3 from S3 was born by Cesarean section, it was healthy with normal body weight (360 g). The two survived puppies (DM-1 and DM-3) expressed EGFP on their claws and skin (Figure 11A) and primary cells from these puppies further expresses EGFP under microscope (Figure 11B). The genetic identities of the cloned puppies with the funder fibroblasts were confirmed by microsatellite parentage analysis (Figure 12). Thus, two puppies were visually confirmed as the transgenic dogs by the EGFP expression on their outer appearances.

Table 10. Summary of embryos transfer

Surrogate ID		Egg donor ID	No. of oocyte retrieved (Mean)	Oocyte stage†	No. of oocyte for NT	No. of fused embryo (%)	Donor cell passage	Clinical pregnancy††
S1		D1	13	M	10	7	6	–
S2		D2	12	M	12	9	6	+
S3		D3	12	A	10	7	6	+
S4		D4	11	I	6	4	6	–
		D5	10	M	10	7	6	
S5		D6	9	M	9	5	10	–
		D7	8	M	8	8	10	
Total	5	7	75 (10.7)		65	47 (72.3)		

†The flushed-out oocytes were categorized as immature (I), mature (M) and aged (A) status

††Clinical pregnancy is determined by ultrasound observation of fetal sac in the uterus four weeks after the embryo transfer.

Table 11. Characteristics of cloned and transgenic puppies

Surrogate ID	No. of embryos	Offspring ID	Gestation length[†]	Bodyweight at birth (g)	Delivery method	EGFP expression	Current status
S2	9	DM-1	61	380	Natural	+	Live
		DM-2	61	640	Natural	–	Dead[‡]
S3	7	DM-3	62	360	Cesarean section	+	Live

[†] Gestation days from the date of embryos transfer

[‡]Death at post-natal day 1

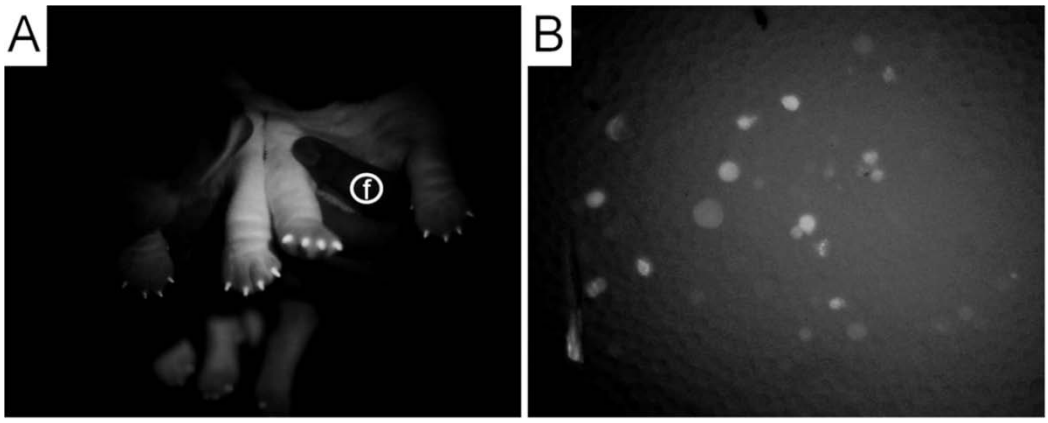

Figure 11. Confirmation of transgenic and cloned puppies

A. 3-month old puppies (DM-1 and DM-3) were exposed under UV light. EGFP expression was observed on the claws and skin. f, human index figure as a negative control.

B. Primary cultured cells from DM-3 express EGFP under fluorescence microscopy.

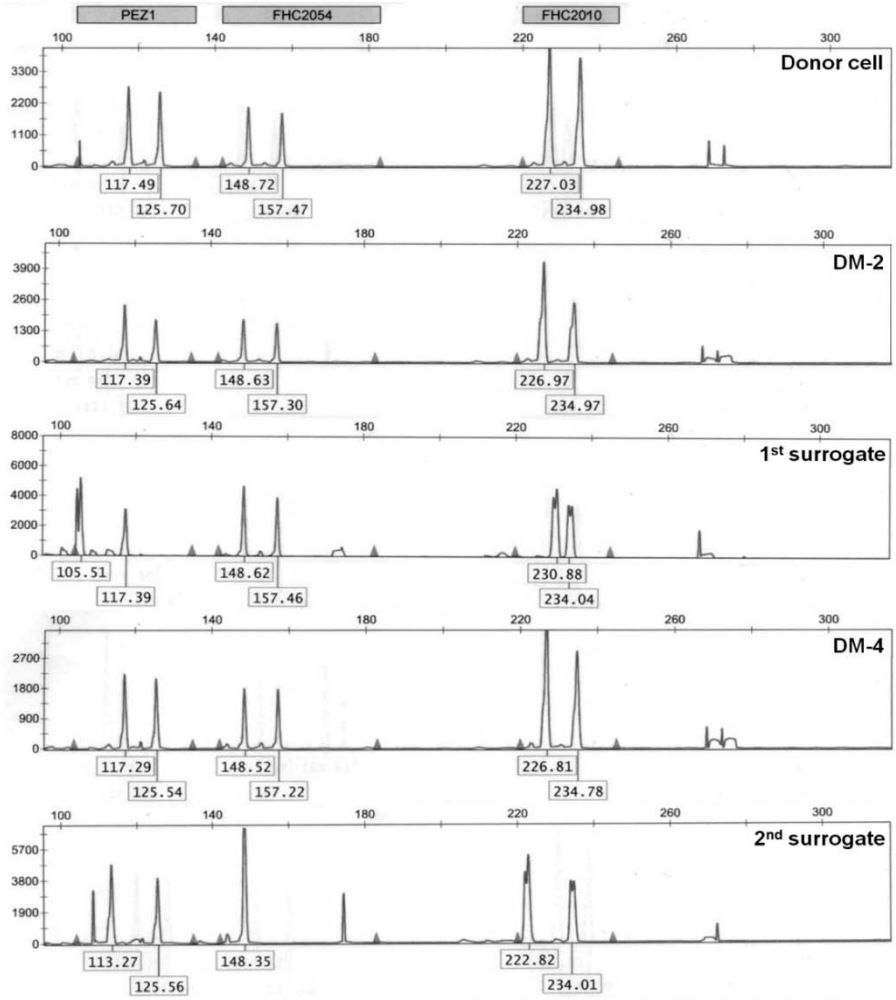

Figure 12-1. Microsatellite analysis of nuclear donor cell, cloned puppies and surrogate

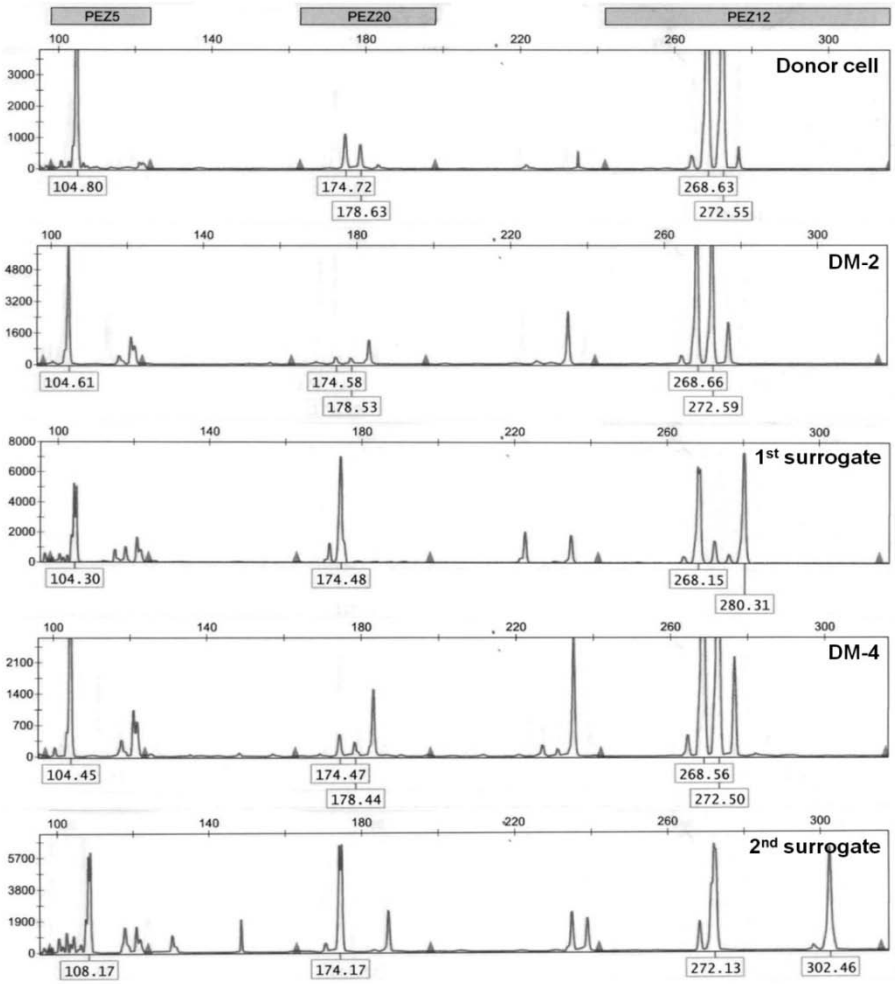

Figure 12-2. Microsatellite analysis of nuclear donor cell, cloned puppies and surrogate

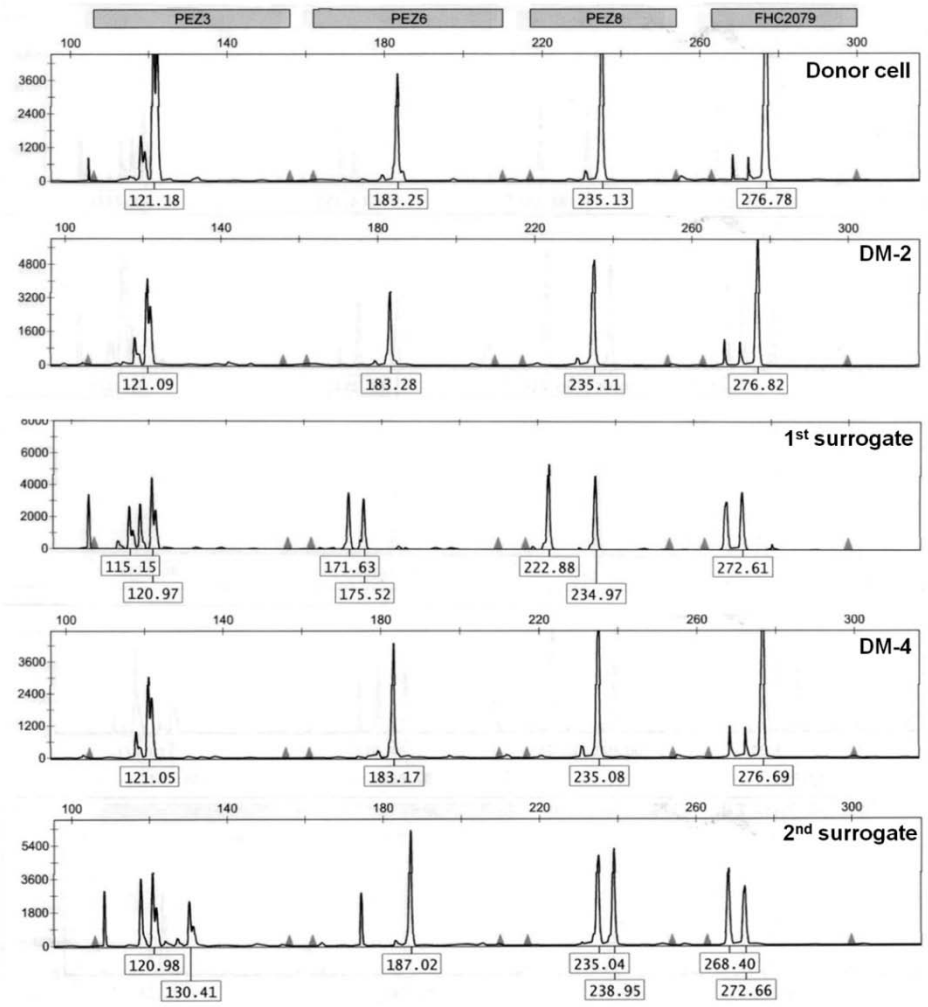

Figure 12-3. Microsatellite analysis of nuclear donor cell, cloned puppies and surrogate

Genotypes at 10 polymorphic microsatellite loci were determined for genomic DNA samples isolated from the nuclear donor cell lines, the cloned puppy, and the surrogate that she delivered. Values represent the base pairs of the amplified microsatellite DNA markers in each sample.

3.4. Characterization of the transgenic and cloned puppies

To confirm chromosomal insertion of the targeting vector, genomic DNAs of three puppies (DM-1, DM-3, and control) were prepared from their umbilical cords and performed PCR based genotyping. DM-1 and DM-3 have both the PEPCK expression cassette (2.7 Kb) and naïve PEPCK gene (5.2 Kb) but the age-matched control have only naïve gene (Figure 13A). The integration of the selection cassette was further confirmed by a PCR (Figure 13B). In addition, the functional activity of PEPCK gene derived from the targeting vector were estimated by real-time PCR (Figure 14A) and immunoblotting assay (Figure 14B) using liver biopsy sample from DM-3. PEPCK mRNA expression was 2-times higher than what of wild-type control and its protein levels was induced in DM-3. Although the current transgenic dog (DM-3) overexpressed PEPCK gene were observed, it has not presented any symptom like T2DM so far. Further investigations are needed for further follow up the DM-3 until onset diabetes-like symptom because this disease is late onset disease.

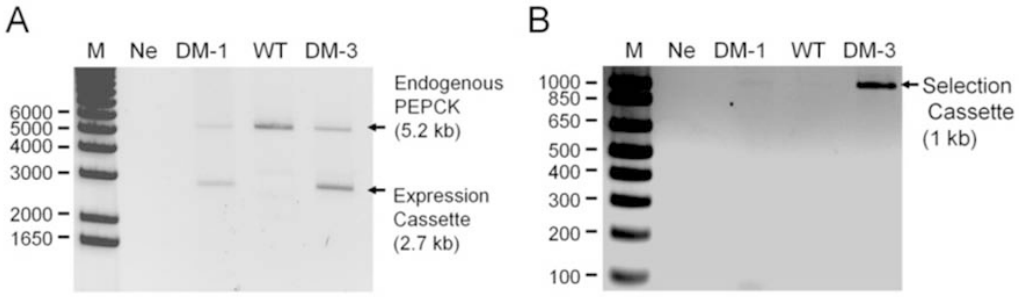

Figure 13. Confirmation of PEPCK gene from cloned puppies

Genomic DNAs were obtained from umbilical cords of DM-1, DM-3, or age-matched wide-type (WT) puppy. The chromosomal insertion of PEPCK expression cassette were confirmed using primer a and b described in Figure 9. (A). This insertion was further confirmed by a nested PCR (B).

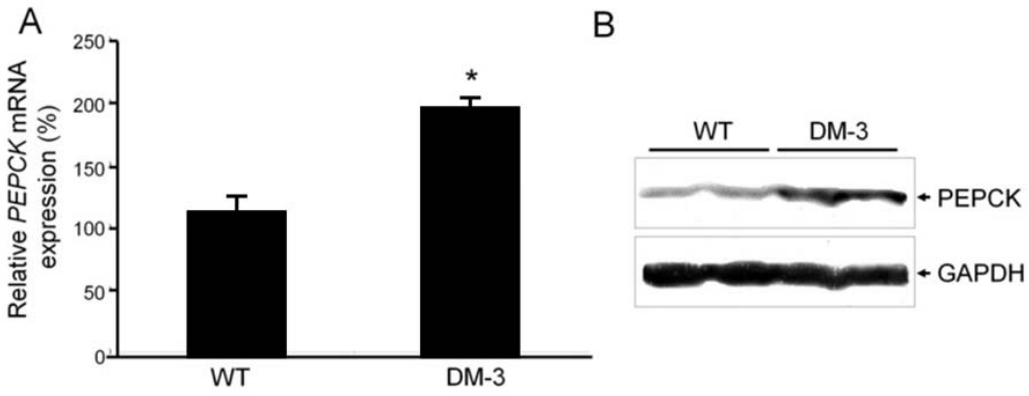

Figure 14. Confirmation of PEPCK gene expression in liver

Canine PEPCK mRNA expression was measured by real-time PCR (A) and its protein expression was detected by anti-PEPCK antibody (B) using liver biopsy samples. The quantitative PEPCK mRNA expression was calculated by the delta Ct method using the expression of GAPDH as internal control; the data are presented to show the percent in expression of the control dog compared with the diabetes dog. Data represents the mean ± SEM of three independent experiments, each performed in triplicate. *P<0.05 vs. WT; M, molecular weights; Ne, PCR reaction without genomic DNA.

4. Discussion

Domestic dogs offer many advantages compared with experimental rodent models due to their homogeneous populations, approach to surgical operation, medical care, comparable organ sizes, cohabiting with humans, and pathophysiological similarities to humans. They are therefore useful for understanding the molecular basis of the pathogenesis of vascular and neural lesions, the actions of therapeutic agents, and genetic and environmental influences (Tsai *et al*., 2007). Despite the genetic and physiologic usefulness for animal model, studies of transgenic dogs have lagged behind those using laboratory rats and mice as experimental models of human disease. The reason for the poor development of canine transgenic technique is that the scarcity of available canine oocytes has limited and canine *in vitro* maturation system have not been established (Luvoni *et al*., 2005). Here, two transgenic and cloned puppies and one dog (DM-3) over-expressed PEPCK mRNA and protein in the liver that might be causing type-2 diabetes mellitus-like symptom were generated.

Efficient techniques of genetic engineering are essential for the successful production of transgenic animals. Several studies have reported that virus-mediating gene delivery system are useful method to modify genetic information of target animal (Bosch *et al*., 2006; Gómez *et al*., 2009). Although viral system performed high chromosomal integration rates, it caused unwanted side effects such as neoplastic transformation (Sokol *et al*., 1996). On the other hand,

liposome-mediating gene delivery system was relatively safer and easier than the viral system however it presented low transfection efficiency with long-term selection (Chen *et al*., 2002; Nikcevic *et al*., 2003). In the present study, the liposome-mediating gene modification to generate transgenic puppies was adopted.

PEPCK is a key gluconeogenic enzyme in the liver and kidneys with an established role in the metabolism of intermediates (Hanson *et al*., 1997). Previously, transgenic mice overexpressing PEPCK were generated and shared key characteristics of T2DM symptoms (Valera *et al*., 1994). In addition, several mice strains overexpressing PEPCK gene were further produced (Sun *et al*., 2002; Hanson *et al*., 2008). Based on these mouse models, the targeting vector containing dog PEPCK cDNA controlled by its promoter regions (-746 nt to + 1 nt) selected by the promoter study were designed. The targeting vector also contained two selection marker genes, EGFP and Neo^r to facilitate the antibiotic screening and the visual confirmation of transformation.

The current cloned and transgenic puppies were confirmed the genetic identification using microsatellite analysis and artificial PEPCK expression using real-time PCR and immunobloting assays. Although the transgenic puppies expressed high level of liver PEPCK compared with wild-type dogs respectively, any symptom like T2DM were observed. Glucose tolerance tests, intravenous injection of glucose solution (500 mg / Kg), were conducted to induce the diabetes-like symptom in the transgenic dogs. Although the transgenic puppies

presented higher level of serum glucose and lower level of serum insulin than wild-type dogs, there was no significantly difference between dogs. The compensatory machinery of pancreatic β-cells and/or the genetic background of breeds might fail this glucose challenging. Beagle is less susceptible to T2DM than other breeds and the onset and progression of T2DM vary according to breed, sex, and age (Fall *et al*., 2007).

Chapter III.

Canine model of Alzheimer's disease over-expressing a mutated human amyloid precursor

1. Introduction

AD is a devastating neurodegenerative disorder that usually affects elderly people (Rocchi *et al*., 2003). Human life expectancy has increased tremendously over the years due to improved public health programs and advances in clinical medicine. At the same time, the incidence of AD is also rapidly increasing. It has been reported that more than 15 million people are suffering from AD and this number is expected to more than double in the next generation (Götz *et al.*, 2004). Studies of AD pathogenesis require the use of animal models that develop some degree of amyloid pathologies in the brain (Head *et al*., 2010). Utilization of animal models is crucial for any biomedical research on human disease processes at the cellular and molecular levels, and for developing new therapies. A transgenic animal model could also be used to examine the pathogenic mechanisms of AD and related disorders such as frontotemporal dementia. Furthermore, these models could help develop vaccines or improve treatment strategies.

AD is considered a multifactorial and polygenic disease in which environmental and genetic factors play a major role. Although several candidate genes have been surveyed, three are believed to be responsible for the rare early-onset familial form of the disease. These include the amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes (Rocchi *et al*., 2003). Genetically modified mice, flies, fish, and worms were developed that reproduce different AD histopathologies such as plaques containing β-amyloid

(Aβ) and Tau-containing neurofibrillary tangles (NFT) (Kragh *et al*., 2009). Recently, production of transgenic mini-pigs carrying the APP695sw transgene using a handmade cloning procedure was reported (Kragh *et al*., 2009). However, AD-like symptoms in a transgenic dog model have not yet been published.

One main purpose of an animal model of AD is to replicate the symptoms, lesions, or causes of the disease. Numerous transgenic murine lines have successfully been used to partially reproduce AD lesions such as extracellular deposits of the Aβ peptide and intracellular accumulation of Tau protein (Jang *et al*., 2007). Mutated human APP (mhAPP) transgenes result in the deposition of Aβ peptide, similar but not identical to the plaques observed in senile humans (Duyckaerts *et al*., 2008). Dogs are extensively used in various biomedical studies as model animals. Development of successful SCNT procedures in dogs has made considerable progress. Consequently, small, medium, and large breed dogs have been cloned from cultured cells as the genetic donor or long-term cryopreserved somatic cells (Lee *et al*., 2005; Hossein *et al*., 2007; Jang *et al*., 2007; Hong *et al*., 2009). Therefore, it has become possible use donor cells to create knock-out and knock-in dogs or animals over-expressing the gene of interest. In the present study, a somatic cell line over-expressing mhAPP were established to generate an AD canine model by SCNT. The transgenic dogs were confirmed to have increased mhAPP expression in the brain resulting in the accumulation of Aβ enlarged ventricles, atrophied hippocampus, and abnormal behavior.

2. Materials and methods

2.1. Animal care and welfare

Standard procedures established by the Sooam Biotech Research Foundation (Seoul, South Korea) for the Accreditation of Laboratory Animal Care were followed strictly, when caring for the dogs used for the present study. All experiments and surgical procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the Sooam Biotech Research Foundation. Pro-estrus dogs (mixed breed, 1-7 y old, 20-25 kg body weight) were obtained from a breeder and reared indoors in separated cages up until oocyte recovery. Following oocyte recovery, the dogs were sent back to the breeder. None of the dogs were used repeatedly. The pregnant recipient dogs were kept in the research facility while the others were returned to the breeder.

2.2. Chemicals

All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), unless otherwise stated.

2.3. Cell culturing and establishment of canine fibroblast cells

Human neuroblastoma IMR-32 cells (Korean Cell Line Bank, Seoul, South

Korea) were maintained in Dulbecco's Modified Eagle Media (DMEM; Invitrogen, Carlsbad, CA, USA) containing penicillin and streptomycin (Invitrogen), and 10 % fetal bovine serum (FBS; Invitrogen). Canine primary culturing of adult and fetal fibroblasts was performed as described in the previous study (Hossein *et al*., 2007). Briefly, fetal fibroblasts were obtained from artificially inseminated embryos on Day 30 of pregnancy, using a trypsinization. Fibroblast cells were cultured in DMEM containing 10 % FBS.

2.4. Construction of the transgenic vector

Various regions of the Thy-1 promoter (from nucleotides -2.0 ~ +2.3 kilo-nucleotides (k nts), +1 = the transcriptional start site) were isolated by a long-range PCR (LA Taq; TaKaRa Bio., Inc., Shiga, Japan) using genomic DNA from beagle fibroblasts as a template. Amplified fragments were ligated into the MluI site of the promoter less pGL3-Basic vector (Promega Co., Madison, WI, USA). Human APP cDNA was prepared by PCR using the Full-Length Mammalian Gene Collection (cat. #6152423; Invitrogen) as a template. The amplified APP gene was mutated using a Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA, USA) and ligated into the NcoI and XbaI sites of the recombinant pGL3-Basicvector containing the Thy-1 promoter. An enhanced green fluorescent protein (EGFP) gene from pIRES2_EGFP (Clontech Laboratories. Inc., Madison, WI, USA) was ligated into the EcoRV and BamHI sites of thepIRES_Neo vector

(Clontech). This selection cassette was inserted into the SalI site of the backbone plasmid containing the dog Thy-1 promoter region and mutant human APP gene. The final construct (pGL3-pThy1-mhAPP-pCMV-EGFP/Neo) can be linearized by NotI digestion (Figure 15). All amplified products obtained at each step of the construct production were analyzed by sequencing analysis (Genotech Co. Ltd., Daejeon, South Korea).

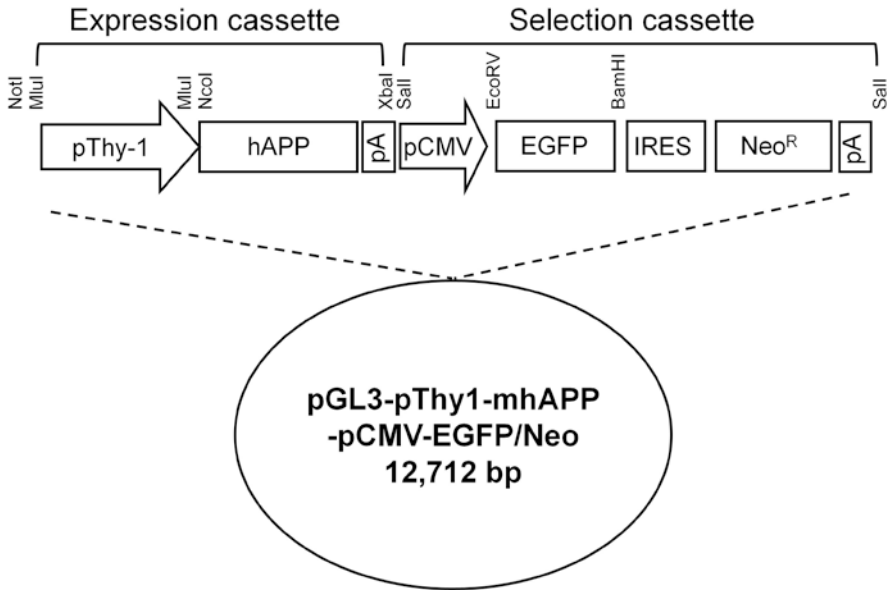

Figure 15. Schematic structure of the transgenic vector, pGL3-Thy1-mhAPP-pCMV-EGFP/Neo

The transgenic vector consists of two expression and selection cassettes. The expression cassette leads to over-expression of mutated human APP (mhAPP) mRNA controlled by the canine Thy-1 promoter (pThy-1). The selection cassette contains genes for enhanced green fluorescent protein (EGFP) and neomycin resistance (Neo) linked with internal ribosomal entry site (IRES) regulated by a CMV promoter (pCMV). The two cassettes were inserted into a pGL3-Basic vector.

2.5. Transient transfection and reporter gene assay

The RSV-lacZ plasmid was co-transfected along with the Thy-1 promoter-luciferase construct into IMR-32 cells to serve as a control for different transfection efficiencies of various luciferase constructs. Briefly, 3×10^5 IMR-32 cells were seeded in 6-well tissue culture plates 1 day before transfection. Next, 3.5 μg of the Thy-1 promoter-luciferase construct and 0.5 μg of the RSV-lacZ plasmid were transiently transfect using Lipofectamine^{TM} 2000, and the cells were incubated for 48 h. Cellular lysates were prepared using 150 μL of reporter lysis buffer, and the luciferase activity was assayed using the luciferase assay system. Luminescence was measured using the Glo Max 20 / 20 Luminometer, and β-galactosidase activity was measured using the β-galactosidase enzyme assay system. The promoter activity was expressed as the percentage of relative luciferase activity (% RLA, luciferase / β-galactosidase activity).

2.6. Laparotomy and oocyte collection

Mature canine oocytes were collected *in vivo* by laparotomy at 72–84 h after ovulation by using standard procedures (Lee *et al*., 2005; Hossein *et al*., 2007). The time of ovulation was determined using serum progesterone concentration and vaginal cytology (Hossein *et al*., 2007). Licensed veterinarians performed all operative and postoperative procedures and care. The reproductive tract was exposed by performing mid-ventral laparotomy under general anesthesia. The

fimbriated end of the oviduct was canalized using a 6-gauge bulbed needle held in place by a surgical ligature. The oviductal lumen at the base of the utero-oviductal junction was cannulated using a 24-gauge hypodermic needle (Angiocath TM Plus; Becton Dickinson, Franklin Lakes, NJ, USA). Approximately 10 mL of TCM 199 collection medium supplemented with HEPES (Sigma-Aldrich Corp.) was used to flush the hypodermic needle. The medium was passed through the oviductal lumen and the bulbed needle before being deposited into a sterile plastic Petri dish. After flushing both the oviducts, each ovarian bursa was incised to pull out the ovary, and corpora lutea in each ovary were counted. Finally, the abdominal incision was closed using a two-layer method, followed by the application of a surgical adhesive along the skin incision.

2.7. SCNT and embryo transfer

SCNT was performed according to a procedure previously described (Lee *et al*., 2005; Jang *et al*., 2007), with some modifications. Briefly, stripped oocytes were enucleated under an inverted microscope with epifluorescence (TE2000-E; Nikon Corp., Tokyo, Japan). By using an injection pipette, a trypsinized fibroblast cell with a smooth surface was transferred into the perivitelline space of an enucleated oocyte. The obtained couplets were fused using two DC pulses of 1.75 kV / cm for 15 μs from a BTX Electro-Cell Manipulator 2001 and were

transferred to naturally synchronized recipients. The embryos were loaded into a Sovereign Tom Cat catheter (Sherwood Medical, St. Louis, MO, USA), with a minimal volume of medium, and were gently transferred into the deep distal two-thirds of the oviduct without insufflating air.

2.8. Pregnancy diagnosis

Approximately 30 days after embryo transfer, the recipients were examined using a portable real-time ultrasonography machine with a 3.5-MHz curved transducer (SSD-900; Aloka Co. Ltd., Tokyo, Japan). Sizes and shapes of chorionic cavities along with embryonic or fetal heartbeats were examined to detect embryonic or fetal death. After confirming pregnancy, this examination was performed after every 7 days to monitor fetal development until birth.

2.9. Genomic DNA extraction and polymerase chain reaction

Canine genomic DNA was isolated using a G-DEX™ II Genomic DNA Extraction Kit. Genomic DNA (100 ng) or plasmid DNA (10 ng) was amplified in a 50 μL PCR reaction containing 1U of LA™ or Ex™-Taq polymerase (TaKaRa) and 10pmol of specific primers. PCR was performed for 35 cycles of denaturation at 94 °C for 30 s and annealing and extension at 68 °C for 1–3 min, followed by a final extension at 72 °C for 30 min. All the primers are described

in Table 12. PCR products were fractionated in an agarose gel, stained with ethidium bromide, and photographed under UV illumination. The photographs were scanned using Gel Doc EQ.

2.10. RNA preparation and quantitative RT-PCR

Total RNA was isolated from the heart, pancreas, liver, lung, kidney, spleen, and cerebrum of two puppies (AM144 and AM145) by using TRIzol reagent. DNA contamination was eliminated by treatment with DNase (Invitrogen). Next,1 μg of total RNA was reverse transcribed into first-strand cDNA by using M-MLV reverse transcriptase and random primers (9-mers). GAPDH was amplified to evaluate RNA degradation and was used as the control for variations in mRNA concentration in the RT-PCR reactions. GAPDH and human APP were quantified by performing 28 and 30 cycles of PCR, respectively. cDNA was amplified in a 20 μL PCR reaction containing 1U Taq polymerase (iNtRON Biotechnology) and 10pmol of specific primers (Table 12). The PCR procedure included denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The PCR products (8 μL) were separated on 2 % agarose gel, stained with ethidium bromide, and photographed under UV illumination. The photographs were scanned using Gel Doc EQ.

Table 12. Primer list

Name	Direction	Sequences (5' to 3')
Human APP cDNA	Forward	CGC GCA GGG TCG CGA TGC TGC CCG
	Reverse	CTG TGG CGG GGG TCT AGT TCT GCA
Thy-1 promoter region	Forward	GAC TGG ACC ATC CTT GCA GCT CAT
	Reverse	GCT CAG TCC TTG ATC TGG GGG TGG
KM to NL (Swedish mutation)	Forward	CGA GAT TCT GAA GTG AAC CTG GAT GCA GAA TTC CGA CAT G
	Reverse	CAT GTC GGA ATT CTG CAT CCA GGT TCA CTT CAG AAT CTC G
V to F (Indiana mutation)	Forward	GGA CTC ATG GTG GGC GGT TTT GTC ATA GCG ACA GTG ATC
	Reverse	GAT CAC TGT CGC TAT GAC AAA ACC GCC CAC CAT GAG TCC
EGFP	Forward	CAC AAC CAT GGT GAG CAA GGG CGA
	Reverse	TTA CTT GTA CAG CTC GTC CAT GCC
Confirming primer A	Forward	CCT TGT GCT GTC TCC CCC TC
Confirming primer B	Reverse	TCA CAA AGT GGG GAT GGG TC
Confirming primer C	Forward	CAT GAA GCA GCA CGA CTT CT
Confirming primer D	Reverse	CCT AGG AAT GCT CGT CAA GA
Confirming primer for human APP	Forward	TCA AGC AAA GAA CTT GCC TAA AGC TGA TAA
	Reverse	AGG AGG AAC AGC CTG CAG AGC G

Proper restriction enzyme sequences were added on the 5' region. KM to NL represents Swedish (K670N and M671L) mutation and V to F means Indiana FAD (V717F) mutations

2.11. Western blot analysis

Proteins (40 μg) were separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated with antibodies against APP and β (dilution 1 : 1,000; catalog # 2450 and 2454; Cell Signaling Technology, Beverly, MA, USA) or β-actin (dilution 1 : 2,000; Santa Cruz Biotechnology). Immunoreactive proteins were visualized by exposure to a radiographic film. Band intensities were quantified by scanning the image using the Gel Doc EQ system, with correction by background subtraction, and were normalized using β-actin as the internal control.

2.12. Immunohistochemical staining

Brain tissues were embedded in paraffin. Sections (5-μm thick) were deparaffinized and hydrated in descending grades of ethanol. The sections were subsequently incubated with an anti-Aβ antibody (dilution 1 : 100, #2454) and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1, dilution 1 : 500, #019-19741; Wako, Osaka, Japan) in 10% normal goat serum for 2 h at room temperature (RT). The sections were treated with biotinylated goat anti-rabbit IgG (Vector ABC Elite Kit, Burlingame, CA, USA) for 45 min at RT. Immunoreactivity was assessed using avidin–biotinperoxidase complex (Vector ABC Elite Kit) prepared according to the manufacturer’s instructions. The peroxidase reaction was developed using a diaminobenzidine substrate kit (SK-

4100; Vector). Primary antibodies were omitted for a few test sections in each experiment to serve as a control. The sections were counterstained with Harris's hematoxylin before mounting.

3. Results

3.1. Construction and functional activity of the transgenic vector

A human APP gene expressing a cassette containing two well-characterized mutations, Swedish (K670N and M671L) and Indiana FAD (V717F), were generated which successfully induces AD-like pathological symptoms in mouse models (Spanopoulou *et al*., 1991; Dudal *et al*., 2004; Spires *et al*., 2005). A canine Thy-1 promoter for selective expression in neural tissues controlled the mutant human APP (mhAPP) gene.

Specific regions of the Thy-1 promoter were selected by a luciferase activity assay in human neuroblastoma IMR-32 cells (Figure 16). The upstream portion (-2.0 to -1.5 kilo-nucleotides (k nts), +1=transcriptional start) of the Thy-1 promoter were deleted because it possessed unknown inhibitory elements, and the neural element (between -2.0 to -2.3 k nts) in intron A was added in because it was essential for promoter activity in the IMR-32 cells. Finally the Thy-1 promoter (-1.5 k nts to +2.3 k nts) was found to have maximum activities similar to the SV promoter in neural cells. The transgenic vector was transiently expressed in IMR-32 cells and induced expression levels of APP transcripts 2-

times higher compared to the empty pGL3-Basic vector. The transgenic vector also contained a selection cassette including EGFP and neomycin resistance genes linked by IRES, and were controlled by the CMV promoter to facilitate selection for further processes.

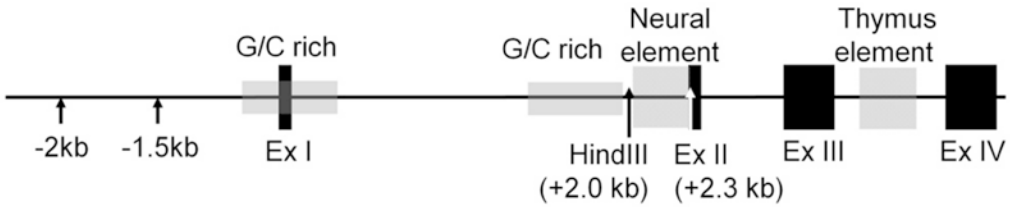

Figure 16. Canine Thy-1 promoter structure

A schematic structure of the canine Thy-1 promoter region which has two tissue-specific (neurons and thymus) expression elements.

3.2. Establishment and confirmation of the somatic cell line as a nuclear donor

Canine fibroblasts were transfected with the linearized transgenic vector and cultured with media containing 350 μg / mL of G418 to select a transgenic clone. The cells resistant to G418 for 4 weeks were further confirmed to express EGFP by fluorescent microscopy. Insertion of the transgenic vector in the transgenic cells was confirmed by PCR (Figure 17). The positive cells were isolated and frozen until SCNT.

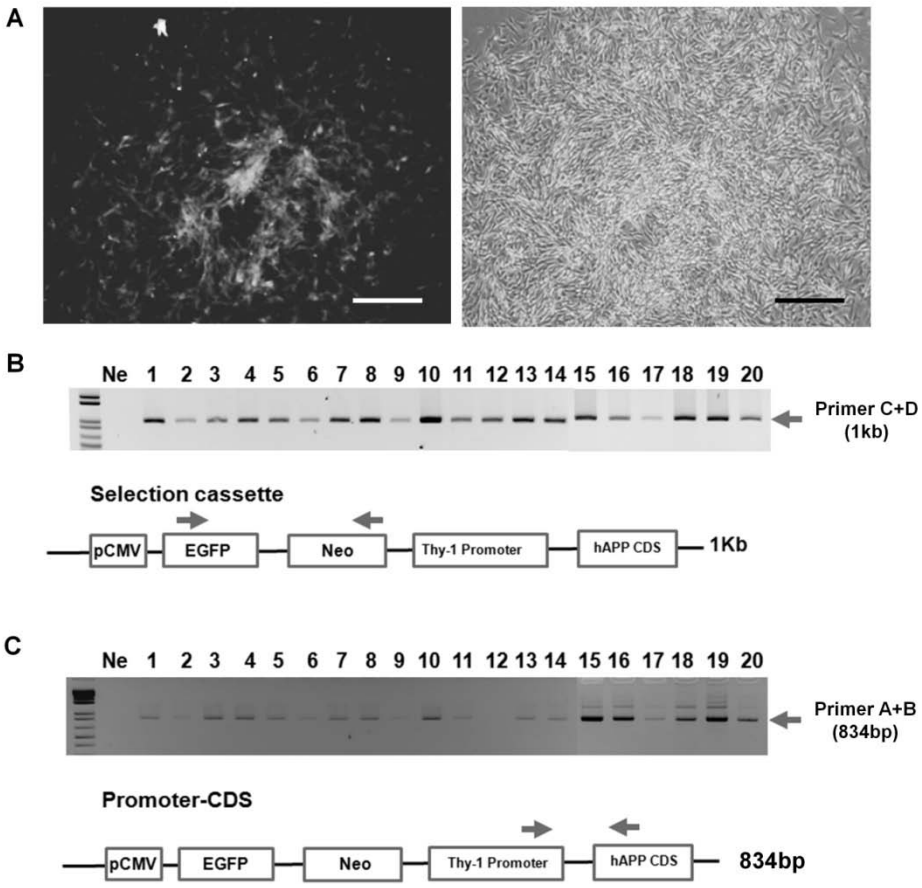

Figure 17. Confirmation of transgenic fibroblasts

Canine fibroblasts and transgenic puppies were confirmed to express EGFP and underwent PCR analysis. A, A representative clone of the transgenic fibroblasts were observed by light and fluorescent microscopes. Bar indicates 500 μm. B, PCR was carried out to analyze the transgenic fibroblast clones. Primers A+B (834 bp) amplified the region of the selection cassette and primer C+Ds (1 kb) produced fragments of the expression cassette. M.W., molecular weight (1-kb ladder).

3.3. Production and characterization of transgenic puppies

SCNT was performed using the transgenic cell line and 332 oocytes matured *in vivo* from 29 donors. Six puppies (AM141 to 146) were born from four out of 17 surrogates. Five puppies (AM141, 142, 143,144, and 146) were confirmed to be transgenic animals by PCR (Figure 18A), and observed to express EGFP in their nails, toes and fur (Figure 18B).

An EGFP-positive puppy (AM144) and EGFP-negative littermate (AM145) were sacrificed to measure mhAPP expression inserted by SCNT. As seen in Figure 19, expression of the mhAPP gene was only observed in organs of the EGFP-positive puppy. Endogenous canine APP mRNA was detected in the organs of both animals. Amplified mhAPP transcription was further confirmed by sequence analysis. Thus, the transgenic puppies were found to successfully express the exogenous mhAPP gene.

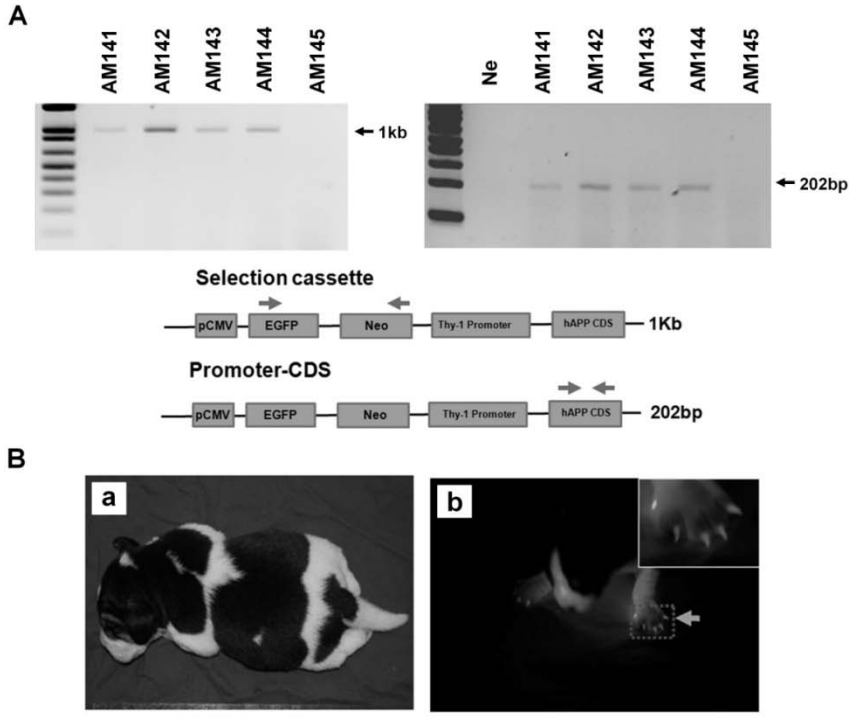

Figure 18. Confirmation of transgene from cloned offspring

A. Six pups (AM141 to AM146) from the surrogates were confirmed to have undergone genetic transmission from the transgenic fibroblast with two primer sets. Primers A+B (834 bp) amplified the region of the selection cassette and primers C+D (1 kb) produced fragments of the expression cassette. M.W., molecular weight; N.C., negative control (no template in the PCR reaction).

B. AM144 died 48 h of after birth. Post-mortem examination revealed that the pup suffered from a severe cleft palate. a, Transmission light photograph; b,EGFP expression in the nails (dotted rectangle), toes, and white fur.

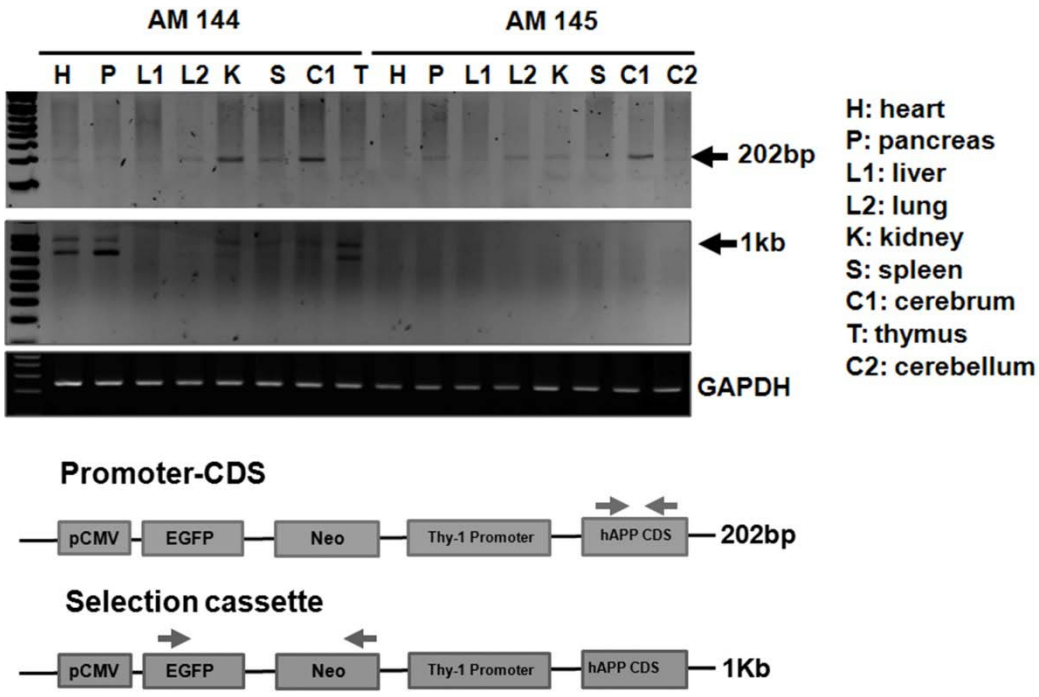

Figure 19. Confirmation of APP gene expression in different organ

Transgenic puppy (AM144) and its littermate (AM145) were sacrificed to measure the level of human APP gene expression. Human APP (upper lane), expression cassette (middle lane), and GAPDH (lower lane, an internal control) gene transcription was detected in various organs by RT-PCR as indicated.

3.4. AD-associated pathology in the transgenic dogs

One of the mhAPP transgenic dogs (AM146) was mated with a wild-type female dog (AF 165) to produce more puppies for observing the development of AD-like symptoms and stabilizing of the mhAPP gene. This mating produced four male and three female puppies (SB16 to 22). All dogs had the transgenic vector in their genomic DNA except for SB21 (Figure 20). When the puppies were 6mo old, one transgenic dog (SB17) presented recurrent behavior disorders like tetanic convulsions. Although seizures are not a common phenotype of AD, recently reports indicated that seizures may be one of phenotypes of AD (Larner *et al*., 2011; Picco *et al*., 2011; Friedman *et al*., 2012; Irizarry *et al*., 2012; Pandis *et al*., 2012). The brain of SB17 was further examined by magnetic resonance imaging. As shown Figure 21, AD-like symptoms, including enlarged ventricles and an atrophied hippocampus, were observed. The symptoms were not common in canine however emerging references have been indicated that ventricle enlargement and hippocampal atrophy are phenotypes of human AD (Ferrarini *et al*., 2008; Nestor *et al*., 2008; Chou *et al*., 2009; Apostolova *et al*., 2012). These results indicated that the transgenic dogs developed human AD-like symptoms.

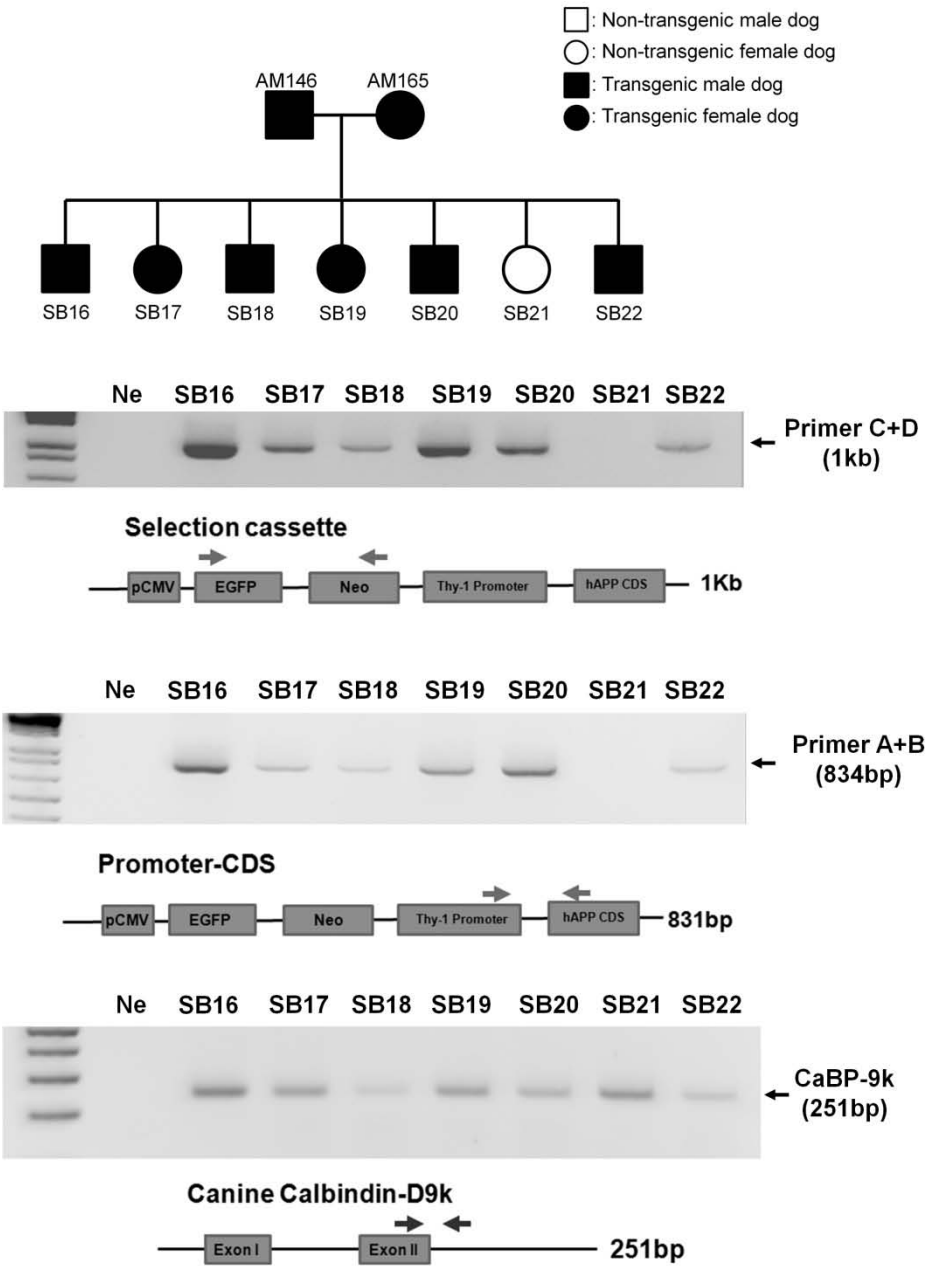

Figure 20. AD-like symptoms in the transgenic dog brain

Figure 20. AD-like symptoms in the transgenic dog brain

Family tree and confirmation of mhAPP transgene integration is shown. One of the mhAPP transgenic puppies (AM146, male) and a non-transgenic animal (AM165, female) were mated. Seven offspring (four males and three females) were confirmed to express the mhAPP transgene with PCR methods using two primer pairs. Primers A+B (834 bp) amplified the region of the selection cassette and primers C+D (1 kb) produced fragments of the expression cassette. SB17 developed abnormal behavior and was sacrificed for further study along with SB21 as a non-transgenic control. CaBP-9k gene was amplified as an input control.

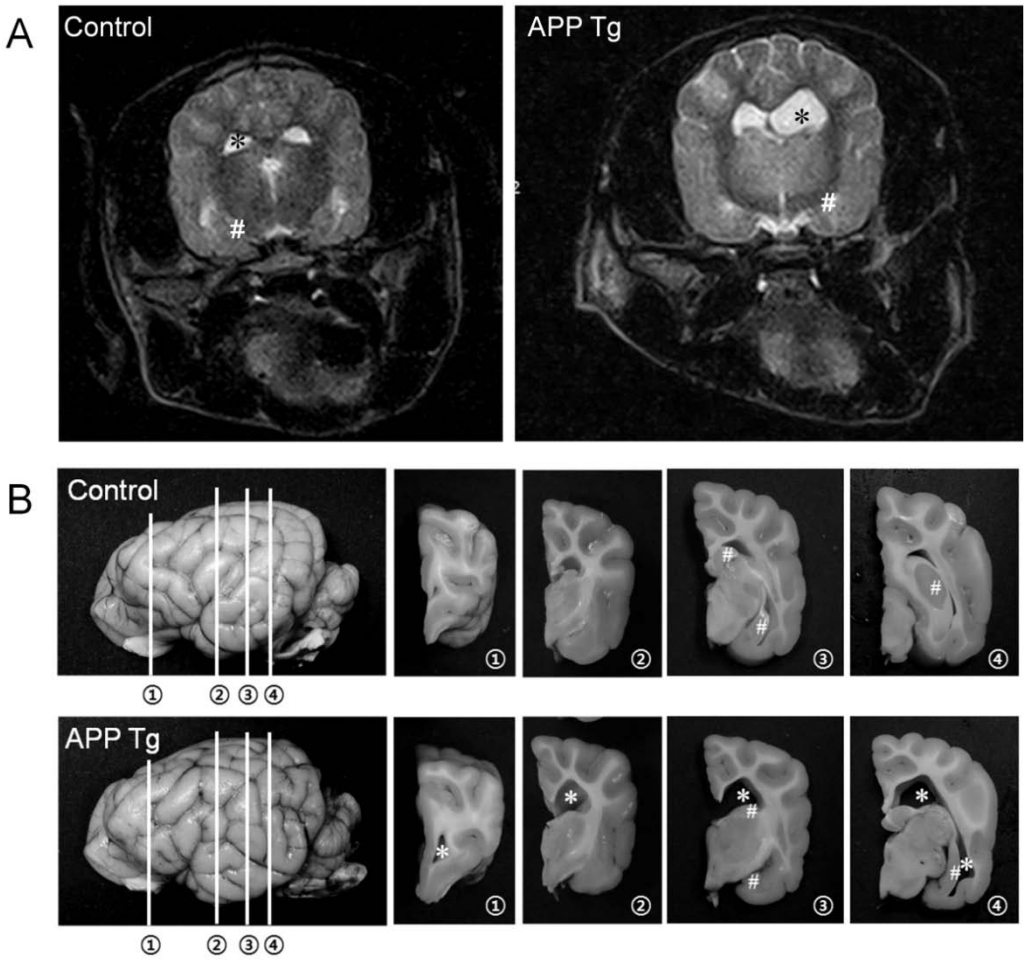

Figure 21. MRI imaging in the transgenic dog brain

A, MRI scans of the transgenic (SB17) and non-transgenic (SB21) dogs were acquired.

B, Gross appearances of the transgenic (SB17) and non-transgenic (SB21) brains were compared. Cross-sections are indicated as circled numbers (1 to 4). *ventricles; #hippocampus; APP Tg, SB17; Control, SB21.

SB17 was sacrificed and pathological AD phenotypes in the entire brain were analyzed. As shown Figure 21B, the cerebral ventricles were greatly enlarged. The hippocampus had also atrophied and degenerated in the transgenic dog compared to the non-transgenic animal as previously documented by MRI. To further characterize this animal, the molecular mechanisms underlying the AD-like symptoms were examined. Increased levels of Aβ, hallmarks of AD, were also observed in the transgenic dog (Figure 22). However the expression of mhAPP from that of endogenous canine APP with the currently available anti-APP sera was not distinguished. This finding implies that overexpression of mhAPP in dogs caused accumulation of the hallmarks of AD, Aβ in the brain resulting in AD-like symptoms such as the enlarged ventricles, atrophied hippocampus, and abnormal behavior.

For additional patho-histological characterization, Aβ accumulation in the hippocampus regions was further measured with an immunohistochemical assay. In the hippocampus, Aβ-positive immunoreactivity was rarely detected in non-transgenic dog (Figure. 23a and d), whereas the immunoreactivity was observed remarkably in the transgenic dog (Figures. 23b and e). Additionally, the microglial activation was also found in the hippocampus of the transgenic dog where the Aβ accumulation was present (Figures. 23c and f). These finding indicated that Aβ accumulation in cells induced an immune response like the previous finding (Glass *et al.*, 2010).

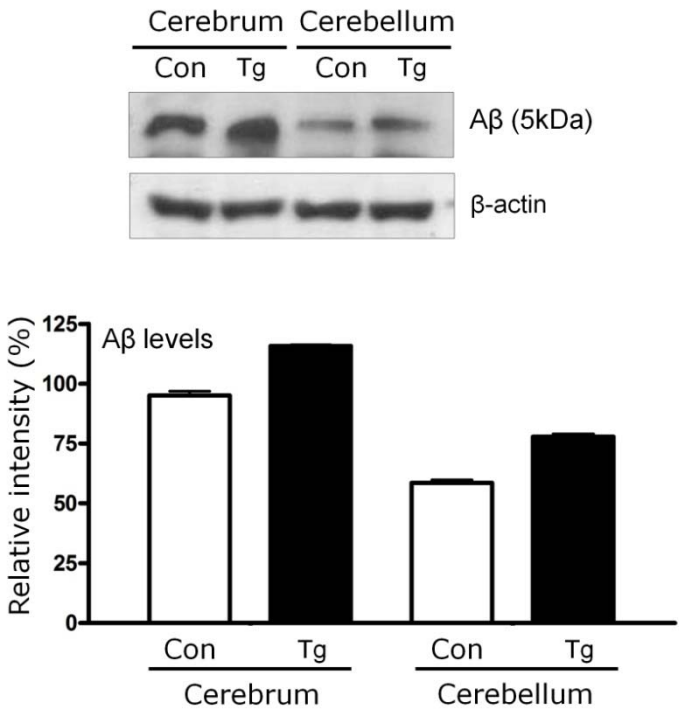

Figure 22. Immunoblotting confirmation of the AD model dog

The transgenic dog (Tg, SB17) presenting abnormal behavior and the age-matched control (Con, SB21) were sacrificed. Expressions of the Aβ were analyzed as indicated. The cerebrum includes the cortex region. Relative protein expression levels are presented as a graph (mean ± SEM of duplicated values from all samples).

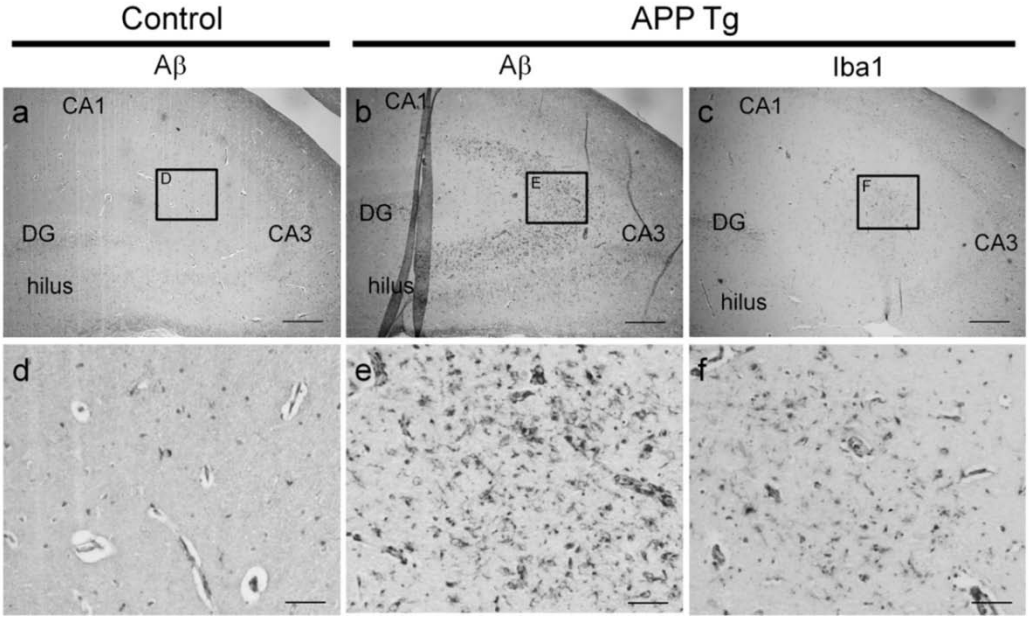

Figure 23. Aβ immunoreactivity in the transgenic dog

The transgenic dog (APP Tg, SB17) presenting abnormal behavior and the age-matched control (SB21) were sacrificed. Spatial expression of the AD hallmark protein Aβ was analyzed. Thin sections were prepared from the hippocampus region. The left (a and b) and middle panels (b and e) show Aβ-specific staining. The right panel (c and f) shows the staining for Iba 1, a microgial marker, using the serial section of the middle one (b and e). Scale bars=300 μm (a-c), 80 μm (d-f), CA, Cornu Ammonis; DG, dentate gyrus.

4. Discussion

In this study, transgenic dogs over-expressing the human APP gene containing two well-known AD-associated mutations, Swedish (K670N and M671L) and Indiana FAD (V717F), were generated which inhibited proteolytic processing by α-secretase. The animals were produced with a non-viral gene delivery system to avoid unknown side effects of the viral protein contamination in the chromosome. mhAPP was successfully over-expressed in the brain of the transgenic animals and the protein end-product, Aβ accumulated in the brain. The transgenic dog developed abnormal neurological symptoms like tetanic convulsions along with enlarged ventricles and atrophied hippocampus similar to humans with AD. In addition, Aβ plaque-like structures, a patho-histological hallmark of AD, were detected in the frontal cortex regions, and significant Aβ accumulation was further observed in the cortex and hippocampus. Thus, the transgenic dogs are good candidates for replacing the present rodent model to study the pathophysiological characteristics of AD and performing pharmaceutical research to treat this disease.

AD is the most common dementia disorder in humans. The major pathological hallmarks of this disease are abnormal accumulation of extracellular amyloid plaques and intracellular NFTs in the vulnerable brain regions. The NFTs primarily contain paired helical filaments of hyperphosphorylated forms of microtubule-associated tau protein. The amyloid plaques mainly contain Aβ, a peptide containing 40–42 amino acids that is derived from APP by sequential

proteolytic cleavage by β- and γ-secretases (Dyrks *et al*., 1988; Sisodia, 1992; Selkoe, 2001). Numerous mouse lines that develop Aβ deposits have been produced (Duyckaerts *et al.,* 2008). Mutated human APP transgenes in mice result in Aβ deposition; however, these lesions are not identical to the Aβ protein in the amyloid plaques of human patients with AD. Besides Aβ deposition, axon dystrophy and dendrite alterations have been observed (Duyckaerts *et al.,* 2008). All APP mutations in transgenic mice increase Aβ 42 levels; however, overexpression of wild-type APP alone does not induce Aβ deposition in mice (Duyckaerts *et al*., 2008). Double transgenic mice (expressing both APP and PS1) develop lesions earlier. These data from transgenic mice have allowed some insights into the kinetics of AD pathogenesis. The connections between AD-associated symptoms, lesions, and increased levels of Aβ oligomers have been difficult to unravel. Thus far, transgenic mouse has been the best animal model for studying AD. However, transgenic dogs with AD-like symptoms will be useful for studying AD in humans.

Successful studies of human diseases need appropriate animal models. The common animal models for AD research are transgenic mice that overexpress a mutant form of the human Aβ precursor protein and/or enzymes implicated in its metabolic processing (Sarasa *et al.,* 2009). However, rodent models are not sufficient to completely elucidate the pathogenesis of AD because of genetic, physiologic, and anatomic differences between mice and humans. Dogs would be more suitable for examining human disorders than mice because they have

evolved physiologically and genetically in proximity to humans (Head, 2007; Siwak-Tapp *et al*., 2008). In addition, canine models naturally develop an age-related cognitive dysfunction that reproduces several aspects of AD (Head, 2007; Siwak-Tapp *et al*., 2008). Numerous studies on dogs, which examined several behavioral paradigms, have revealed subsets of aged dogs with learning and memory impairments (Head *et al*., 1995; Adams *et al*., 2000; Smith *et al*., 2001). Thus, dogs have been identified as a unique model for studying a human disorder such as AD.

Although a canine model is ideal for studying human diseases, no such model has been reported yet because of the lack of canine embryonic stem cells, which are generally required for gene targeting and for producing mature oocytes *in vitro*. From the molecular perspective, the dog is a suitable animal model for AD because APP and most of the enzymatic machinery for processing this factor are highly homologous between dogs and humans. This genetic similarity allows us to expect concordance in regulation of gene expression and disease development in transgenic puppies. This will facilitate the understanding of the disease process and phenotype development over time.

Previously, Hong *et al*. (2009) reported on the production of a transgenic dog expressing red fluorescent protein using retroviral gene delivery methods. Although efficient genetic modification of donor cells is a key prerequisite to produce transgenic animals, viral delivery systems are associated a minor risk of neoplastic transformation (Sokol *et al.*, 1996). To prevent these unwanted effects,

five genetically engineered puppies expressing mhAPP were produced using a liposomal transfection method. Stable transfection of canine fibroblasts with a liposomal reagent is a very effective method that can replace viral gene delivery techniques. In the present study, the expression and selection cassettes were successfully integrated into genomic DNA and effectively expressed in tissues after SCNT. This non-viral, SCNT-mediated gene delivery system is a safer protocol for creating animal disease models than viral gene delivery.

Dogs naturally develop Alzheimer's like senile plagues similar to human and the plaques were initially observed between the ages of 8 and 9 years (Martin *et al*., 2011). The pathological features in aged dogs having AD like symptoms are quite similar to human's phenotypes such as neuronal loss and cognitive deficit. These progressive studies suggest that canines are the best model for human AD medication. Although the aged canine model has several advantages, more than 8 years to develop AD symptom is still major obstruct for general usage in a research field. Hence transgenic canines having mutated human APP to early onset AD phenotype in the present study are demonstrated.

In conclusion, a canine model of AD to replace the current rodent models was successfully produced. This is important as canines possess a higher intelligence than mice and are large enough to undergo surgical procedures in pre-clinical studies. This canine model expressing the mhAPP gene produced by SCNT may be used for understanding the pathological and developmental characteristics of AD, and may aid in the development of novel therapeutic model for AD.

General conclusion

This report has explored the various facets of the transgenic dog; from which breed is reliable to produce cloned dogs to which candidate genes can be used to reproduce the cloned dogs reflecting the human disease using transgenic technique.

Somatic cells from various breeds from small to large-sized dogs were employed as donor nuclear for cloning. They were successfully reprogrammed into the oocytes, leading to the production healthy pups. The different genetic background may affect the efficiency of somatic cell cloning, the BW and the gestational length after the embryos transfer. Furthermore, the meiotic maturation of recipient oocyte and the passages of donor nuclei did not affect the cloning efficiency. However, the reconstructed embryos from immature oocytes used as the recipient cytoplasts had neither attached nor implanted, while those from aged oocytes had maintained a full term pregnancy.

Beagles, which were used as the selective breed, showed the higher pregnancy output, making them relatively favorable for the further rounds of transgenic dog models. Fetuses were surgically obtained on Day 25 after natural breed. PEPCK gene that participates in the induction of gluconeogenesis for T2DM models, and APP gene that contributes to the induction of amyloidogenesis for AD models were isolated and transfected into the canine fetal fibroblasts using the liposomal carrier. The PEPCK or APP stably overexpressing cell lines were established and used as donor nuclei for the nuclear transfer.

For producing T2DM dog model, three puppies were born and confirmed genetically identical to the donor with the microsatellite analysis. Among them, one puppy was confirmed to show the presence of exogenous PEPCK gene using PCR. Using RT-PCR, relatively high expression of PEPCK gene was examined in the liver biopsy. However, no diabetic symptoms were observed in the dog.

For producing AD dog model, the second generation was produced from the cloned transgenic male and female. One of the transgenic dogs in the second generation showed spontaneous tetanic convulsion that were examined AD-like symptoms such as enlarged ventricles, atrophied hippocampus, and β-amyloid plaques in the brain. Although the presence and expression of the inserted genes from the dog were indentified, the current transgenic dog has not yet presented the exact symptoms interested. Therefore, further in-depth studies are needed to probe the functions of the transgene and its patterns of genetic expression in different organs.

Coupling with transgenic technologies, ART provide powerful tools for the development of genetically living organisms for the agricultural and medical applications and the analysis of gene functions. Domestic dogs, as mentioned in previous chapters, provide valuable animal models to study various human diseases. In the present study, the transgenic cloned dogs that carried the exogenous genetic constructs were successfully generated, but those animal models are yet to show the typical phenotypes of T2DM and AD.

Two major reasons could be postulated for coming up the undesirable symptoms in this dog model. Although numerous successes in producing transgenic animals using the overexpression of the exogenous genes for the genetic transformation of donor cells, the development of the intended phenotype with the gene expression in the target organ is still largely low. It may be attribute to the inefficient delivery of the genetic materials to the nucleus and undesirable integration of transgenes, leading to random insertion into the host genome, and misexpression of the transgene as result of proximal genetic control elements, or local chromatin folding (Horn *et al.,* 2002; Wang *et al.,* 2003). Moreover, two representative diseases researched in the thesis are considered polygenic and multi-factorial disease. Pathophysiology of those diseases in dog unlike those in mice is assumed to be more complex and variable. Therefore, the diversity of mechanisms can be orchestrated in compensating for the disease condition originated from the expression of a single exogenous gene.

Cloned embryos derived from various dog breeds could give birth to healthy offsprings into medium-sized recipients. The cell lines derived from different breed could influence the efficiency of somatic cell cloning and fetal survival after embryo transfer. The canine model generated in the present study could not perfectly embody the symptoms of the T2DM and AD model. However, the study of generations of cloned pups carrying transgene helped lay the foundation for the novel model to better understand human disease.

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Abstract in Korean

국문초록

사람 Phosphoenolpyruvate Carboxykinase와 Amyloid Precursor Protein 유전자가 도입된 형질전환 체세포 복제 개 생산

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역사적으로 개는 반려 동물로서 인간에게 친근한 존재이며, 최근에는 사람의 질병을 연구하는 질환 모델 동물 또는 신약 개발에 전임상 동물로서 중요시 되고 있다. 본 연구의 목적은 1) 개 복제 효율에 영향을 미칠 수 있는 여러 인자를 확인하고 2) 당뇨병과 치매 유발에 각각 관여하는 phosphoenolpyruvate carboxykinase (PEPCK)와 amyloid precursor protein (APP) 유전자가 도입된 복제 개를 생산하고 그 특성을 분석하는 것이다.

첫 번째 연구에서는 10개의 품종을 대상으로 체중에 따라 소형견, 중형견, 대형견, 초대형견으로 구분하고 각 품종에서 채취한 체세포를 이용하여 핵이식란을 생산한 후 대리모에 이식하였다. 이식 후 30일의

수태율과 분만율에는 유의적 차이가 없었다. 이식한 복제란 대비 생존한 분만자견의 비율은 초대형견(2.4%), 대형견(1.0%), 중형견(0.9%), 소형견 (1.1%)로 초대형견에서 유의적으로 높았다 (P<0.05). 하지만 품종간 이식한 복제란 대비 생존한 분만자견 비율은 유의적 차이가 없었다. 각 품종에 따른 초기 유산율과 후기 유산율은 차이가 없었다. 임신 기간은 소형견 (58.8±0.3일)이 초대형견 (59.8±0.1일), 대형견 (60.7±0.3일), 중형견 (58.8±0.3일)에 비해 유의적으로 짧았다 (P<0.05). 복제자견의 분만 체중은 소형견 (209.2±10.5g)과 중형견 (412.7±15.3g)에 비해 대형견 (551.4±28.1g)과 초대형견 (575.4±18.8g)에서 유의적으로 높았다 (P<0.05). 초대형견과 소형견이 중형견과 대형견에 비해 산자수가 증가하는 경향을 보였으나 산자수는 이식한 핵이식란의 수와는 연관성이 없었다. 공여핵원의 계대수와 수여난자의 성숙정도가 수태율과 분만율에는 영향을 주지 않았다. 다만, 미성숙 유래 핵이식란은 이식후 수태가 되지 않음을 확인하였다.

두 번째 연구에서는 비글견의 태아 섬유아세포를 배양하여 포도당 신생과정을 조절하는 중요한 인자로 알려진 PEPCK를 과발현하는 세포주를 공여핵원으로 사용하여 핵이식을 수행하였다. 핵이식란을 5 마리의 대리모에 이식한 결과 그 중 2 마리로부터 3 마리의 복제자견이 생산되었다. 3 마리의 복제자견 중 2 마리에서 녹색 형광이 발현되었고 PEPCK 유전자가 존재함을 확인하였다. 이 중 한 마리에서 간 조직을 채취해 PEPCK mRNA와 단백질이 과발현 되었다. 하지만, 직접적인 당뇨증상은 발견되지 않았다.

세 번째 연구에서는 비글견의 태아 섬유아세포를 이용하여 변이 사

람 APP를 과발현하는 형질전환 세포주를 확립하였고, 이 세포를 활용한 핵이식을 통해 암컷과 수컷 형질전환 개를 생산하였다. 성 성숙에 도달한 암수 복제 개를 교배시켜 7 마리의 자견을 생산하였다. 이들 중 한 마리가 6 개월령에서 간헐적인 전신 경련 증상을 보였고 MRI 결과 뇌실 확장과 해마 위축이 확인되었다. 병리조직학적인 검사 결과 β-amyloid plaque이 관찰되었다.

본 연구 결과 개에서 복제 효율 및 복제 자견의 생산과 관련된 번식 지표는 체세포 공여견에 따라 달라짐을 알 수 있었다. 또한 개에서 핵이식 기법에 의해 PEPCK와 APP 유전자 도입 형질전환 자견을 생산하였다. 이 연구의 결과는 개복제 효율을 높이고, 당뇨병과 알츠하이머병 같은 유전적 질환의 모델 동물로서 형질전환 복제 개의 활용 가능성을 제시하였다.

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주요어: 개, 체세포 핵이식, 당뇨병, PEPCK, 알츠하이머 병, APP, 배아 이식

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