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The promise of dog cloning

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Abstract. Dog cloning as a concept is no longer infeasible. Starting with Snuppy, the first cloned dog in the world, somatic cell nuclear transfer (SCNT) has been continuously developed and used for diverse purposes. In this article we summarise the current method for SCNT, the normality of cloned dogs and the application of dog cloning not only for personal reasons, but also for public purposes.

Additional keywords: normality, reproductive technique, somatic cell nuclear transfer.

Introduction

The first animal cloned from somatic cells, Dolly the sheep, represented a great innovation in biotechnology (Campbell *et al.* 1996). Since then, cloning technology has developed rapidly and the cloning of many species, including the mouse (Wakayama *et al.* 1998), pig (Polejaeva *et al.* 2000), rabbit (Chesné *et al.* 2002) and cat (Gómez *et al.* 2003), using somatic cell nuclear transfer (SCNT) was reported within a decade. The dog was considered difficult to clone because of its specific reproductive features and low rate of oocyte *in vitro* maturation. Nevertheless, through many trials and errors, the first cloned dog, Snuppy, was born in 2005 (Lee *et al.* 2005). At the time when Snuppy was born, it was predicted that 'cloning technology could become a useful research tool for studying the genetics of outcrossed populations' (Lee *et al.* 2005). SCNT used in dog cloning has actually been used for the propagation of the genetics of valuable individuals and the production of disease models as a biomedical resource (Kim *et al.* 2012; Oh *et al.* 2016). This article chronologically reviews the principles, methods and results of dog cloning research conducted in recent decades. We then review the productive outcomes of SCNT in dogs and foresee future advances in dog cloning.

Overview of dog cloning

The most significant difference between normal fertilisation and cloning is the genetic information in the resulting offspring. In normal fertilisation, the spermatozoon and oocyte fuse into a zygote, which develops further to form the blastocyst. After implantation and pregnancy, offspring are born containing a combination of genes from the father and mother. In cloning, although the cloned offspring has no shared genetic information with the biological oocyte donor and recipient, the nucleus of a somatic cell is inserted into an enucleated oocyte to obtain a cloned embryo. Through SCNT, cloned offspring that share identical genetic information with the somatic cell donor can be born.

To accomplish cloning of dogs, there are difficulties that need to be overcome that are related to the unique reproductive physiology of the dog. Dogs have a mono-oestrous, polyovulatory and non-seasonal reproductive cycle. Dogs need approximately 4 months of obligatory anoestrus to prepare for the next pregnancy, in contrast with the short periods of pro-oestrus (a rise in oestrogens) and oestrus (allow mating) of 13–16 and 4–12 days respectively. Moreover, there is a 2-month period of dioestrus during which progesterone (P4) levels are high, a typical characteristic of dogs (Johnston *et al.* 2001). This means that *in vivo*-matured oocytes from natural ovulation can be collected every 6–7 months. Dogs ovulate immature oocytes at the germinal vesicle stage and the meiotic resumption of these oocytes is delayed (Johnston *et al.* 2001). The oocyte needs an approximate 72-h maturation period in the oviduct for fertilisation to be successful (Holst and Phemister 1974; Reynaud *et al.* 2005).

In addition to problems regarding the reproductive cycle, it is more difficult to collect oocytes from dogs because the quantity of oocytes used for experiment is lower than for other domestic animals, such as cows and pigs, whose ovaries can be obtained from abattoirs; in addition, the efficiency of IVM for canine oocytes is very low (Luvoni *et al.* 2005). Recently, several protocols using exogenous gonadotrophins, dopamine agonists and gonadotrophin-releasing hormone (GnRH) agonists have been reported to induce oestrus in bitches (Fontaine *et al.* 2011). However, it is difficult to use to SCNT in dogs because the

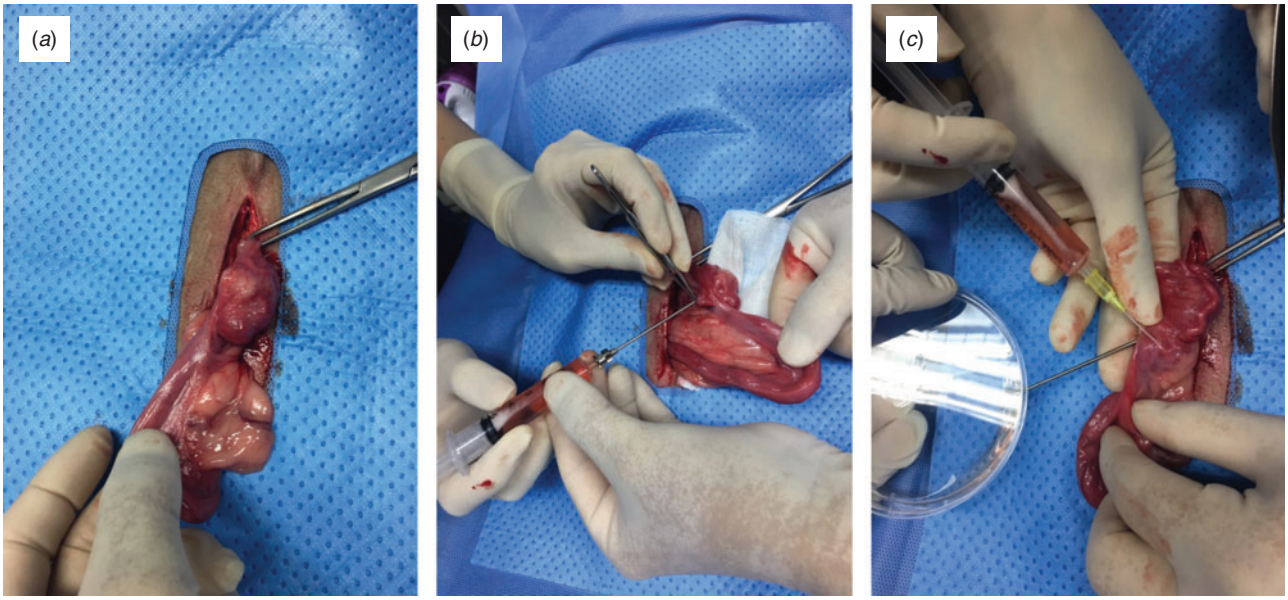

Fig. 1. Surgical method used to collect canine oocytes. (*a*) The uterine horn and ovary are exposed and the suspensory ligament is fixed with Allis tissue forceps. (*b*) An inverted flanged bulb needle is inserted in the oviducal opening, which can be found within the ovarian bursa. (*c*) An intravenous catheter is inserted from the uterotubal junction to the caudal portion of the oviduct, and HEPES-buffered tissue culture medium is used to flush out oocytes.

number of oocytes is less than that of natural ovulation. Thus, *in vivo*-matured oocytes derived from oestrus batches are used for SCNT.

Determining the timing of natural ovulation of oocyte donors is crucial for canine oocyte recovery (Reynaud *et al.* 2005). Ovulation can be predicted using serum P4 concentrations and vaginal smears. Serum P4 concentrations increase rapidly above 2 ng mL^{-1} during the preovulatory LH surge, and luteinisation of preovulatory follicles occurs immediately (Reynaud *et al.* 2015). Canine oocyte ovulation occurs when the P4 concentration reaches 4.0–9.0 ng mL^{-1} (Kim *et al.* 2010; Reynaud *et al.* 2015).

Methods of dog cloning

Oocytes and donor cell collection

We have developed an efficient technique for oocyte recovery in dogs by flushing the fallopian tube (Lee *et al.* 2005). The day of ovulation in a dog can be determined by analysis of daily increases in serum P4 concentrations (Kim *et al.* 2014). After observation of pro-oestrus vaginal bleeding, blood is collected every day or every the other day until the P4 concentration reaches >10 ng mL^{-1}. Ovulation can be regarded as the day when P4 concentrations reach 4–10 ng mL^{-1} (Johnston *et al.* 2001). Approximately 72 h after ovulation, the oocyte donor dog is anaesthetised and the surgical region is prepared aseptically (Kim *et al.* 2014). A midline incision is made 2 cm from the umbilicus, and the uterine horn and ovary are exposed. The suspensory ligament is temporarily fixed outside the abdominal cavity. An inverted flanged bulb needle is inserted into the oviducal opening and kept there using a stay suture. For collection of matured oocytes, an intravenous catheter is inserted into the oviduct near the uterotubal junction to the caudal portion of the oviduct. The intravenous catheter is connected to a syringe containing TCM-199 (Invitrogen) supplemented with 10 mM HEPES, 2 mM $NaHCO_3$, 5 mg mL^{-1} bovine serum albumin (BSA; Invitrogen) and 1% (v/v) penicillin–streptomycin. The medium in the syringe is flushed out from the end of the bulb needle, and the medium containing ovulated oocytes is collected in a Petri dish (Fig. 1). The number of oocytes collected can be compared with the number of corpora lutea (CL) by tearing off the thin ovarian bursa. A retrospective study showed that the oocyte recovery rate (number oocytes recovered/number CL counted) of surgical flushing of the fallopian tube was 94% (Kim *et al.* 2010). The maturation stages of canine oocytes are categorised as immature, mature, early aging, moderate aging and severe aging (Jang *et al.* 2007; Kim *et al.* 2014). Results of experiments cloning female dogs after the cloning of Snuppy showed that cloned puppies could only be successfully produced from the group of oocyte donor dogs that provided mature oocytes (Jang *et al.* 2007). Similarly, in the case of a cloned Sapsaree, a traditional Korean breed, pregnancies only occurred from mature donor oocytes (Jang *et al.* 2009).

Donor cells can be obtained from several cell types, including skin fibroblasts (by simple biopsy), fetal fibroblasts (from fetuses on Day 28 of gestation) and mesenchymal stem cells (from adipose tissue). Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) or RCME-P, a medium for adipose-derived stem cell culture (Biostar) is used as culture medium, with the number of passages of cells used as nuclear donors ranging from two to seven. Cells are harvested when they have reached 80–90% confluence using 0.25% trypsin. In addition, for successful SCNT, there should be cell cycle synchronisation of donor cells. Nuclear reprogramming can be enhanced when donor cells are in the G_0 or G_1 phase (Wilmut *et al.* 1997). It is known that contact inhibition,

chemical treatment and roscovitine treatment (15 μg mL^{-1} for 24 h) has positive effects on cell cycle synchronisation and cloning efficiency (Oh *et al.* 2009).

Somatic cell nuclear transfer

The SCNT process consists of enucleation of the donor oocyte, injection of the donor cell, cell fusion, activation and usually a period of culture before embryo transfer. Enucleation is the step of removing the nucleus from the recipient oocyte. Before starting enucleation, cumulus cells of collected cumulus–oocyte complexes should be removed by moderate pipetting in medium with hyaluronidase. The first polar body and metaphase spindle of denuded oocytes are removed by micromanipulation. Cultured somatic cells derived from the donor animal are injected into the perivitelline space of each enucleated oocyte. The cell–oocyte couplet is placed between microelectrodes in mannitol medium and fused with two direct current pulses of 3.8–4.0 kV cm^{-1} for 15 μs using an Electro-Cell Fusion apparatus (NEPA GENE; Park *et al.* 2011). Activation is one of the procedures in natural fertilisation after the spermatozoon penetrates the oocyte. Similarly, in SCNT embryos, development is initiated after oscillations of intracellular calcium, which are originally initiated by spermatozoa. In dog cloning, this activation process is induced artificially using 10 μM ionophore and 6-dimethylaminopurine treatment for 4 h (Lee *et al.* 2005).

Embryo transfer

In the case of canine embryos, a method for *in vitro* embryo culture has not been developed, largely because of the inaccessibility of oocytes and lack of a successful method for the IVM of oocytes. Therefore, in SCNT, immediately after activation, presumptive zygotes are surgically transferred to the oviducts of recipient dogs. The oestrous cycle of recipient females should either be synchronous with or 1 day earlier than the oocyte donor dog. As mentioned earlier, we can determine the timing of ovulation in the oocyte donor and the recipient using P4 concentrations. Pregnancy rates were similar in recipients that were synchronous with the oocyte donor and in those that were in advanced synchrony (Kim *et al.* 2010). In addition to cycle synchronisation, parity of the recipient is another factor affecting pregnancy rates. Nulliparous dogs had a higher delivery rate than multiparous females; therefore, we recommend using nulliparous dogs as surrogates for SCNT embryos (Kim *et al.* 2010). For embryo transfer, the oviduct of a recipient is exposed using the same technique as used for recovery of oviducal oocytes. Cloned embryos (i.e. presumptive zygotes) are loaded into a 3.5-Fr Tom Cat Catheter (Sherwood) and the catheter is inserted into the opening of the oviduct. On average, 15 cloned embryos are transferred into each recipient. Pregnancy is diagnosed by ultrasonography. After pregnancy is confirmed, the delivery date is predicted as 63 days after ovulation and 60 days after embryo transfer.

Normality of cloned dogs

From analyses of the health and reproduction of cloned dogs, it has been demonstrated that growth patterns, including bodyweight, height, bone development and blood composition, of SCNT cloned dogs are identical to those in non-cloned dogs. The skull, pelvis and lumbar vertebrae were measured by radiography and haematological and serum biochemical values were collected from three female cloned dogs (Park *et al.* 2010). Cloned dogs and the donor dog had similar growth patterns (bodyweight, height and bone). In addition, cloned dogs had similar clinical characteristics as control dogs and age-specific results of the haematological analyses in both cloned dogs and age-matched controls were within the normal reference ranges of healthy dogs. Fatal side effects regarding the health of cloned dogs have not been reported.

Reproduction ability is also normal in cloned male and female dogs (Park *et al.* 2009). Sperm motility in a cloned male dog was within the normal range and the spermatozoa of this dog were able to fertilise matured oocytes, resulting in embryo development to the 8-cell stage. Similarly, cloned female dogs showed standard reproductive cycling of P4 concentrations and vaginal cytology. After sperm and hormone analysis, artificial insemination (AI) was conducted between cloned dogs. Ten puppies were born by natural delivery after AI between cloned male and female dogs, and these puppies grew similarly to non-cloned dogs.

Moreover, it was recently revealed that genetically identical cloned dogs have similar learning, memory and exploratory abilities (Shin *et al.* 2016). Genetically identical beagles, cloned by SCNT, were subjected to behavioural examination. For learning and memory similarities, variations in time to reach the obligatory preferred site were evaluated between clones and control dogs. Not only did clones show a significantly lower time to reach the obligatory preferred sites than control dogs, but they also showed less variation compared with controls. For exploratory similarities, a permutation test to analyse the significance of variation in values between clones and controls was conducted and the cloned dogs showed significantly less variation in the open field test. It was concluded that cloned dogs show similar cognitive and exploratory patterns, sharing behavioural phenotypes related to the genotypes of the original donors (Shin *et al.* 2016). In addition, another study analysed puppy aptitude using the Volhard test on two cloned dogs derived from an elite rescue dog (Battlett 1979). The two cloned puppies were classified as belonging to the same type, accepting humans and leaders easily. That study indicated that dogs cloned using donor cells derived from one elite dog have similar behavioural tendencies (Oh *et al.* 2016).

Applications of dog cloning for preservation of endangered species, pet dogs and elite working dogs

Since 2005, when Snuppy, the first cloned dog in the world, was born, the canine SCNT technique has been established (Lee *et al.* 2005), making it possible to use canine SCNT in various fields (Fig. 2). The uses of cloning in canids during the early days were for the conservation of endangered species (Oh *et al.* 2008; Kim *et al.* 2012) and the cloning of pet dogs (Jang *et al.* 2008; Park *et al.* 2011).

Cloning of endangered species

Species extinction is a universal issue and the number of endangered species continues to increase. To overcome this,

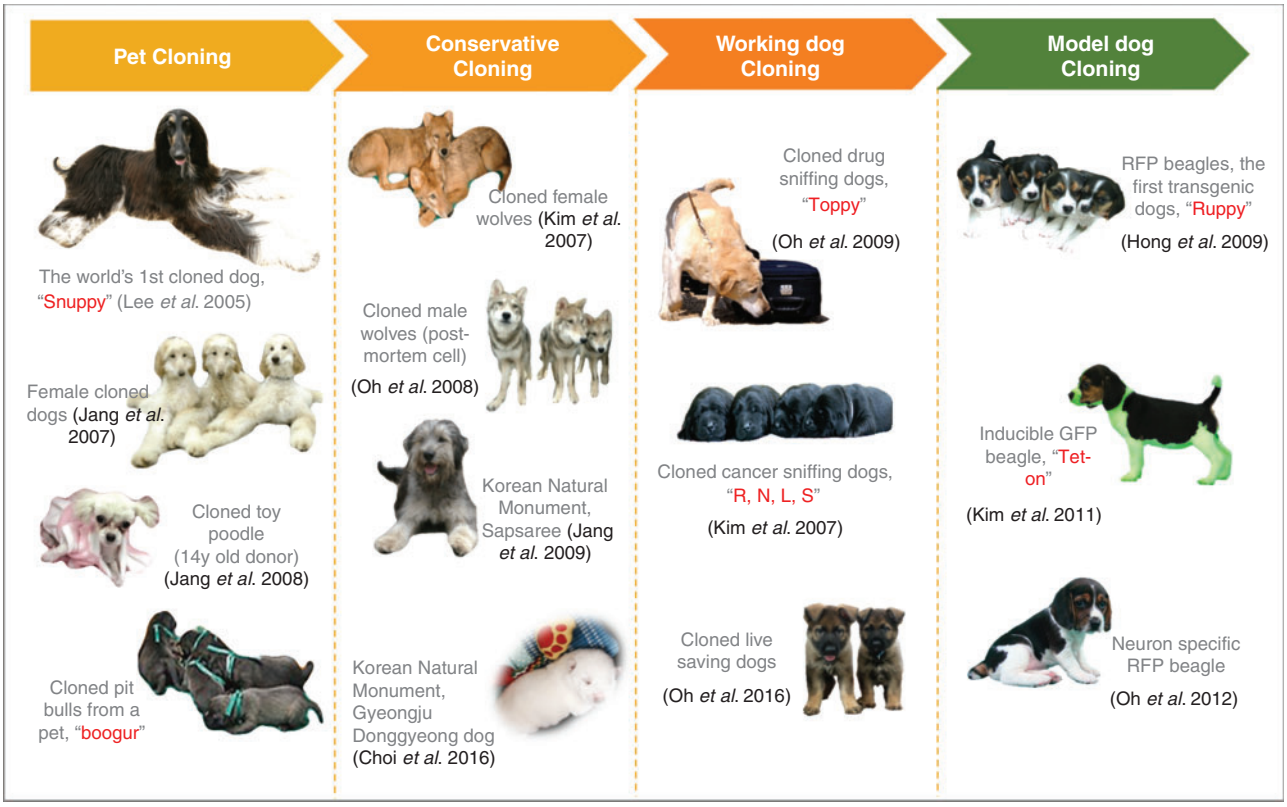

Fig. 2. Milestone of canine somatic cell nuclear transfer in various fields.

assisted reproductive technology (ART) for endangered species has been developed, including SCNT, an efficient method for the preservation of species. The grey wolf has been considered a threatened species in many countries and the Korean grey wolf has been successfully cloned (Kim *et al.* 2007). It was not possible to find grey wolf recipients because of their low numbers. As a result, intraspecies SCNT (iSCNT) using a somatic cell from a wolf, an oocyte from a dog and a dog recipient has been used. The process of iSCNT was same as original dog cloning. Microsatellite analysis revealed that the cloned wolves had the same genetic background as the cell donor wolf (Kim *et al.* 2007). However, the mitochondrial (mt) DNA in cloned wolves was the same as the oocyte donor dog, not the cell donor wolf. In the case of the Sapsaree breed, which is not listed as an endangered species but is worth preserving as a national treasure in South Korea, mixed-breed dog oocytes were used in SCNT (Jang *et al.* 2009). The Gyeongju donggyeong dog, another Korean national breed, was also cloned by SCNT and its phenotype was successfully conserved (Choi *et al.* 2016). As for the grey wolf, the genetic information of the clones was identical to that of the nuclear donor dog, whereas the mtDNA was inherited from the oocyte donor dog. Thus, we have demonstrated that dog SCNT is useful for preserving endangered canid species.

Cloning of pet dogs

The basic significance of cloning pet dogs is fulfilling the emotional needs of owners to preserve time with their favourite companion dogs. People want to share their lives longer with their dog but dogs have a shorter life span than humans and, as they age, they can possibly suffer from senile diseases. For that reason, the demand for cloning of pet dogs is increasing. A 14-year-old toy poodle (Jang *et al.* 2008) and five pit bull terriers cloned from the skin cells of a 15-year-old dog named Booger were cloned in 2008. The clones of Booger have significance in that they have realised the dreams of many owners, demonstrating the commercial application of dog cloning technology (http://news.bbc.co.uk/2/hi/asia-pacific/7542338.stm, accessed 5 October 2017). Cloning of old, small breed dogs has also been demonstrated: ear fibroblasts were obtained from a toy poodle, whereas the oocyte donors and recipients were large breed dogs (Jang *et al.* 2008). Through the SCNT process, one puppy from a recipient was delivered by Caesarean section. Analysis of the cloned poodle revealed that its genetic information was identical to that of the nuclear donor dog and its mtDNA was from the oocyte donor. A telomere length assay was conducted in an age-matched control poodle, the donor poodle and clone poodle, revealing that there was no significant difference in telomere length among three dogs. Interestingly, there was also no evidence of aging in the toy poodle cloned using 14-year-old donor cells (Jang *et al.* 2008). Therefore, it is concluded that telomere length is not an indicator of aging in the toy poodle and the genetic age of cloned dogs is not the age of the cell donor dog, rather it is reset to the beginning.

Cloning of elite working dogs

There are working dogs in human society, including drug sniffer dogs, quarantine dog and cancer-sniffing dogs. These elite dogs can use their remarkable sniffing ability in public service fields. Optimal utilisation and selection of elite dogs that show the desirable characteristics of boldness and concentration will require significant expenditure of time and effort supported by adequate financial resources. Despite substantial investment, the final selection rate is less than 30% and the top of top elite dog is highly rare. To overcome the supply limitations of elite dogs, dog cloning technology could be a possible solution. The technique has generated seven cloned beagles, Toppy, from a retired elite drug sniffing dog named 'Chaser' in the Korean custom service (Oh *et al.* 2009), and two cloned dogs from an elite rescue dog (Oh *et al.* 2016). The sniffing abilities of the seven Toppy dogs were evaluated (Choi *et al.* 2014). The Toppy were trained with the same number of control dogs; the Toppy had the exact same genetic information as the elite drug sniffing dog, whereas the control dogs were the offspring of sniffer dogs. Surprisingly, all seven Toppy were selected with high scores, in contrast with the control group, of which three of the seven trained dogs were selected (Choi *et al.* 2014). In the 6 months after the seven Toppy clones were added to airport security, the drug detection rate increased sixfold, at the same time saving the budget for selecting elite dogs. Thus, outstanding abilities can be passed on to the next generation by cloning identical dogs that inherit identical genetic material.

Dog cloning can be used to produce elite dogs for biomedical purposes, as demonstrated by the cloning of an elite cancer-sniffing dog. Marine, known as a cancer-sniffing dog, had the unique ability to detect human cancer. A Japanese research group evaluated the cancer-sniffing ability of Marine and reported that Marine showed 94% sensitivity and 99% specificity for colorectal cancer screening (Sonoda *et al.* 2011). Marine was cloned to preserve her ability, which was the only option because she had been neutered after suffering from pyometra. Four dogs were cloned from marine's skin cells and were trained the as same as Marine. The cloned dogs showed similarly excellent ability to detect cancer, with 93.9% sensitivity and 99.5% specificity for the detection of breast cancer (Kim *et al.* 2015). Thus, elite working dogs in diverse fields can be propagated by using the SCNT technique with donor cells obtained from small pieces of tissue.

Applications of dog cloning for human disease models

As the dog cloning protocols were established and stabilised, the ultimate aim of dog cloning was to apply the technique to develop disease models. It has been reported that current animal models are poor predictors of patient responses, which leads to failure in drug discovery (Perrin 2014). In contrast, dogs have common disease features to humans, including histological appearance, tumour genetics, molecular targets, biological behaviour and responses to conventional therapies (Paoloni and Khanna 2008). Therefore, a new concept of disease model animals, including large animal models, has to be developed. Dogs receive exceptional medical care, have comparable organ sizes to humans and generally cohabitate with their human owners, minimising different environmental effects. Dogs also have a very similar physiology and disease pattern compared with humans. In addition, for biomedical resources, dogs are easy to handle and communicate with humans. The Mendelian inheritance in animals database indicates 405 types of canine inherited disorders could be used as potential models for human disease because they have similar characteristics to those of human diseases, including histological appearance, genetic distance, biological behaviour, symptoms and response.

The possibility of using cloned dogs as models of human disease was demonstrated by the reporter gene system. Using fetal fibroblast cells transfected with red fluorescent protein (*RFP*) genes, which can be used as a reporter gene under the cytomegalovirus promoter, the first transgenic cloned dogs expressing *RFP* genes were constitutively cloned (Hong *et al.* 2009). Unlike control beagles, in transgenic beagles the red fluorescence is expressed in the whole body and organs. In addition, the transgenic cloned dogs exhibited normal reproductive ability and the foreign gene was successfully inherited by their offspring, and the transgene was stably inserted into their genome (Hong *et al.* 2011). As a next step, methods for an inducible transgenic system have been developed, including a tetracycline-inducible vector system. Consequently, inducible transgenic cloned dogs expressing green fluorescent protein genes have been produced (Kim *et al.* 2011). This inducible dog model can be widely used for a variety of biomedical research studies. An early example of cloning of dogs as a disease model was the production of a transgenic dog that expressed a neuron-specific transgene under the control of human synapsin promoter (Oh *et al.* 2012). After SCNT using neuronal-specific RFP-expressing cells, three cloned pups were delivered. In pups that were 4 years of age, the RFP protein was observed in samples of the nervous system collected postmortem, including the brain, spinal cord and peripheral nerves, but RFP expression was not detected in non-neuronal tissue such as the heart, kidney and skin (H. J. Oh, K. Ra, M. J. Kim, G. A. Kim, E. M. N. Setyawan, S. H. Lee and B. C. Lee, unpubl. data). This study revealed that the human synapsin promoter works in neuron cells in the dog and will be a useful tool for producing a human neuronal disease model.

The marked increase in average life expectancy over the course of the 20th century ranks as one of society's greatest achievements. With increased life expectancy, the anti-aging field has been growing rapidly. The major model systems used to study human aging are human cells, the fruit fly *Drosophila melanogaster* and rodents, such as mice and rats. However, the small size and short life cycles of these organisms is very limiting for anti-aging studies. For this reason, we used dogs in an anti-aging study recently. In the elderly, progressive loss of skeletal muscle mass and function accompanied by weight loss, weakness, decreased mobility and impaired locomotion are commonly observed. In humans, there is an approximate 1% decrease in muscle mass every year after the age of 30 years, and the rate of decline accelerates after 65 years of age (Nair 2005). As an anti-aging model, three transgenic beagles expressing peroxisome proliferator-activated receptor (PPAR) α in a muscle-specific manner were produced (H. J. Oh, K. Ra, M. J. Kim, G. A. Kim, E. M. N. Setyawan, S. H. Lee and B. C. Lee, unpubl. data). Transgene insertion in the genome of these dogs

and higher expression of PPARα transcripts were confirmed. A significantly faster decline in serum glucose concentrations after the oral glucose tolerance test was seen in transgenic dogs, which may result from the high rates of β-oxidation in their muscle. Analyses of exercise ability and muscle mass in these dogs are now underway.

In addition, the knockout technique has been used to generate human Duchenne muscular dystrophy (DMD) dogs. DMD is an X-linked disease caused by mutations in the *DMD* gene and loss of the protein dystrophin. Numerous animal models of DMD have been developed over the past 30 years. The mdx mouse is the most commonly used model in studies of DMD pathogenesis and treatment development. However, the clinical syndrome in the golden retriever muscular dystrophy (GRMD) dog is more severe than in mice and shows better alignment with the progressive course of DMD in humans. Thus, canine DMD is regarded as a better model of human DMD (Duan 2015) than other animal models. To generate a DMD dog, cytoplasmic injection of Cas9–sgRNA (a genome editing method enabled by Cas9 nuclease and single-guide RNAs (sgRNAs) efficiently manipulating target loci) in canine embryos was used by targeting dystrophin exon 6 (H. J. Oh, K. Ra, M. J. Kim, G. A. Kim, E. M. N. Setyawan, S. H. Lee and B. C. Lee, unpubl. data). The highest homologous mutation rate after Cas9 cytoplasmic injection into zygotes at different stages was seen in the 4-cell group, but the heterogeneous mutation rate in the 2-cell group was higher than in the 4-cell group. Cas9 cytoplasmic injected embryos were transferred into recipients to generate knockout dogs. Unfortunately, the generation of knockout dogs failed, with only one pup showing a positive mutation based on the T7e1 assay (H. J. Oh, K. Ra, M. J. Kim, G. A. Kim, E. M. N. Setyawan, S. H. Lee and B. C. Lee, unpubl. data). This pup is mosaic and it is considered that this mosaicism occurred as a result of the injection of two or four cells, not one cell. Furthermore, in order to produce a *DMD*-knockout dog, SCNT will be performed using donor cells with *Cas9* mRNA and sgRNA for dystrophin.

Conclusion

The development and establishment of dog cloning technology is a notable achievement in the field of biotechnology. With time, SCNT technology has become more efficient and the application of dog cloning will be broadened in the future. Cloning of elite dogs is already acknowledged as efficient and stable utilisation of this technology. The combination of transgenic or knockout methods and SCNT will be key to generating human disease dog models. Further research into transgenic dogs will become more active, considering their importance in providing resources to solve disease problems. Thus, dog cloning is obviously improving and further developments are highly expected.

Conflicts of interest

The authors declare no conflicts of interest.

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