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Interspecies Somatic Cell Nuclear Transfer Technique for Researching Dog Cloning and Embryonic Stem Cells

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ABSTRACT : Large quantities of high-quality recipient oocytes with uniform cytoplasm are needed for research in the promising field of somatic cell nuclear transfer (SCNT) and embryonic stem cell research. In canines, however, it is difficult to obtain large quantities of oocytes because each donor produces a limited number of mature oocytes *in vivo*. Although *in vitro* maturation (IVM) is considered an alternative approach to oocyte production, this technique is still too rudimentary to be used for the production of high-quality, uniform oocytes in large quantities. One technique for overcoming this difficulty is to use oocytes obtained from different species. This technique is known as interspecies SCNT (iSCNT). This review provides an overview of recent advances in canine - porcine interspecies SCNT. (**Key Words :** Canine, Interspecies, Porcine, Somatic cell Nuclear Transfer)

INTRODUCTION

Dogs are useful models for human drug discovery and basic research because the physiology, disease presentation, and clinical responses of dogs are more similar to humans than various other traditional animal models. More than half of the approximately 400 known hereditary canine diseases have an equivalent human disease, including cardiomyopathies, muscular dystrophy, and prostate cancer. Moreover, the dog is the most prevalently used species in early transplantation research (Starkey et al., 2005).

Thus far, as reproductive technology in dogs, studies related to time of insemination, artificial insemination, hormonal profiling, semen freezing, embryo transfer and *in vitro* production of embryos have been done for breeding and genetic conservation (Yamashiro et al., 2007; Sugimura et al., 2008; Yamashiro et al., 2009). On the other hand, as highly-developed technology, development of human disease models using a transgenic approach that employs somatic cell nuclear transfer (SCNT) has been eagerly anticipated. Furthermore, dogs may serve as an appropriate animal model for a new strategy of therapeutic cloning using embryonic stem cells (ESCs) derived from SCNT embryos (ntESCs). Thus far, there have been reports on the successful cloning of puppies by somatic cell nuclear transfer (SCNT) (Lee et al., 2005; Jang et al., 2007; Jang et al., 2008a; Kim et al., 2008). However, the efficiency of the procedures involved has been reported to be extremely low. To improve the cloning efficiency of canine SCNT techniques, it is important to optimize the various elements used, such as the types of donor cells, the recipient oocytes, the activation protocol, the *in vitro* culture system and several other variables. For many mammalian species, the SCNT techniques have already been optimized by monitoring the types of donor cells used (Wakayama and Yanagimachi, 2001; Lagutina et al., 2005) and selecting procedures for cell passage (Kubota et al., 2000; Li et al., 2003; Zhao et al., 2007; Zhang et al., 2008); treatment of the SCNT embryos with histone deacetylase inhibitors such as trichostatin A (TSA) (Kishigami et al., 2006) and scriptaid (Thuan et al., 2009); and allowing early-stage SCNT embryos to aggregate in order to increase the total number of cells at the blastocyst stage have also been undertaken (Boiani et al., 2003; Terashita et al., In press). As most recently study, deletion of Xist on active X chromosome of donor cells showed in normal global gene expression (Inoue et al., In press). These procedures have resulted in improvement of cloning efficiency. However, there is a lack of information on whether these optimization procedures can be applied to canine SCNT techniques.

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To optimize canine SCNT techniques, it is necessary to prepare large quantities of high-quality recipient oocytes with uniform cytoplasm. However, it is difficult to obtain many large quantities of oocytes because each donor produces a limited number of mature oocytes *in vivo* (average, ~10) (Hossein et al., 2007). Although *in vitro* maturation (IVM) is considered an alternative approach to oocyte production, this technique is still too rudimentary to be used for the production of high-quality, uniform oocytes in large quantities (Saikhun et al., 2008).

Interspecies SCNT (iSCNT), which involves the use of oocytes obtained from different species, is recommended as an approach for overcoming the abovementioned difficulties (Beyhan et al., 2007). This technique has been used widely (a) for studies on the mechanisms underlying nuclear-cytoplasmic interactions, (b) in attempts to rescue highly endangered species, and (c) for the production of ntESCs (Tecirlioglu et al., 2006; Beyhan et al., 2007). In a recent study, Illmensee et al. suggested that an interspecies bioassay can be performed using bovine oocytes in order to assess the preimplantation potential of various types of human adult somatic cells to develop into embryos (Illmensee et al., 2006). Additionally, Jang et al. indicated that canine iSCNT embryos derived from bovine oocytes can develop to the blastocyst stage (Murakami et al., 2005; Jang et al., 2008b). However, the rate of blastocyst formation in iSCNT performed with these oocytes is low (2.8%). Therefore, the possibility of using recipient oocytes of other species for canine iSCNT is being explored. On the other hand, domestic pig oocytes have been used for iSCNT of other species such as tiger and sheep and, successful development of the oocytes to the blastocyst stage has been reported (Hashem et al., 2007; Uhm et al., 2007). In addition, we demonstrated in a previous study that optimized oocytes derived from domestic pigs in a slaughterhouse can be used to produce cloned miniature pigs (Wakai et al., 2008; Sugimura et al., 2009b).

Recently, we demonstrated that the use of porcine oocytes can induce blastocyst formation in iSCNT embryos cultured in porcine zygote medium-3 and proposed that iSCNT using porcine oocytes could provide a bioassay system for evaluating the developmental competence of canine somatic cells (Sugimura et al., 2009a). This review provides an overview of our current knowledge on the subject of canine-porcine iSCNT.

Preparation of large quantities of high-quality recipient oocytes with uniform cytoplasm for canine iSCNT

Although several factors may be responsible for the success of iSCNT, cross-talk between the donor nucleus and the recipient cytoplasm could be considered as a particularly essential factor (Mastromonaco et al., 2007). Hence, culture conditions and preparation of oocytes that function as recipient cytoplasts as well as the species employed appear to be the main factors for the success of iSCNT. Several iSCNT studies were performed using rabbit or bovine oocytes. The resulting iSCNT embryos of several species including cat, human, rhesus monkey, pig, and camel developed to blastocysts with high efficiency. However, this was not the case with canine embryos (Beyhan et al., 2007). On the other hand, another report, described successful production of canine iSCNT embryos using bovine oocytes, although the rate of blastocyst formation in iSCNT performed with these oocytes was found to be low (Murakami et al., 2005; Jang et al., 2008b). This may be due to the ability of the culture medium to support embryonic development of interspecies units and/or intrinsic characteristics of bovine oocytes (Dominko et al., 1999; Beyhan et al., 2007). When porcine oocytes were used as recipient cytoplasm, we found that the canine iSCNT embryos efficiently developed to the blastocyst stage (Figure 1), which is in contrast to previous reports. The cytoplasm was derived from oocytes treated with dbcAMP and has high uniformity (Sugimura et al., 2010). Interestingly, we could not observe development to the blastocyst stage when oocytes were used as recipients without dbcAMP (unpublished data). Although the reasons for the improvement in development to the blastocyst stage by dbcAMP treatment are unclear, one can speculate that the iSCNT success could be due to the production of high-quality cytoplasm that provides a compatible environment for the reprogramming of canine somatic cells.

Culture media for producing canine-porcine interspecies SCNT embryos

It has been suggested that energy metabolism of interspecies iSCNT differs from that of allogeneic SCNT embryos because of disrupted cellular processes associated with mitochondrial function (Mastromonaco et al., 2007). Furthermore we have suggested that the donor nucleus might be responsible for the overall metabolic activity as identified by oxygen consumption in SCNT embryos (Sugimura et al., 2010). Thus, it was considered that optimization of culture conditions will be needed for success of the iSCNT technique (Dominko et al., 1999). However, there is limited information regarding culture conditions or media for canine iSCNT embryos.

We recently demonstrated that canine iSCNT embryos generated using porcine oocytes as recipients can be induced to develop to the blastocyst stage by culturing them in PZM-3 (Sugimura et al., 2009a). Lee et al. demonstrated that the development of canine iSCNT embryos generated using porcine oocytes as recipients was arrested at the eight-cell stage when the embryos were cultured in modified NCSU-23 (mNCSU-23) (2008). In our study, the porcine parthenotes and SCNT embryos also developed to

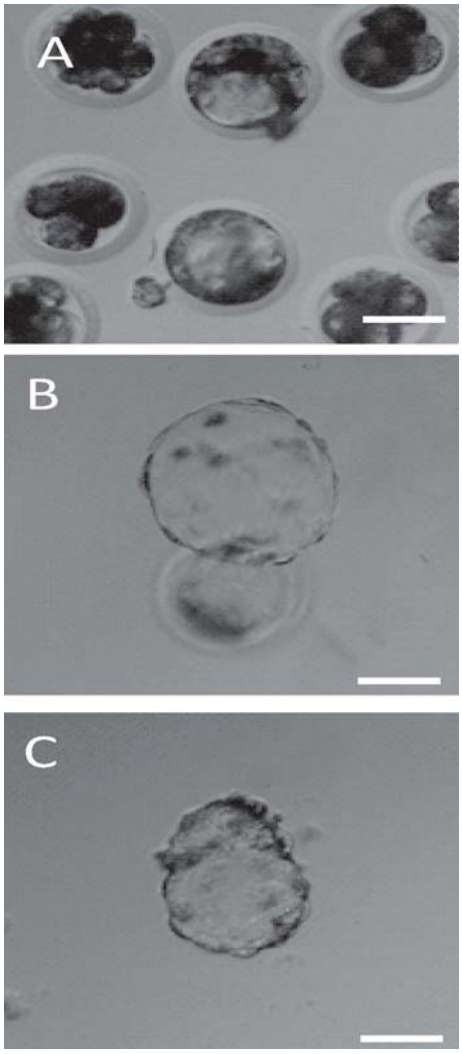

Figure 1. Porcine-porcine (A) and porcine-canine (B and C) SCNT embryos on culture Day 7 (blastocyst stage). Some of iSCNT blastocysts derived from dewclaw cells were hatched on culture Day 7 (hatched blastocyst stage) (C). Scale bars = 100 μm.

the blastocyst stage when cultured in NCSU-23 and mNCSU-23. However the iSCNT embryos failed to develop to this stage when cultured in these media. mNCSU-23 and PZM-3 differ in composition. The former contains high concentrations of phosphate, whereas the latter contains low concentrations of phosphate as well as essential and nonessential amino acids. Previous studies have reported that the presence of phosphate in media used to culture hamster (Ludwig et al., 2001) and rat embryos (Matsumoto and Sugawara, 1998) decreases the *in vitro* developmental competence of the embryos in a concentration-dependent manner. Moreover, the presence of essential and nonessential amino acids in the culture medium is known to significantly improve the developmental competence of embryos of many mammalian species (Liu and Foote, 1995; Thuan et al., 2002). For canine SCNT embryos, an *in vitro* culture system conducive to blastocyst formation has not yet been developed and the optimal composition of a culture medium that can be used for this purpose remains unclear. However, we hypothesize that these compositions may improve the developmental competence of canine iSCNT embryos.

Assessment of the competence of different types of canine somatic cells to develop into embryos and the effects of cell-passaging procedures

The types of donor cells and treatments can have a significant impact on the cloning efficiency (Ogura et al., 2000; Gibbons et al., 2002; Inoue et al., 2003). To optimize donor cells, it is necessary to prepare large quantities of recipient oocytes with uniform cytoplasm. To overcome this problem, we used porcine oocytes as recipients for canine iSCNT. Fibroblasts obtained from the tail tip and dewclaws of male (Mp) and female poodles (Fp) were used as donor cells (Figure 2). Among the iSCNT embryos generated using the cells of either Fp or Mp, the rate of blastocyst formation and the total number of cells at the blastocyst stage was significantly higher for embryos derived from dewclaw cells than for those derived from tail-tip cells. Although this finding cannot be definitively explained, it indicates that the types of donor cells used (e.g., embryonic blastomeres, embryonic stem cells, cumulus cells, fetal fibroblasts, and adult fibroblasts) influence the preimplantation competence of murine SCNT embryos to develop to the blastocyst stage (Wakayama and Yanagimachi, 2001). In addition, the effect of the donor-cell type on the developmental outcome of SCNT embryos has

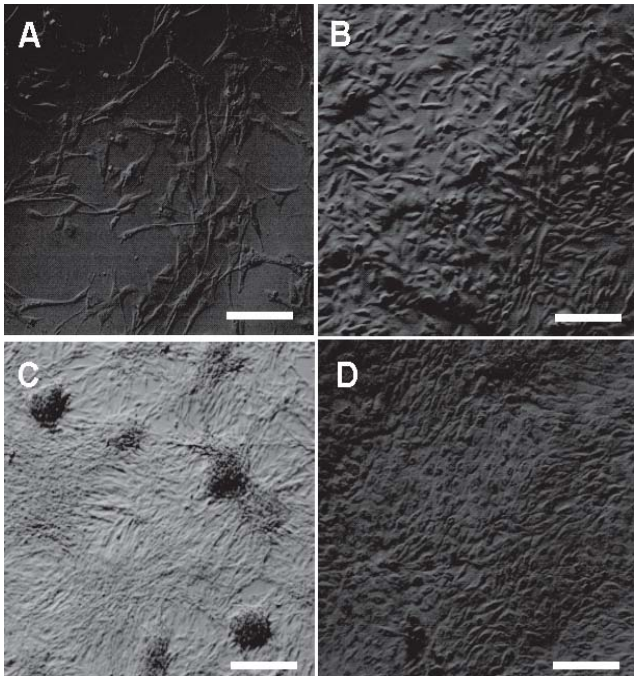

Figure 2. Primaly canine somatic cells derived from tail tip (A-B) and dewclaw (C-D). Scale bars = 100 μm.

been extensively investigated, particularly in the case of cattle and sheep (Wilmut et al., 1997; Kato et al., 2000). A previous study has suggested that the use of embryonic (Rideout et al., 2000) and mesenchymal stem cells (Colleoni et al., 2005; Jin et al., 2007) as donors improves the survival efficiency of SCNT embryos. Postnatal stem cells have been recently isolated from bone marrow (Pittenger et al., 1999), exfoliated deciduous teeth (Miura et al., 2003), and hair follicles (Jaks et al., 2008). In addition, Nakamura et al. have reported that stem cells can be found in the basal layer of the nail matrix adjacent to the nail bed (Nakamura et al., 2008). Therefore, we hypothesize that the dewclaws of animals may contain relatively undifferentiated somatic cells, and that these cells may be able to develop into blastocysts and to improve cell proliferation at the blastocyst stage in each of the iSCNT embryos derived from Fp and Mp.

Next, we evaluated the effect of the cell-passaging technique on the competence of iSCNT embryos to develop into blastocysts. It has been reported that the developmental competence of SCNT embryos is affected by cell-passage (Giraldo et al., 2008). The population doubling time of the cells has an effect on histone acetylation status (Enright et al., 2003) and mRNA expression patterns (Wrenzycki et al., 2001). Additionally, Kubota et al. reported that the developmental competence of bovine SCNT embryos derived from cells obtained from later passages (10 and 15) is higher than that of embryos derived from cells obtained from an earlier passage (5) (Zhang et al., 2008). In contrast, Li et al. reported that the rate of nuclear remodelling in reconstructed horse oocytes decreased significantly when fetal fibroblasts obtained from later passages were used as donor cells (Li et al., 2003). We demonstrated that the competence for development to the blastocyst stage decreases when cells in later passages are used as donors (Sugimura et al., 2009a). Furthermore, we found that the life-spans of dewclaw-derived cells that can develop to the blastocyst stage are longer than the life-spans of tail-tip cells. It is known that cells cultured *in vitro* become more prone to development of chromosomal abnormalities with prolonged culture. Slimane-Bureau et al. demonstrated a direct correlation between the incidence of chromosomal abnormalities in donor cells and in SCNT embryos (Slimane-Bureau et al., 2002). Moreover, in SCNT embryos of argali sheep, poor development and many chromosomal abnormalities are observed when cells obtained from passages 7-10 are used as donor cells (White et al., 1999; Loi et al., 2001). We propose the following explanation for this finding: Both genetic and epigenetic alterations may affect nuclear remodelling and these alterations are likely to increase as cells progress through different passages. Consequently, the competence for development to the blastocyst stage may decrease when donor cells in later passages are used. Another possible explanation is that compared to dewclaw-derived cells, tail-tip-derived cells may be more sensitive to the culture conditions.

Effect of histone deacetylase inhibitors (HDACis) on iSCNT embryos

Aberrant epigenetic modification such as DNA methylation and histone acetylation has been reported in SCNT embryos of numerous species (Yang et al., 2007a). To alter the anomalous epigenetic status, SCNT embryos were treated with histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA) (Kishigami et al., 2006; Yang et al., 2007b), valproic acid (VPA) (Miyoshi et al., 2010) and scriptaid (Thuan et al., 2009). These treatments enabled efficient development of embryos to the blastocyst stage after activation and also improved the extent of subsequent full-term embryonic development. Furthermore, the rate at which ntESCs are obtained from TSA-treated cloned blastocysts after activation is three times higher than the rate at which these cells are produced from untreated blastocysts (Kishigami et al., 2006). However, to the best of our knowledge, there have been no reports on the effects of HDACi on canine SCNT embryos. Hence, we evaluated TSA treatment and found that the beneficial effects depend upon the donor cell type. Our finding was consistent with a previous report by Kishigami et al. who demonstrated that the competence of TSA-treated embryos for development to the blastocyst stage varies depending on the types of donor cells used (2006).

Outgrowth formation of canine-porcine interspecies embryos

Outgrowth formation appears to be linked to the competence of implantation and/or establishment of embryonic stem cells. To evaluate the competence of the blastocysts derived from different types of donor cells to develop further, we examined the formation of outgrowths in these blastocysts. Only the iSCNT embryos that were derived from dewclaw cells of Mp developed outgrowths (Sugimura et al., 2009a). Previously, Tecirlioglu et al. reported observations of outgrowth of iSCNT embryos by using an aggregation technique (2006). Furthermore, we suggested that aggregation of miniature pig SCNT embryos at the four-cell stage can be a useful technique for improving the quality of miniature pig SCNT blastocysts(Terashita et al., in press). Thus, we attempted to encourage the aggregation of iSCNT embryos at four-cell stage. As a result, outgrowth formation in the SCNT blastocysts derived from the dewclaw cells of Fp and Mp was induced and improved, respectively, by aggregation of the iSCNT embryos (Sugimura et al., 2009a). Boiani et al. demonstrated that blastocyst aggregation enhances cell-to-cell communication and thus facilitates the development of

outgrowths during expression of the pluripotent stem cell marker *Oct4* (Boiani et al., 2003). Furthermore, Ohta et al. investigated tetraploid embryos and found that the number of cells at the blastocyst stage and the implantation rate of ESC-derived mouse embryos improve upon aggregation of three of such embryos. It was presumed that this is due to an increase in the number of trophectoderm (TE) cells, which may enhance placentogenesis and/or the implantation ability of the embryos (Ohta et al., 2008). Thus, blastocyst aggregation may promote the subsequent development of blastocysts, enhance the implantation ability of canine SCNT embryos, and produce new canine embryonic stem cells.

CONCLUSION

Based on our recent data, it appears that iSCNT performed using porcine oocytes as recipient cytoplasm could provide a novel bioassay system for evaluating the developmental competence of canine somatic cells, improving the strategies for cloning of dogs, and producing canine embryonic stem cells (Figure 3). Further work will be needed to produce canine ESCs using iSCNT, such as mitochondrial DNA studies, investigations of the nuclear-cytoplasm interaction, as well as activation of the embryonic genome and embryonic metabolism. Nonetheless, this strategy has a tremendous amount of potential for developing a novel model for disease and for investigational cell therapy in humans and canines.

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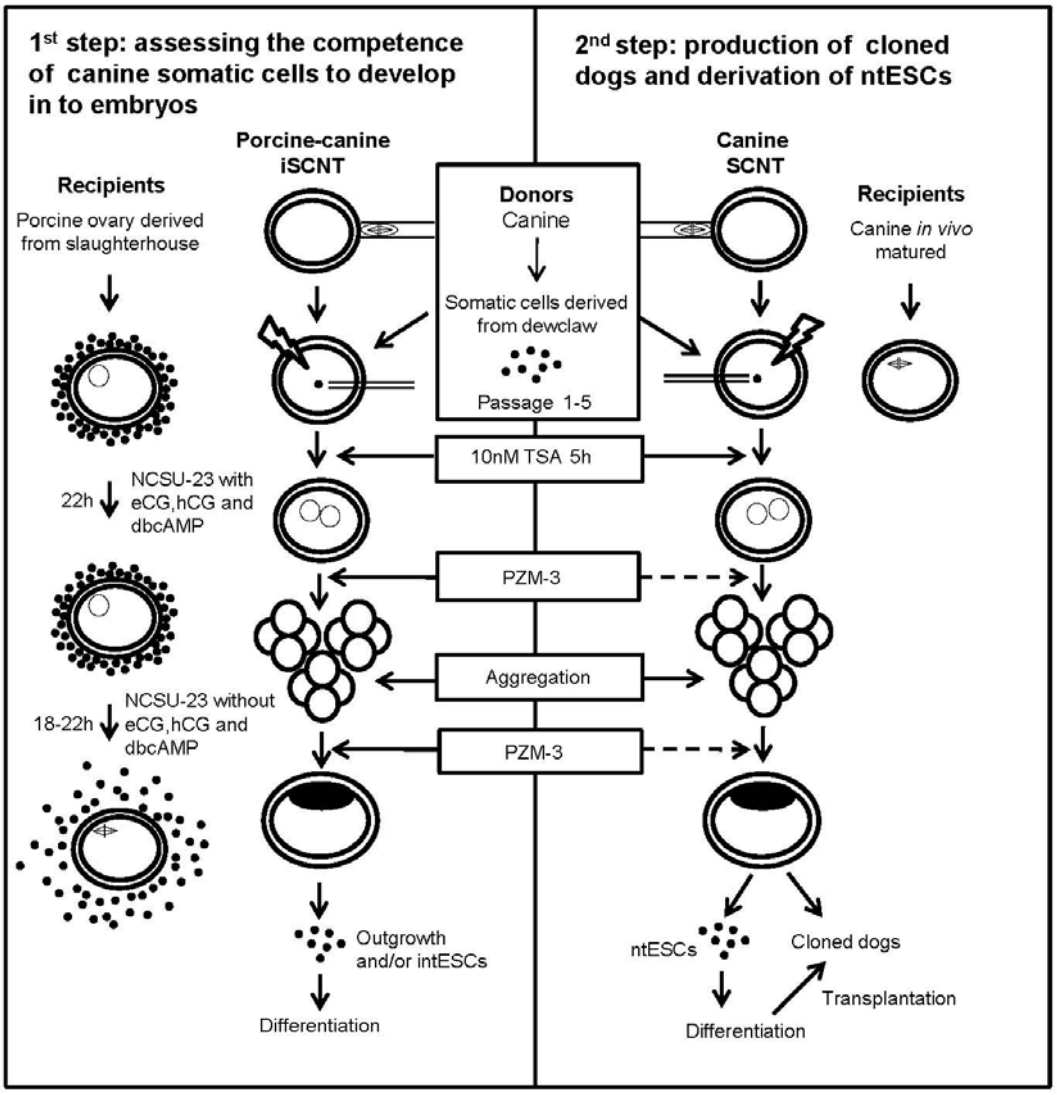

Figure 3. Schematic representation of the novel bioassay system for production of cloned dogs and derivation of ntESCs.

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