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Functional consequences of mitochondrial mismatch in reconstituted embryos and offspring

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Abstract. Animal cloning technology has been developed to produce progenies genetically identical to a given donor cell. However, in nuclear transfer protocols, the recipient oocytes contribute a heritable mitochondrial genomic (mtDNA) background to the progeny. Additionally, a small amount of donor cell-derived mitochondria accompanies the transferred nucleus in the process; hence, the mtDNAs of two origins are mixed in the cytoplasm (heteroplasmy) of the reconstituted oocyte. Herein, I would like to introduce some of our previous results concerning five key considerations associated with animal cloning, including: mtDNA heteroplasmy in somatic cell nuclear transferred (SCNT) animals, the variation in the transmission of mtDNA heteroplasmy to subsequent generations SCNT cows and pigs, the influence of mtDNA sequence differences on mitochondrial proteins in SCNT cows and pigs, the effects of the introduction of mitochondria derived from somatic cells into recipient oocytes on embryonic development, and alterations of mtDNA heteroplasmy in inter/intraspecies nuclear transfer embryos.

Key words: Cattle, Mitochondria, Nuclear transfer, Pig

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Introduction

The nuclear transfer technique has potential agricultural applications for replicating food animals with desired genetic traits, animal transgenesis, or the conservation of endangered species. Techniques to obtain genetically identical progenies have started with the production of twins via the surgical division of a fertilized egg, and developed into the nuclear transfer method, which aims to produce multiple identical offspring using somatic cell nuclei and enucleated oocytes. As animals inherit most or all their mitochondria through maternal transmission, mitochondrial genetics (mtDNA) will not be the same if the recipient oocytes harbor different mtDNA sequences. While the primary purpose of cloning technologies is to produce progeny genetically identical to a given donor cell, mtDNAs originate from host oocytes, and often, the resultant progenies derived from nuclear transfer are not genetically identical with regards to the mtDNA composition. Pigs show a few intra-breed mutations in their mtDNA sequences; the mtDNAs of the European and Asian breeds show a clear difference. In the case of cattle, base substitution frequently exists among each maternal line. Therefore, genetically different recipient oocytes are often used for nuclear transfer; hence, the probability that the mtDNA will be identical to that of the donor cells is extremely low [1–3].

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Somatic cell nuclear transfer (SCNT) is employed to modify the mtDNA of individuals from the next generation. It is possible to replace the undesired mtDNA/cytoplasm to one of a desired background via the nuclear/pronuclear transfer technique. A recent technological development called mitochondrial replacement therapy (MRT) prevents the transmission of mtDNA defects into the second generation [4–6]. However, during SCNT, small amounts of donor cell-derived mitochondria are mixed during nuclear transfer to the host ovum, resulting in a state where two types of mtDNAs are present (heteroplasmy). The proportion of heteroplasmy in SCNT animals fluctuates due to the random distribution or selective replication/degradation associated with cleavage or cell division [3]. Therefore, we investigated the influence of differences in mitochondrial and nuclear origins/mitochondrial heteroplasmy on embryonic development and individual production traits from a multifaceted perspective.

Transmission of mtDNA Heteroplasmy to Subsequent Generations of SCNT Cattle and Pigs

Since mtDNA is present in the cellular cytoplasm (peripheral to the cell's nucleus), two types of mtDNAs derived from the donor cell (D-mtDNA) and the recipient oocyte are mixed after nuclear transfer. We investigated the proportions of mtDNA heteroplasmy in SCNT calves (n = 46) and their subsequent generation (n = 53), and SCNT pigs (n = 4) produced using Chinese pig (Meishan; M) nucleus and European pig (Landrace, etc.; L) ooplasm, and their first-generation (n = 46) and second-generation offspring (n = 18) [7]. The sequences of mtDNA of the M and L breeds are sufficiently different to enable RFLP detection.

In SCNT pigs, D-mtDNA (Meishan type) was detected at a proportion of 0.1–1.0%; most of the resultant mtDNA originated from the host

oocyte ooplasm (European type). In 25 progenies of the NT founder pigs, D-mtDNA was detected in only one female pig. Surprisingly, the proportions of D-mtDNA in tissue samples of heteroplasmic pigs varied widely (0 to 44%). Furthermore, D-mtDNA has been reported to be transmitted to 89% of the next-generation progeny of the heteroplasmic female pig [7]. Similarly, in SCNT cattle lineages, D-mtDNA was heritable, with proportions ranging as high as 46% in the subsequent generations [2]. These results demonstrated that a very small population of D-mtDNAs could persist in the SCNT animals and could be transmitted to the progeny. Germline transmission of mtDNA in animals following SCNT resulted in varying proportions of heteroplasmy in their offspring, due to the "bottleneck effect" on the mtDNA population in the germ cells (Fig. 1) [3, 8–10]. Clearly, it is possible to introduce significant levels of D-mtDNA into maternal lineages with germline persistence in subsequent generations. The different tissue-specific patterns observed in individual animals may be due to a functional competition between the mtDNAs in different organs or interactions between the mtDNA and nuclear genomes.

In our previous work, D-mtDNA has been reported to be damaged by ROS during *in vitro* cell culture under serum starvation conditions [11]. In fact, the injection of somatic mitochondria from cells subjected to serum starvation affected the embryonic development [12]. In terms of mitochondrial function, normal mtDNA can complement the mitochondrial function in the presence of a minor contamination of mutated mtDNAs [13]. Thus, mutated mtDNAs could be eliminated by mitochondrial turnover, which involves mitochondrial biogenesis, mitochondrial dynamics, and selective autophagic removal of dysfunctional mitochondria (mitophagy) [14]. The segregation pattern observed here shows that the D-mtDNA was not completely eliminated, and could duplicate and be inherited via the bottleneck effect.

Mitochondrial Proteomic Approach for SCNT Cattle and Pigs

As noted earlier, SCNT has great potential for agricultural applications for replicating food animals with desirable genetic traits, animal transgenesis modeling using comparative mammalian species, and the conservation of endangered species. Although nuclear transfer is widely recognized as an effective technique for producing economically important and value-added domestic animals, it has not yet reached an economically feasible state. SCNT technology is currently struggling, with extremely low experimental efficiencies, and even when it is successful and live progenies are generated, various abnormalities, including large offspring syndrome, frailty, immunodeficiency, etc., have been observed. Such difficulties and variability in developmental outcomes are a major obstacle to the practical applications of SCNT. Aberrant reprogramming of donor somatic cell nuclei during animal cloning may result in many severe problems in the cloned organisms [15]. Genetic differences in the recipient oocyte cytoplasm resulting from nuclear transplantation and the mitochondrial mismatch between somatic cells and egg cells may influence the health, longevity, and uniformity of the progeny.

Mitochondria are involved in ATP synthesis, and controlling cellular functions such as apoptosis, cell proliferation, and cell cycle via ROS [16]. In addition, mtDNA encodes important proteins in the electron transport system. Accordingly, many abnormalities observed in SCNT fetuses and offspring may be caused by deficiencies in mitochondrial function. We attempted to investigate the differences of protein expression profiles in SCNT cattle and pigs.

The influence of potential nuclear-cytoplasmic incompatibility post SCNT is largely unknown. Solubilized hepatic mitochondrial proteins from SCNT pigs produced via the microinjection of M fibroblasts into L oocytes were comprehensively analyzed by fluorescent labeled two-dimensional difference gel electrophoresis (2-D DIGE) [17]. The 2-D DIGE analysis revealed the differential expression of three proteins among the M and L breeds, all of which showed high expression in M pigs. Alteration of the mitochondrial protein expression levels was observed in adult SCNT pigs and was not reflective of differences among the breeds. In addition, the expression levels of two novel proteins including HMG-CoA synthase were identified as common differences in the SCNT pigs; these proteins were encoded by nuclear genomes. This indicates that the mtDNA type had no effects on the protein expression.

A comprehensive analysis of solubilized hepatic mitochondrial proteins was performed to assess the effects of differences in the origins of recipient oocytes on the productivity and identity of bovine SCNT recipients. 2D-DIGE analysis revealed the differential expression of three proteins in case of SCNT cattle and seven proteins in case of SCNT calves (died within 10 days of birth), compared to the controls ($P < 0.05$). All SCNT calves in the study showed some morphological abnormalities. The protein expression profiles of SCNT calves varied widely among individuals, and the most differences were detected particularly in calves with large-offspring syndrome (body weight at birth, 70 kg). Apoptosis-related proteins with different expression patterns were also identified in the hepatic mitochondria of SCNT calves post mortem. Two protein spots (apoptosis-related proteins) were detected among the lean and control calves. The mitochondrial protein profiles varied widely among the SCNT cattle even if they were generated from the same donor cell line. One SCNT cow, which was produced using oocytes collected from a slaughterhouse cow-derived ovary that was cultured overnight in PBS at 15°C, showed the greatest profiling disparity compared to the other adult clones produced using oocytes obtained from anesthetized cows via the surgical ovum pick-up technique. From these results, it is likely that mtDNA heteroplasmy did not influence the mitochondrial protein expression.

Embryonic Development after Introducing Foreign Somatic Cell Mitochondria into Oocytes

The physiological and morphological status of mitochondria differed greatly between somatic cells and oocytes [18, 19]. Earlier, the effects of these parameters on the embryonic development after nuclear transfer were unknown. We investigated whether the injection of somatic cytoplasm or mitochondria influenced the parthenogenetic development. Centrifugally fractionated mitochondria were obtained from the somatic cells of wild-type mice with different mtDNA sequences or after serum starvation treatment. In addition, mitochondria purified from serum-starved bovine fibroblasts were microinjected into MII-stage oocytes. The exogenous mitochondria originating from donor cells were observed to directly affect the parthenogenetic

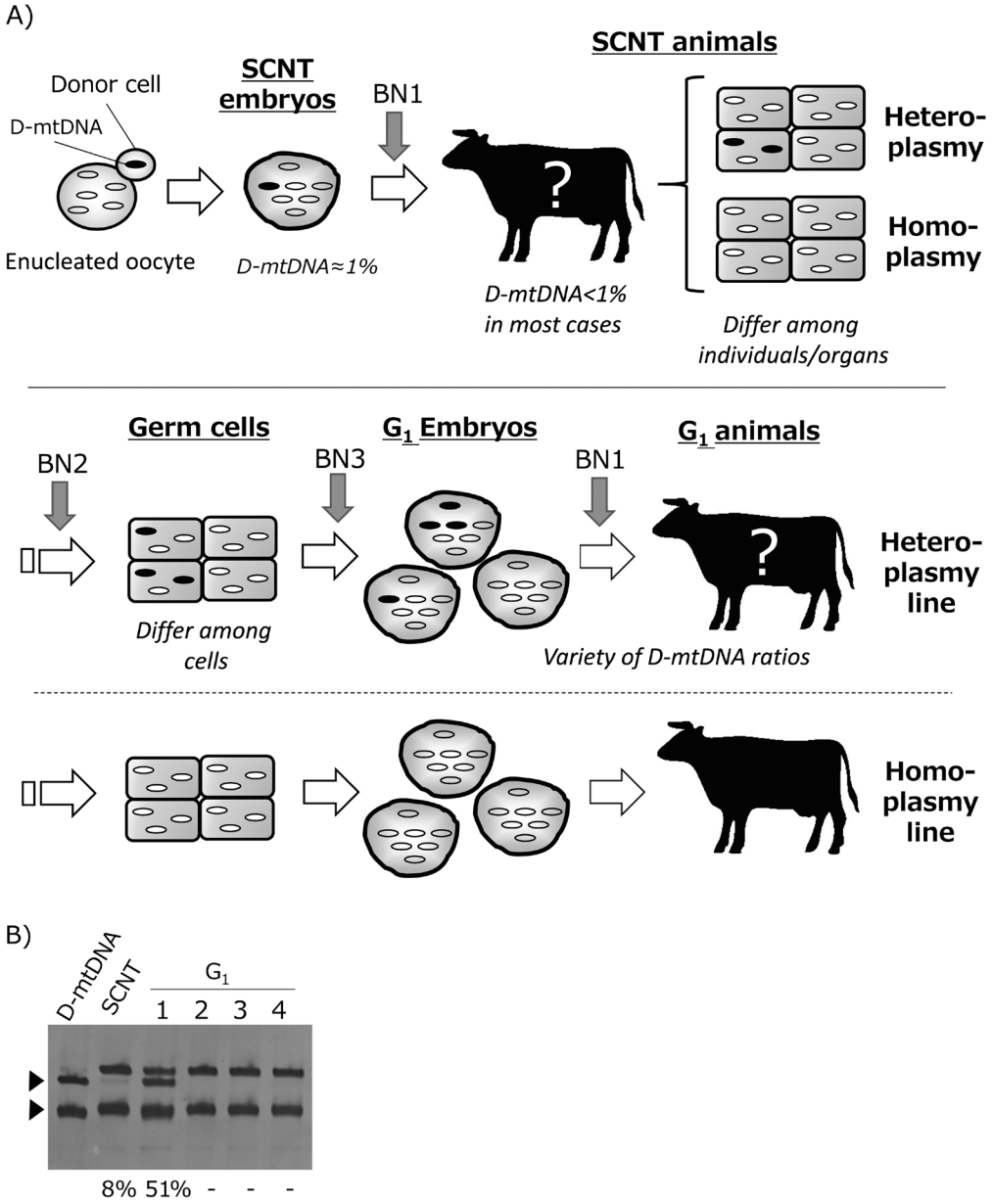

Fig. 1. Mitochondrial DNA (mtDNA) bottleneck in an SCNT maternal lineage [3]. A) Schematic features of the mtDNA bottleneck associated with SCNT cattle and pigs. mtDNA in an SCNT embryo was randomly or positively selected during embryonic development (bottleneck 1; BN1) and through a maternal germline (bottleneck 2; BN2). The results of the bottleneck transmission, i.e., the somatic cell mtDNA (D-mtDNA) may or may not be inherited to the next generation (G_1) of the embryos or animals. Maternal germlines with mtDNA heteroplasmy can show a range of D-mtDNA ratios in G_1 embryos and animals, including mtDNA homoplasmy. Maternal germline with mtDNA homoplasmy can result in the presence of mtDNA homoplasmy in G_1 embryos and animals. B) D-mtDNA transmission in SCNT heifers and their G_1 calves, as determined by PCR-mediated single-strand conformation polymorphism (PCR-SSCP) analysis based on the D-loop [2, 3]. The SCNT heifer (SCNT) and one of the G_1 offspring (G_1-1) showed mtDNA heteroplasmy, with D-mtDNA contents of 8% and 51%, respectively. The other three G_1 animals (G_1-2, 3, and 4) demonstrated mtDNA homoplasmy.

development. Long-term serum starvation (greater than 7 days in culture) of the donor cells prior to nuclear transfer also influenced the morphology of the mitochondria and parthenogenetic development. Unlike cytoplasmic or mitochondrial injections, ooplasm injection did not affect the embryonic development.

Mitochondrial Heteroplasmy in Interspecies (Buffalo-bovine) Nuclear Transfer

SCNT offers the possibility of preserving endangered species; however, till date, there has been very limited success in this regard.

Current methodologies result in the production of nuclear–cytoplasmic hybrids, owing to the limited availability of oocytes from wild animals. Despite numerous attempts of SCNT in a wide variety of species, the number of live births of SCNT-derived offspring is still limited to instances that combine the divergent genetic composition of closely related species, such as gaur–bovine SCNT embryos. Buffalo–bovine SCNT embryos demonstrated the development to the blastocyst stage [20, 21]; however, neither pregnancy nor natural delivery was reported. The developmental ability of buffalo-bovine embryos is much lower than that of buffalo-buffalo SCNT embryos [20]. Since the mtDNA sequences of buffalos and cattle are clearly different, interspecies SCNT poses several problems, including mitochondrial/genomic DNA incompatibility and embryonic genome activation of the donor nucleus by the recipient oocyte. Quantitative analysis of mtDNAs during embryonic development would be a useful method to investigate nuclear-mitochondrial interactions after nuclear transfer.

Mitochondrial fractions were obtained from swamp buffalo ear fibroblasts by differential centrifugation and microinjected into bovine oocytes to assess the parthenogenetic development [22]. Bovine embryos injected with the buffalo mitochondria were developed to the blastocyst stage and then analyzed for the mtDNA copy number by real-time PCR. The results demonstrated that the buf-mtDNA copy number was constant during the embryonic development process until the blastocyst stage. Buffalo-bovine SCNT embryos were then reconstructed using water buffalo (swamp type) fibroblasts and bovine enucleated oocytes; however, they did not develop beyond the 16-cell stage [23]. Nonetheless, the donor cell and recipient cytoplast mtDNAs of the buffalo-bovine SCNT embryos were maintained until the 16-cell stage. The exogenous buffalo mtDNA injected into the bovine oocytes was not selectively destroyed; indeed, they were maintained throughout the early development stage until the blastocyst stage. It is doubtful whether the exogenous intergeneric or somatic cell mitochondria would be recognized by the mitophagy process in the recipient cells, which is observed in case of sperm mitochondria.

Concluding Remarks

In order to produce identical cloned animals with desired mtDNA composition, it is necessary to completely eliminate the mtDNA of recipient oocytes; this allows for the amplification of only the donor cell mtDNA. There is a possibility that mitochondrial heteroplasmy could be naturally eliminated; however, it is not a process that is easily controlled or manipulated with current cloning technologies – even through a single generation. Recently, mitochondrial function and dysfunction have been the subjects of various studies on ovarian ageing and metabolic stress modeling. To remove the mutated mtDNA from the ooplasm, MRT has been proposed to represent an effective treatment for both mitochondrial dysfunction and age-related infertility. Another technique that is currently being developed for the selective reduction of mutated mtDNA in the germline involves genome editing [24]. Our previous data based on the animal cloning technique could also provide important insights into these evolving techniques. Further studies are needed to clarify the intracellular characteristics of SCNT animals and elucidate the cellular mechanisms that limit the practical use of cloning techniques, while simultaneously contributing to our basic biological understanding of epigenetics and nuclear–cytoplasmic interactions during embryonic development.

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