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Inheritance of mitochondrial DNA in serially recloned pigs by somatic cell nuclear transfer (SCNT)

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ABSTRACT

Somatic cell nuclear transfer (SCNT) has been established for the transmission of specific nuclear DNA. However, the fate of donor mitochondrial DNA (mtDNA) remains unclear. Here, we examined the fate of donor mtDNA in recloned pigs through third generations. Fibroblasts of recloned pigs were obtained from offspring of each generation produced by fusion of cultured fibroblasts from a Minnesota miniature pig (MMP) into enucleated oocytes of a Landrace pig. The D-loop regions from the mtDNA of donor and recipient differ at nucleotide sequence positions 16050 (A→T), 16062 (T→C), and 16135 (G→A). In order to determine the fate of donor mtDNA in recloned pigs, we analyzed the D-loop region of the donor's mtDNA by allele-specific PCR (AS-PCR) and real-time PCR. Donor mtDNA was successfully detected in all recloned offspring (F1, F2, and F3). These results indicate that heteroplasmy that originate from donor and recipient mtDNA is maintained in recloned pigs, resulting from SCNT, unlike natural reproduction.

1. Introduction

Since the first somatic cell (SC)-cloned lamb was reported, successful SC cloning has been achieved in several species via SC nuclear transfer (SCNT), including pigs [1–4]. The SCNT has potential agricultural applications, such as genetically engineered transgenic animals (e.g., bioreactor, xenotransplantation) or the conservation of endangered species. However, various questions and problems with regard to SCNT exist, such as abnormal development of cloned fetuses, anatomical abnormalities of cloned offspring, and postpartum mortality [5,6].

Mitochondria are maternally inherited organelles that are responsible for the production of cellular energy in the form of ATP. Mature oocytes from different mammalian species contain an average of 1.74–9.5 $\times$ 10^5 mitochondrial DNA (mtDNA) copies, and a threshold of approximately 1 $\times$ 10^6 mtDNA copies must be exceeded for fertilization to ensue in mouse, human and pig [7–9]. Furthermore, during normal fertilization, sperm mitochondria are destroyed and maternal mitochondria are transmitted to the offspring [10,11]. SCs have a low mtDNA copy number (from several hundred to several thousand) compared with oocytes [12]. Therefore, the mitochondrial genomes of the resultant SCNT clones may have two different origins and be slightly heteroplasmic, based on the quantitative participation of the mtDNA from the two partners. Quantitative studies on SCNT-derived fetuses and offspring revealed the level of heteroplasmy to be between 0 and 13%, indicating the neutral transmission of mtDNAs of the nuclear donor and oocyte recipient [13–15]. However, higher contributions of up to 40% attributed to the replicative advantage of donor mtDNA have also been reported [14]. In our previous study, the foreign cytoplasmic genome from donor cells was not destroyed, up to the blastocyst stage following SCNT [16]. However, the fate of mtDNA in cloned progeny is unclear because no donor mtDNA could be detected in any of the 10 sheep cloned previously [17].

The mtDNA has a short segment, which is called the displacement loop (D-loop). Many studies have focused on the mitochondrial D-loop region which is the most variable part of mtDNA [18]. Mitochondrial D-loop DNA sequences have provided significant insights into the genetic diversity and past migration history of cattle, sheep, goats and pigs [19–22].

In the present study, we demonstrated successful serial SCNT through the third generation and investigated the fate of foreign mtDNA during serial cloning via detection of polymorphisms in the D-loop using allele-specific PCR (AS-PCR) analyses. Fetal fibroblasts were collected from cloned pig ear skin to produce

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subsequent generations of cloned pigs. A third-generation cloned pig was successfully produced, and donor-specific mtDNA in the cloned pigs from each generation was detected.

2. Materials and Methods

2.1. Animal ethics statement and Chemicals

All procedures in this study were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes approved by the Institutional Animal Care and Use Committee (IACUC approve number: 2010–006, D-grade), Dankook University. Unless otherwise noted, all chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of donor cells

All ear fibroblast cells obtained from specific pathogen-free Minnesota miniature pigs were used as donor cell for SCNT. Briefly, small pieces of ear skin tissue were washed in Dulbecco's phosphate-buffered saline (Invitrogen, Carlsbad, CA) and minced with a surgical blade on a 100 mm Petri dish. Cells were then dissociated from tissues in 0.25% trypsin–EDTA (Invitrogen) for 10 min at 39 °C. The digested cells were cultured for 6 to 8 days in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 20% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), 1 mM L-glutamine, 100 units/mL penicillin, and 0.5 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 in 95% air. To obtain donor cells passaged for 1–5 generations, ear fibroblasts were cultured until confluent and subcultured at intervals of 2–7 days by trypsinization for 5 min using 0.25% trypsin–EDTA until used for SCNT.

2.3. Production of recloned pigs

SCNT was carried out as previously described [23]. Briefly, oocytes were enucleated and one ear fibroblasts were transferred into the perivitelline space of an enucleated oocyte in HEPES-buffered TCM-199 supplemented with 0.4% bovine serum albumin (BSA) and 7.5 μg/mL cytochalasin B. Fusion of cell–oocyte couplets was accomplished by two direct current pulses (1.5 kV/cm for 40 s) in an electrical fusion solution that contained 0.3 M mannitol, 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$. Following electrical stimulation, reconstructed oocytes were cultured in NCSU23 supplemented with 4 mg/mL fatty acid-free BSA and 7.5 μg/mL cytochalasin B for 3 h to suppress extrusion of the second polar body. Oocytes were then cultured for 4 days in NCSU23 containing 4 mg/mL fatty acid-free BSA and transferred to NCSU23 containing 10% FBS and cultured for another 4 days. All SCNT embryos were cultured at 39 °C in a humidified atmosphere containing 5% CO_2 in 95% air. On the next day of SCNT, reconstructed embryos were surgically transferred to naturally cycling surrogate pigs on the second day of standing estrus.

2.4. Preparation of DNA from fibroblasts of recloned pigs

To determine whether the cloned pigs have the same mtDNA as the donor, ear fibroblasts were collected from different founder pigs produced by SCNT. Genomic DNA samples were extracted from the ear skin fibroblasts of the donor Minnesota miniature pig (MMP), the ear skin of recloned pigs, and the ear skin of recipients in 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% SDS, 0.5 mg/mL protein K, and phenol/chloroform.

2.5. Sequencing of pig mitochondrial D-Loop region

The D-loop region of pig mtDNA was amplified by PCR. PCR primers specific for pig mtDNA were synthesized based on the reference genotypes of *Sus scrofa* (GenBank: AP003428.1). The primer sequences were as follows: forward (F) 5′-CATCGAAAACAACCTATTAA-3′ (from 15276 to 15295 bp) and reverse (R) 5′-ATAGCAC CTTGTTTGGATTG-3′ (from 16092 to 16111 bp). The PCR protocol was 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min in a thermal cycler (C1000 Thermal cycler with dual 48/48 fast reaction module; Bio-Rad Corporation). The resulting D-loop fragments were sequenced using an ABI 377 automated DNA sequencer (Retrogen, San Diego, CA).

2.6. AS- PCR and real-time PCR

To determine the fate of donor mtDNA in recloned pigs, allele-specific primers were designed to exclude the possible amplification of a false mitochondrial allele from recipient oocytes. Allele-specific primers did not react to mtDNA from recipient oocytes but only to donor mtDNA. The primer sequences for AS-PCR were as follows: sPM, (F) 5′-TATGTGACCCCAAAAATTTA-3′ and (R) 5′-GTTTCACGCGGCATGGTAA-3′; lPM, (F) 5′-CACTAGATCACGAGCTTAAT-3′ and (R) 5′-GACGGCCATGGCTGAGTCC-3′; and UNIV, (F) 5′-CCCATAAAATTGCGCACAAA-3′ and (R) 5′-TAGAAACCCCCACGGTTTAT-3′. Each reaction consisted of initial denaturation at 94 °C for 3 min, and 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min using a thermal cycler. To determine the ratio of donor mtDNA in recloned pigs, real-time PCR was performed using SensiMix (Bioline, London, UK) in a Rotergene- 3000 real time PCR machine (Corbett Research, Cambridge, UK). Real-time PCR consisted of initial denaturation at 95 °C for 15 min, and 50 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 15 s.

2.7. Statistical analyses

All experiments were repeated at least three times, and statistical analyses were performed using the Student's *t*-test or analysis of variance analyses followed by the Duncan's multiple comparison test. Differences with $p < 0.05$ were considered significant. Results are expressed as the mean ± SEM of triplicate independent samples.

3. Results

3.1. Diversity in the D-loop region of mtDNA

To examine the diversity of mtDNA between recipient pig (RCP) and MMP, the D-loop region of mtDNA was used for the analysis of mtDNA heteroplasmy. First, D-loop region was amplified using specific primers (SusF and SusR), whose synthesis was based on the published reference sequence (REF; recording number, AP003428) using genomic DNA as a template (Fig. 1A). These PCR-amplified D-loop products were sequenced, and the analyzed polymorphisms were compared with the MMP and published REF sequence (recording number, AP003428). Sequence comparisons in the D-loop regions of mtDNA revealed one nucleotide difference between reference pig (*S. scrofa domestica*) and Landrace pigs in Korea, and two nucleotide differences between the reference pig and MMP (Fig. 1B). An analysis of the chromatograms showed that the sequences of mtDNA D-loop region contained nucleotide differences at three places between the RCP and the MMP. Automated DNA chromatographs showed differences in the mtDNA

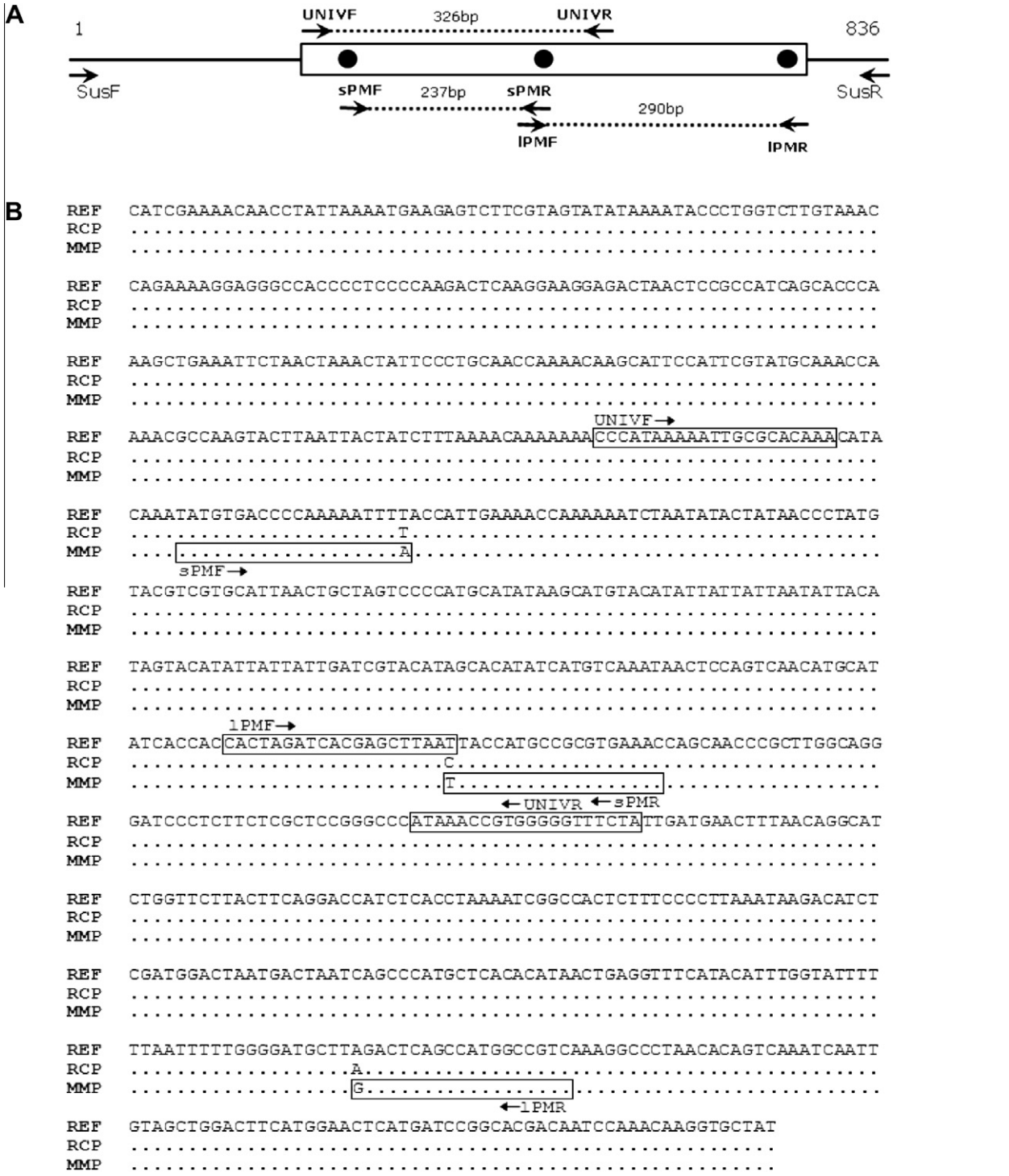

Fig. 1. Displacement loop (D-loop) region. Schematic representation of the D-loop and allele-specific region of mtDNA (A). DNA sequences of the 796 bp PCR-amplified region (B). The polymorphisms were compared with the published reference sequence (REF; recording number AP003428). Dots display identity with the RFE sequences. RCP, recipient pig; MMP, Minnesota miniature pig.

sequences at 264, 463 and 714 bp in the D-loop region of recipient and donor cells. The nucleotide at the 264 bp position was thymine (T) in the RCP but adenine (A) in the MMP. At position 463 bp, the nucleotide of RCP was cytosine (C) but the nucleotide of the MMP was T; at peak of 714 bp, the RCP was A, but the MMP was guanine (G) (Fig. 2A). Differences in the D-loop region sequence were observed commonly in the same species. The diversity in D-loop sequences appears to be related to species specificity. The diversity of the D-loop region of mtDNA was used for the analysis of mtDNA heteroplasmy. As shown in Fig. 1B, three primer pairs were designed for AS-PCR analysis. The UNIV primer pairs universally amplified the mtDNA D-loop regions from the RCP and MMP, and the sMP and lMP oligonucleotides were MMP-specific primers. UNIV primers amplified 326 bp mtDNA in both strains, but the sMP and lMP sets were only able to amplify sequences in the MMP (Fig. 2B).

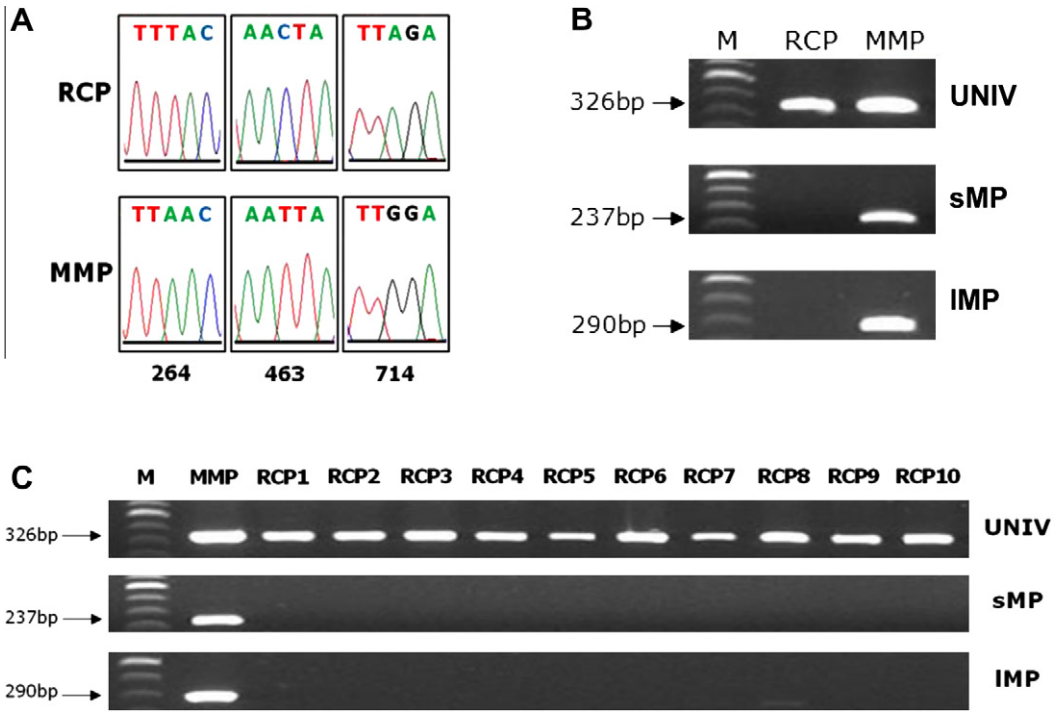

Fig. 2. Automated DNA chromatograms in D-loop region and AS-PCR products. (A and B) Automated DNA chromatographs show the differences of mtDNA sequences at 264, 463 and 714 bp in the D-loop region of recipient and donor cell (A). AS-PCR products amplified by allele-specific primers (B). (M; DNA marker, UNIV; universal, sMP (short length of MMP) and lMP (long length of MMP); allele-specific). (C). Ten ovaries supplying recipient oocytes (RCP1–10) were compared with MMP from donor tissue. The amplified D-loop products show the difference of sequence between other recipient pig (RCP) and donor pig (MMP) in mitochondrial DNA (mtDNA).

To confirm the specificity of AS-PCR using the sMP or lMP primer set in RCP oocytes, we analyzed the mtDNA genotype of 10 random pig samples. UNIV could amplify mtDNA of RCP 1 ~ 10 and the MMP, but sMP and lMP could not amplify the mtDNA of RCP 1 ~ 10 (Fig. 2C). Taken together, these results suggest that the foreign mitochondrial genome from donor cells was not destroyed during SCNT in pigs.

3.2. Production of serially recloned pigs by SCNT

To examine the fate of donor mtDNA during the reproduction of recloned pigs, we performed serial SCNT using fibroblasts. The first-generation cloned pigs (F1) were produced using fibroblasts as nuclear donors. Fibroblasts from ear skin biopsies of F1 were used as nuclear donor cells for second-generation cloned pigs (F2). The third-generation of cloned pigs (F3) was produced using fibroblasts that were obtained from F2 ear skin. The content of serial recloning through three generations was determined by microsatellite analysis. The comparison of DNA genotypes using microsatellite DNA analysis determined whether serially recloned pigs have the same genotype as the donors. The microsatellite markers SW1026 (Chr 2), SW288 (Chr 14), SW21 (Chr 9), and SW2411 (Chr 16) were used, and the results of analysis showed obvious differences between recloned pigs and recipient (Fig. 3A). These results demonstrated that serially recloned pigs originate from the donor MMP fibroblasts and no genetic relationship exists between recloned pigs and recipient oocytes.

3.3. Transmission of donor mtDNA by serial SCNT

To examine the fate of donor mtDNA inheritance from serially recloned offspring, AS-PCR was performed to analyze the heteroplasmy of the D-loop region of mtDNA. As expected, only recipient DNA was amplified by UNIV primers and DNA from serially recloned pigs was amplified by UNIV, sMP and lMP primers. The serially recloned fibroblasts had similar genotypes to the SC donor pigs (Fig. 3B). The results of AS-PCR showed the genotype of heteroplasmy: both donor and recipient mtDNA. Such heteroplasmy was transmitted into serially recloned pigs as seen in subsequent SCNT experiments. To investigate the amount of donor mtDNA in serially recloned pigs, mtDNA heteroplasmy was analyzed by real-time PCR. Total amounts of mtDNA decreased in F1, whereas amounts of mtDNA slightly increased in F2 compared with RCP mtDNA. Donor-specific mtDNA also decreased in the F1 generation. However, the amounts of donor-specific mtDNA increased in a generation-dependent manner (Fig. 3C and D). These results demonstrated that the total mtDNA amounts were changed during serial SCNT, and donor mtDNAs were existed up to third generation.

4. Discussion

In this study, the fate of mtDNA was examined during serially cloned pigs by SCNT using fetal fibroblasts obtained from ear skin. We demonstrated that donor-specific mtDNA was detected in serially cloned pigs. These results suggest that donor somatic cell-derived mtDNA remains in the cytoplasm of recloned pigs by serial SCNT.

The mitochondrial genome has a short segment of non-coding region called the D-loop, which interacts with several nuclear-encoded, mtDNA-specific transcription and replication factors that mediate mtDNA replication [24–26]. These sequences have genetic diversity among various species [27]. In the present study, we found three differences in the D-loop among different pig species. Additionally, results from chromatograms and AS-PCR analysis showed that a donor-specific DNA fragment was not amplified in recipient oocytes by sMP and lMP. These findings suggest that the designed donor-specific primer sets confirmed the existence of MMP mtDNA in the cells.

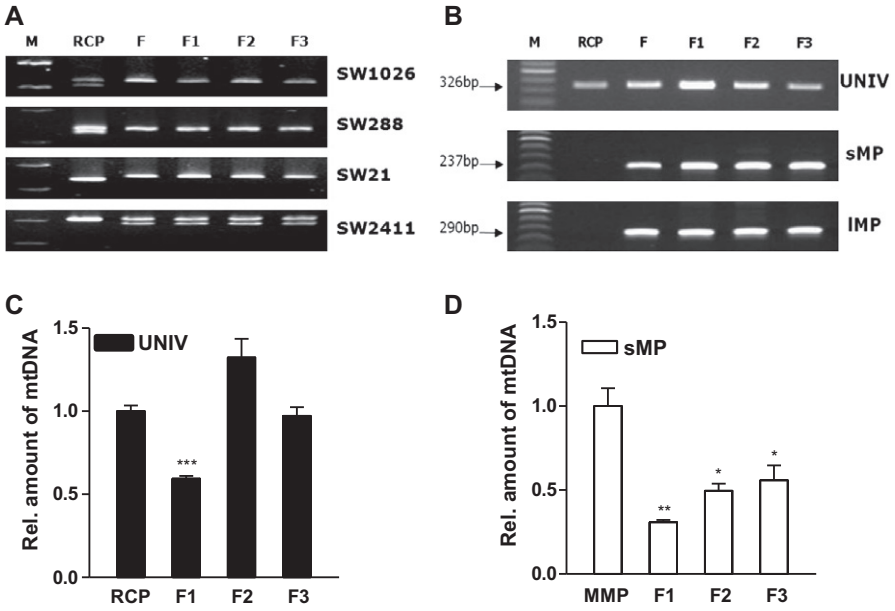

Fig. 3. Recloned pigs produced from fibroblasts using SCNT, and PCR analyses D-loop region and mtDNA amounts. (A) The comparison of DNA genotypes using microsatellite DNA PCR analysis using unique microsatellite marker SW1026, SW288, SW21 and SW2411. Marker size = 100 and 200 bp. (B) AS-PCR analysis of donor-specific D-loop region. The fibroblasts of recloned pigs were compared with RCP from recipient oocytes. (C-D) Quantitative analysis of mtDNA amounts by real-time PCR. To investigate mtDNA amount of recloned pig, the analysis of PCR was examined in both universal D-loop region (UNIV) (C) and specific D-loop region (sMP) (D) using specific-primers. (F; minnesota miniature pig as primary donor, F1; first generation, F2; second generation, F3; third generation). (UNIV; universal primers, sMP; allele-specific primers).

Several studies reported mtDNA heteroplasmy in nuclear transfer embryos and offspring produced by embryonic and somatic cell transfer [13,14,28]. In our previous study, donor mtDNA was studied during embryonic development using SCNT, which involved injection of cumulus cells into enucleated oocytes. Donor mtDNA present in the embryo was found to mix with recipient-mtDNA [16]. However, we could not confirm the fate of mtDNA in a repeated SCNT experiment. In the present study, we found that mitochondria derived from donor fibroblasts remain in the recipient cytoplasm in all offspring during three continuous SCNT experiments. Both total and donor mtDNA amounts were significantly reduced after primary SCNT. In addition, donor mtDNA was slightly increased during serial SCNT through the third generation. Based on our data, we carefully suggest that donor mtDNA play an essential role in the survival of recloned animals. Further studies are needed to precisely define the role of donor mtDNA in recloned animals.

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