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Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep

Matthew J. Evans¹, Cagan Gurer¹, John D. Loike², Ian Wilmut³, Angelika E. Schnieke⁴, and Eric A. Schon^{5,6}

¹Integrated Program in Cellular, Molecular and Biophysical Studies, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032, USA

²Department of Physiology and Cellular Biophysics, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032, USA ³Roslin Institute, Roslin, Midlothian, EH25 9PS, Scotland ⁴PPL Therapeutics and Roslin Institute, Roslin, Edinburgh EH25 9PP, UK ⁵Department of Neurology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032, USA ⁶Genetics and Development, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, New York 10032, USA

Abstract

Eukaryotic cells contain two distinct genomes. One is located in the nucleus (nDNA) and is transmitted in a mendelian fashion, whereas the other is located in mitochondria (mtDNA) and is transmitted by maternal inheritance. Cloning of mammals^{1–6} typically has been achieved via nuclear transfer, in which a donor somatic cell is fused by electroporation with a recipient enucleated oocyte. During this whole-cell electrofusion, nDNA as well as mtDNA ought to be transferred to the oocyte^{7,8}. Thus, the cloned progeny should harbour mtDNAs from both the donor and recipient cytoplasms, resulting in heteroplasmy. Although the confirmation of nuclear transfer has been established using somatic cell-specific nDNA markers, no similar analysis of the mtDNA genotype has been reported. We report here the origin of the mtDNA in Dolly, the first animal cloned from an established adult somatic cell line, and in nine other nuclear transfer-derived sheep generated from fetal cells. The mtDNA of each of the ten nuclear-transfer sheep was derived exclusively from recipient enucleated oocytes, with no detectable contribution from the respective somatic donor cells. Thus, although these ten sheep are authentic nuclear clones, they are in fact genetic chimaeras, containing somatic cell-derived nuclear DNA but oocyte-derived mtDNA.

Several aspects of mitochondrial genetics are relevant to the understanding of mitochondrial transmission during nuclear transfer. First, there are approximately 100,000 copies of mtDNA (a double-stranded DNA circle of ~16–17 kb) in a mammalian oocyte^{9,10}, but only a few thousand copies in a typical mammalian somatic cell¹¹. Second, there is no mtDNA replication until the blastocyst (~100-cell) stage of embryogenesis⁹; thus, each of the 20 cells of the inner cell mass that ultimately develop into the fetus contains approximately 1,000 copies of mtDNA. Third, during normal sexual reproduction, mitochondria (and mtDNAs) are maternally transmitted¹²; paternal mitochondria enter the oocyte, but are eliminated rapidly by an unknown mechanism during the first few zygotic cell

divisions^{13,14}. Fourth, although an individual member of a species normally harbours a single mitochondrial genotype (homoplasmy), an animal can harbour two mtDNA genotypes (heteroplasmy) if a mutation in the germ line of the mother passes into the progeny. Finally, any two genetically unrelated homoplasmic individuals differ at approximately 0.3% of the nucleotides in their mtDNAs, with most mutations located in the highly polymorphic D-loop region (which is the control region in mtDNA that is devoid of structural genes).

There are three possible outcomes in animals cloned by nuclear transfer via whole-cell electrofusion: homoplasmy of donor somatic cell mtDNA; homoplasmy of recipient oocyte mtDNA; or heteroplasmy due to mixing of donor and recipient mtDNAs. The determination of the mitochondrial character of cloned sheep required the analysis of mtDNA from three sources: (i) the nuclear donor somatic cells; (ii) the recipient oocyte; and (iii) the nuclear transfer-derived cloned sheep. We used DNA sequencing of the D-loop region and PCR/RFLP analysis based on polymorphisms found between donor and recipient samples to differentiate the mtDNA genotypes (both qualitatively and quantitatively) among the three relevant sources.

We analysed DNA from three donor cell types: OME, an adult mammary gland primary epithelial cell culture⁵; and PDFF2 and BLWF1 (ref. ⁶), fetal fibroblast primary cell cultures derived from day-35 and day-25 fetuses, respectively. We also analysed DNA from tissues (blood, skeletal muscle, placenta and milk) obtained from ten nuclear transfer-derived sheep, including Dolly (we confirmed that the nuclear DNA in all the samples matched the nuclear DNA isolated from the appropriate somatic donor cells; data not shown). Unfortunately, the animal husbandry regime, the practicalities of the oocyte collection regime and the use of intermediate recipients precluded identification of the Scottish Blackface (SB) oocyte donor for any individual nuclear-transfer lamb. (During each nuclear-transfer session, oocytes derived from eight individual ewes were pooled. Following recovery, the donor ewes were sold to a slaughterhouse. No tissue samples were retained for specific comparison.) To overcome this problem, we analysed the D-loop region (Fig. 1a) of tissues from four randomly selected SB sheep as a representative sample. A 544-bp segment of the 1.2-kb sheep D-loop¹⁵ from OME, PDFF2 and BLWF1 donor cells and from the SB samples was amplified by PCR from total genomic DNA and sequenced (Fig. 1b). Relative to a reference D-loop sequence¹⁵, we found 40 polymorphisms, 4 of which generated useful restriction endonuclease recognition sites (Fig. 1b).

On RFLP analysis, all somatic donor cell and representative oocyte-recipient SB samples displayed the predicted patterns (Fig. 2). The same region of the D-loop was amplified from the DNA of the ten nuclear transfer-derived sheep. RFLP analysis of these amplified products showed that all ten were homoplasmic for the mitochondrial genotype of the SB recipient oocyte in all tissues examined, with no evidence for the presence of donor mitochondria-derived mtDNAs (Fig. 2).

An A→G polymorphism at nt 223 (Fig. 1b) destroys an *AseI* site in OME (genotype A; Fig. 2). This *AseI* site is present in OME-derived sample 4 (Dolly; genotype B). Conversely, nuclear transfer-derived sheep 5 is genotype A, whereas its nuclear donor BLWF1 is genotype B. Similarly, samples 1, 2, 3, 6, 7 and 10 are genotype B, whereas their nuclear donor PDFF2 is genotype A. The *AseI* genotypes of nuclear transfer-derived sheep 8 and 9 were uninformative, as the same genotypes were present in both their respective nuclear donors and the SB samples.

A C→T polymorphism at nt 288 (Fig. 1b) creates an additional *BsrGI* site in the amplified D-loop region of PDFF2 (genotype D; Fig. 2). PCR/RFLP analysis of PDFF2 gave the expected restriction pattern with this enzyme (Fig. 2). All seven nuclear transfer-derived

sheep derived from PDFF2 (1, 2, 3, 6, 7, 9 and 10; Fig. 2) showed no evidence of this polymorphism (all had genotype C).

A T→C polymorphism at nt 418 (Fig. 1b) creates a new *DpnII* site (genotype E; Fig. 2) in one of four representative oocyte recipients (SB-C), but this specific site is not present in the other three oocyte recipients or the three nuclear donors (that is, they are genotype F). Of the ten nuclear transfer-derived sheep, seven were also genotype F, whereas three (sheep 4, 5 and 9) contained a different informative polymorphism (T→A at nt 167, creating a *DpnII* site; genotype G) not present in donor or oocyte samples (Fig. 1b). Each of these three sheep was derived from a different nuclear donor (OME, BLWF1 and PDFF2, respectively), but none of the donors had genotype G. This indicates that the mtDNA genotypes of the nuclear transfer-derived sheep do not match those of their nuclear donors (presumably, genotype G is present in the SB population, although it was absent in the four SB samples analysed here).

Finally, sequencing of the OME-derived PCR fragment revealed a C→T polymorphism at nt 550 (Fig. 1b) that creates a new *Tsp509I* site (genotype J; Fig. 2). Dolly (Fig. 2, sheep 4) is derived from the OME cell line, yet showed no evidence of this mtDNA polymorphism (Fig. 2, genotype H).

We performed 48 RFLP analyses, of which 26 yielded informative results (that is, we were able to distinguish the mtDNA genotype of the somatic donor cells from that of the representative oocyte-recipient sheep breed). All 26 indicated that the nuclear transfer-derived sheep were homoplasmic for the oocyte recipient mtDNA, and none of the 22 non-informative RFLPs contradicted this conclusion. We estimate that the limit of detection in our methodology was at least 99.5–99.9%. We based this range on quantitations performed using three different methods.

The finding of mtDNA homoplasmy in all ten nuclear transfer-derived sheep was unexpected. Because the nuclear transfer method via electrofusion almost certainly introduces donor cytoplasm, including mitochondria, into the cytoplasm of the recipient SB oocyte^{7,8}, one would expect to observe at least some contribution of donor-derived mtDNA in the clones (heteroplasmy). To estimate the lower limit of the potential contribution of mtDNA from donor cytoplasm, we quantitated the number of mtDNAs in OME cells by dot blot analysis (data not shown). We estimated that OME cells contain 2,000–5,000 mtDNAs per cell, values only slightly lower than those in transformed human somatic cells¹¹. Although we did not analyse the BLWF1 and PDFF2 donor cells, we assume they had similar amounts of mtDNA. The mtDNA composition of each fetal sheep derived by nuclear transfer should reflect that of the original electrofused donor/recipient cell—assuming that the inheritance of somatic donor mtDNAs is similar to that of maternal germline mtDNAs, and that there was homogeneity of cytoplasmic mixing—in which case we should have observed 2–5% of donor-derived heteroplasmic mtDNA in the samples.

Our failure to detect donor mtDNAs in any of the examined tissues from the nuclear transfer-derived sheep implies that random partitioning of mtDNAs (ref. ¹⁶) did not occur. This may be due to the failure of the donor mitochondria to enter the ooplasm following electrofusion, to skewed segregation of donor mtDNAs during the cloning process or to some unknown effect of maintaining donor cells in G₀ for five days before nuclear transfer⁵. We favour a scenario in which an active mechanism operates to destroy the donor mitochondria in the recipient ooplasm, similar to what is thought to happen to sperm-derived mitochondria in fertilized ova in both normal human reproduction^{12,14} and in intraspecific (but not in interspecific^{13,17,18}) mouse crosses^{13,19}.

Our conclusion that the nuclear transfer-derived sheep were homoplasmic is based on the analysis of specific tissues in each animal. We analysed one tissue (either blood or skeletal muscle) from each animal, and in two animals we also analysed a second tissue type (milk from sample 1 and placenta from sample 4, Dolly). One or two tissues, however, may not accurately reflect the mtDNA composition of the entire animal. For example, wide variations in heteroplasmy were detected in different progeny and in different tissues of mice produced from fertilized eggs containing exogenous oocyte-derived mitochondria generated by pronuclear karyoplast fusions with enucleated zygotes^{20,21} or injections of heterologous oocyte cytoplasm into recipient oocytes²¹⁻²³. In addition, heteroplasmic patients harbouring pathogenic mutations in human mtDNA (ref. ²⁴) sometimes contain significantly different proportions of the mutation in different tissues, as in mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), where the proportion of mutated mtDNA in blood is usually lower than that in other tissues²⁵. In other mitochondrial disorders, however, such as myoclonus epilepsy with ragged-red fibers²⁶ (MERRF) and maternally inherited Leigh syndrome²⁷ (MILS), the mtDNA mutation is distributed relatively homogeneously in all tissues. Of the sheep samples examined, we obtained three (2, 7 and 10) from skeletal muscle, a long-lived post-mitotic tissue which might be more likely to exhibit heteroplasmy, if present in the animal²⁴. Our conclusions, however, are tempered by the fact that we were unable to obtain samples of other sheep tissues, such as brain and heart, that are frequently affected in human mitochondrial diseases²⁴. Although we cannot eliminate the possibility that there is heteroplasmy in other unexamined tissues of the cloned animals, the uniformity of the results from multiple sample sources supports the conclusion that the clones were homoplasmic for oocyte-derived mtDNA.

These results have implications for future attempts to correct maternally inherited mitochondrial genetic disorders by, for example, nuclear transfer involving a somatic or germline cell from a woman harbouring a pathogenic mtDNA mutation (but normal nuclear DNA) and a recipient enucleated oocyte^{28,29} (containing normal cytoplasm). If the experience with cloned sheep is any guide, we would predict that the human mitochondrial genotype will be determined by the recipient ooplasm.

Methods

Sheep samples

We isolated total DNA from three somatic nuclear donors (OME cultured mammary gland cells derived from a Finn Dorset sheep⁵; BLWF1 fibroblast cells derived from a day-25 Black Welsh Mountain fetus⁶; and PDFF2 fibroblast cells derived from a day-35 Poll Dorset fetus⁶); four representative Scottish Blackface sheep (SB-A, -B, -C and -D); and ten nuclear transfer-derived sheep^{5,6}. Nuclear transfer sheep 1, 2, 3, 6, 7, 9 and 10 were from PDFF2; 5 and 8 were from BLWF1; and 4 (Dolly) was from OME. We isolated DNA samples from blood (sheep 1, 3, 4, 5, 6, 8 and 9), tongue muscle (2, 7 and 10), milk (1) and placenta (4).

D-loop analyses

We amplified a 544-bp region of the sheep D-loop¹⁵ with forward primer 96F (nt 96–113; ref. ¹⁵) and reverse primer 639B (nt 639–621) with *Taq* DNA polymerase (Perkin Elmer). Conditions were 94 °C for 30 s, 66 °C for 30 s and 72 °C for 30 s, for 30 cycles, followed by 7 min at 72 °C, performed on a model 9700 thermocycler (Perkin Elmer). We sequenced the PCR products using the fmol system (Promega). We performed multiple amplifications from the original samples to minimize the possibility of identifying PCR-induced mutations; none were detected.

RFLP analyses

We amplified samples by PCR in the presence of [α - 32 P]dCTP, digested them with appropriate enzymes and electrophoresed the digested products through 8% non-denaturing polyacrylamide gels. After drying the gels, we visualized and quantitated the labelled fragments in a phosphorimager (BioRad Model GS363) or on X-ray film. We estimated the limit of detection for potential heteroplasmy using three different methods: (i) RFLP analysis of PCR-amplified mixtures of two known genotypes was able to distinguish heteroplasmy to a dilution of 1:200 (that is, detection limit of at least 99.5%); (ii) RFLP analysis of PCR fragments (amplified from skeletal muscle DNA isolated from clone sample 2 (PDF2)) subcloned into pCR2.1 (Invitrogen) showed only a single genotype in 190 randomly picked clones (that is, detection limit of at least 99.5%); and (iii) phosphorimager quantitation of the ratio of the signal derived from an authentic RFLP fragment to that of the signal located in the region of the gel in which a predicted heteroplasmic fragment should appear was ~1,000:1 (that is, detection limit of 99.9%), and was confirmed by measuring the signals from known serial dilutions of two labelled DNA samples.

Quantitation of mtDNA in somatic cells

We used serial dilutions of the PCR-amplified sheep D-loop region as standards in dot blot analyses of serial dilutions of OME-derived total DNA isolated from a known number of OME cells. We probed the blots with the sheep D-loop region, labelled by random priming and quantitated the dot intensities as above. The D-loop probe detected only authentic mtDNA, as confirmed by the detection of a single 16.5-kb hybridizing band in Southern-blot analysis of *Pst*I-digested sheep total DNA (data not shown).

Microsatellite analysis

We used ovine nuclear microsatellite marker pairs (5' and 3') MAF33, FCB304, FCB11 and MAF209 to establish the nuclear genotype of the various samples, as described³⁰.

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Fig. 1.

The sheep D-loop region. **a**, Map of the region, flanked by the genes encoding tRNA^{Pro} and tRNA^{Phe}, showing four 75-bp tandem repeats (boxed), the PCR-amplified region (primers indicated above arrows) and the sequenced region. Nucleotide numbering (below map and in **b**) is according to ref. ¹⁵. **b**, DNA sequence of the 544-bp PCR-amplified region. Polymorphisms compared with the published reference sequence¹⁵ are shown. The 75-bp repeats (plus 13 bp of a fifth repeat) are bracketed. The underlined regions indicate the five restriction sites used in the RFLP analyses (restriction sites in bold). Note that a T→A polymorphism at nt 167, not present in the 7 samples shown here, created an additional *DpnII* site in 3 nuclear-transfer sheep samples.

Fig. 2. PCR/RFLP analyses of the 544-bp PCR-amplified D-loop fragments, showing the maps with the predicted digestion fragment sizes, in bp, for each of the 4 indicated restriction endonucleases. Autoradiograms of the respective gels are to the right of each set of maps. The first group of autoradiograms (nearest the maps) shows the RFLP patterns in the four representative oocyte donor samples. In each of the three other groups of autoradiograms, the first lane shows the RFLP pattern in the nuclear donor (bold), followed by the pattern in the nuclear-transfer sheep (NTS) derived from that donor; the tissue analysed (B, blood; S, skeletal muscle; M1, M2, milk (replicates); P1, P2, placenta (replicates)) for each sample (numbered) is indicated above each NTS lane. The mtDNA genotypes (A–J) are indicated at left. Genotypes are shown below the oocyte and donor lanes; only the genotypes below the NTS lanes that were informative for the origin of the indicated mtDNA genotype are shown (bold). Gel fragment sizes in bp are indicated (right).