Non-balanced mix of mitochondrial DNA in cloned cattle produced by cytoplast-blastomere fusion

Ralf Steinborn[a,b,*], Valeri Zakhartchenko[c], Eckhard Wolf[d], Mathias Müller[a,b], Gottfried Brem[a,b,e]

[a]*Department of Animal Biotechnology at IFA, A-3430 Tulln, Austria*
[b]*Institute of Animal Breeding and Genetics, University of Veterinary Sciences, Veterinärplatz 1, A-1210 Vienna, Austria*
[c]*Bavarian Research Centre of Reproductive Biology, Hackerstr. 27, D-85764 Oberschleißheim, Germany*
[d]*Department of Molecular Animal Breeding and Genetics, Ludwig-Maximilian University, Veterinärstr. 13, D-80539 Munich, Germany*
[e]*Ludwig-Boltzmann Institute for Immuno-, Cyto- and Molecular Genetic Research, Veterinärplatz 1, A-1210 Vienna, Austria*

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Abstract We have investigated the transmission of parental mitochondrial DNA (mtDNA) in three clones of born cattle obtained by *intra*specific cytoplast-blastomere fusion. Using allele-specific TaqMan PCR a low level transmission of blastomere mtDNA (DB mtDNA) into the cloned offspring was detected, thereby generating a heteroplasmic population of mtDNA. The amount of DB mtDNA was 13% and 18% in two animals of a clone which derived from a 24-cell morula and 0.6% and 0.4% in two calves of clonal origin derived from a 92-cell morula. These values are in accordance with the tendency expected for neutral mtDNA segregation that the fewer cell divisions that have occurred in the donor embryo, the higher the amount of DB mtDNA. We also found a strong decrease of DB mtDNA which was about three orders of magnitude in the third clone derived from a 52-cell morula stage.

Key words: Cloning; Cattle; mtDNA; Control region; Allele-specific quantitation; Allele-specific TaqMan polymerase chain reaction

1. Introduction

Embryo cloning of rabbits [1], sheep [2,3] and cattle [4–7] has been successfully used to produce genetically identical offspring. Cloning may offer a way of dissecting the non-genetic aspects of complex diseases [8]. It may also assist in gene farming [9], in saving endangered species [10] and in maintaining endangered populations. This technology is also a valuable tool in embryological studies using the mouse model system [11].

Bovine mtDNA polymorphisms were linked to beneficial and detrimental milking performance, reproduction and health [12] and nuclear-mitochondrial interaction was found to be correlated with fitness/heterosis in *Drosophila* [13,14]. mtDNA mutations are also associated with a number of human diseases [15].

The objective of this study was to monitor the fate of both types of parental mtDNA in cloned calves after birth which were produced by *intra*specific cytoplast-blastomere fusion. It was investigated whether different developmental stages of donor morulae would contribute different amounts of mtDNA to the cloned offspring. The transmission of parental mtDNA was investigated on the basis of polymorphisms in the control region (CR) of the mitochondrial genome. In order to reach exact and sensitive allele-specific quantification of parental mtDNA in the cloned calves we developed an allele-specific TaqMan polymerase chain reaction (AS-TaqMan PCR).

2. Materials and methods

2.1. Oocyte maturation, donor embryos, recipient cytoplasts and embryo transfer

In vitro production of morula stage embryos, cytoplast-blastomere fusion, in vitro culture and transfer of cloned blastocysts into recipient cows was carried out as described previously (for references see Steinborn et al., this issue).

2.2. mtDNA analysis of cloned calves derived from DBs of different developmental stages

Morulae of 24, 52, and 92 cells were used for the production of three clones of cattle (C24, C52, and C92), which belonged to different breeds: Holstein-Friesian, Brown Swiss and Simmental respectively. Each clone consisted of two individuals: C24-1 and C24-2, C52-1 and C52-2, and C92-1 and C92-2. As a source of DB mtDNA in cloned calves, blood from the original embryo donor (for C92) or the sexually produced F_1 offspring of the original embryo donor (for C24 and C52) was used. Total DNA used for mtDNA sequencing, in the TaqMan PCR and in the AS-TaqMan PCR was isolated from blood and was finally dissolved in 100 μl buffer as described [16].

Parental mtDNA was differentiated by screening the CR for single nucleotide substitutions. From each sample of parental mtDNA we sequenced a 974 bp mtDNA fragment amplified with oligonucleotides CO1 and CO3 (see below) and subcloned into the pTAg vector (R&D Systems, Germany). For each parental type of mtDNA we sequenced two independent PCR products by automated sequencing (LI-COR, USA) to confirm the point mutations used for allele-specific (AS) PCR and AS-TaqMan PCR. Based on the analysis of fused early embryos which has revealed the predominance of the RC mtDNA (Steinborn et al., this issue), the mtDNA of the cloned animals was regarded as the RC mtDNA. Therefore, the analysis of cloned calves was possible, although information about the exact origin of the enucleated oocytes (RCs) used in the three cloning experiments was not available.

2.3. PCR, AS-PCR and oligonucleotides

PCR and AS-PCR were performed as described (Steinborn et al., this issue) using the oligonucleotides (CO1 and CO3) and the AS primer AS2. Details concerning their sequence are summarised in Table 1 including also details about primers and the fluorogenic probe used in the AS-TaqMan assays (see below). In addition to the NTC the AS-PCR included as a second type of control the NAC (no amplification control): total cellular DNA of cattle which lacked the

*Corresponding author (b). Fax: (43) (1) 250775690.
E-mail: RaSt@i122server.vu-wien.ac.at

Abbreviations: DB, donor blastomere; RC, recipient cytoplast; CR, control region; AS-TaqMan PCR, allele-specific TaqMan polymerase chain reaction; NTC, no template control; NAC, no amplification control; T_a, annealing temperature

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Table 1
Oligonucleotides for PCR analysis

Oligo	5′-3′ sequence	5′-3′ position
CO1	cac cat caa ccc cca aag ct	15747–15766
CO3	ttg ggt taa gct aca tca ac	383–364
AS2	taa tta tat gta tta tgt acG(a) gg	16079–16057
AS5	acc att gac tgt aat gtc T(g)at	189–169
AS6	gcc cca tgc ata taa gca G(a)gt	16022–16042
AS7	gca agt aca tga cct cta C(t)ac	16037–16057
TM1	ctt aat tac cat gcc gcg tga	16159–16179
TM2	cca gct aca ata gat gct ccg	131–111
TMP	ttg acg gcc ata gct gag tcc	99–79

The position refers to the numbering in the GenBank accession number V00654. Nucleotides generating a mismatch in the template DNA are printed as capital letters. The corresponding nucleotide of the reference sequence is given in parentheses. The probe TMP consists of an oligonucleotide with the 3′-quencher dye TAMRA (6-carboxy-tetramethylrhodamine) and the reporter dye FAM (6-carboxyfluorescein) attached to the 5′ end using nonextendible hybridization.

mitochondrial allele under study was used. If the desired type of mtDNA was not available a plasmid bearing the appropriate allele was used. These plasmids were originally produced for sequencing (CR in pTAg, see above) and were quantified by TaqMan™ PCR (see below).

2.4. Allele-specific quantitative PCR in real time (AS-TaqMan PCR)

The ratio of parental mtDNA was analysed by AS-TaqMan PCR (R. Steinborn, M. Müller and G. Brem, unpublished). This approach represents a further development of two established methods, the use of allele-specific oligonucleotides [17,18] and TaqMan PCR (PE Applied Biosystems, Germany) [19–21].

Allele-specific primers were designed instead of conventional oligonucleotides to exclude a possible amplification of a false 'mitochondrial allele'. Therefore, an additional mismatch at position 3 from the 3′ end of each primer was introduced [17]. Details concerning the design of allele-specific primers are given in Table 1. Two sets (each set in triplicate) of real-time experiments were necessary to quantify the percentage of DB mtDNA compared to the total population of mtDNA molecules: (i) the determination of the total amount of mtDNA by TaqMan PCR (for details see Steinborn et al., this issue) and (ii) the AS-TaqMan PCR of interest. In the AS-TaqMan PCR of interest the distal primer (TM1) used in the TaqMan PCR described (Steinborn et al., this issue) which is distant to the fluorogenic probe was substituted by an AS primer. The replacement of the proximal primer (TM2) by the AS primer AS5 leading to an increased distance

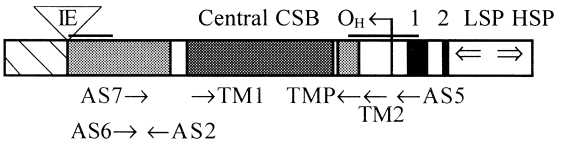

Fig. 1. Oligonucleotides for AS-PCR and AS-TaqMan PCR. Schematic representation of the localisation of the fluorogenic probe (TMP), conventional (TM1 and TM2) and allele-specific (AS2 and AS5–AS7) primers within the bovine mtDNA CR ([40], modified). The primers CO1 and CO3 are located outside the CR (not shown). The shaded and hatched areas correspond to evolutionarily conserved regions. The underlined regions indicate the cloverleaf-like secondary structure. IE, insertion element; O_H, replication origin of the H strand; HSP and LSP, promoters of the H and L strands; 1 and 2, conserved sequence blocks (CSBs). For more details of the functional elements of bovine mtDNA CR see [26].

between the fluorogenic probe and the proximal primer (11 bp versus 71 bp) was also successful. The localisation of the oligonucleotides and the fluorogenic probe used in the AS-TaqMan PCR is illustrated in Fig. 1.

The reliability of AS-TaqMan PCR was demonstrated in preliminary experiments mimicking the in vivo situation of mtDNA heteroplasmy. Different ratios of parental mtDNA were mixed and subsequently quantified. Total cellular DNA isolated as described [16] was diluted 1:8 prior to AS-TaqMan PCR. The absence of AS-TaqMan PCR inhibitors from the DNA isolations was documented by the comparison of threshold values obtained for the 1:8 dilution and for a 1:64 dilution of the same sample.

In addition to the no template control (NTC) the AS-TaqMan PCR included as a second type of control the NAC (no amplification control): total cellular DNA of cattle which lacked the mitochondrial allele under study was used. If the desired type of mtDNA was not available a plasmid bearing the appropriate allele was used. These plasmids were originally produced for sequencing (CR in pTAg, see above) and were quantified by TaqMan PCR (see below).

3. Results and discussion

The transmission of parental mtDNA in three pairs of cloned calves after birth was assayed by AS-PCR and by AS-TaqMan PCR. The clones were generated from donor embryos with differing numbers of blastomeres: 24 cells, 52 cells, and 92 cells. All three clones contained both types of

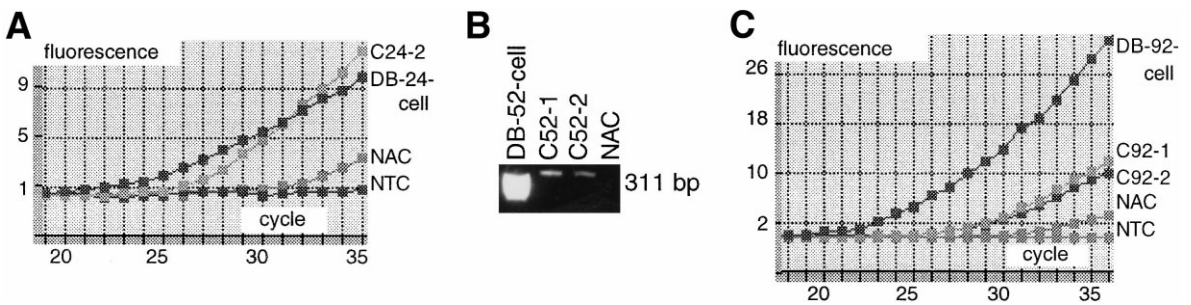

Fig. 2. Non-balanced mix of parental mtDNA in three cattle clones (C24-1 and C24-2, C52-1 and C52-2 and C92-1 and C92-2) shown by AS-TaqMan PCR (A and C) and AS-PCR (B). The first PCR cycles in A and C are not shown since the fluorescence signal was still below the threshold for detection. Total cellular DNA was isolated from blood. The number of donor blastomeres (DBs) of the three representative morulae, i.e. the donor embryos, was 24 cells (A), 52 cells (B), and 92 cells (C). The CR of RC mtDNA was sequenced (Table 2). A: Primers AS6 and TM2, $T_a = 70°C$, 4 mM $MgCl_2$. Ratios of the non-balanced mix are summarized in Table 3. The sample C24-2 showing a higher fluorescence rate of increase achieves endpoint plateau at a higher fluorescence level than would be expected from the input DNA. This phenomenon may be attributable to late cycle inhibition [21] and demonstrates that PCR endpoints are not suitable for quantification. B: Primers CO1 and AS2, $T_a = 44°C$, 1.5 mM $MgCl_2$, hot start. NAC, total bovine DNA with the mtDNA allele nt 16057G which lacked the allele nt 16057C. This G to C transversion was used to quantify the ratio of both alleles in the two cloned C52 animals by AS-TaqMan PCR (primers AS7 and TM2, $T_a = 59°C$, 4.5 mM $MgCl_2$, for complete results see Table 3). C: Primers AS5 and TM1, $T_a = 62°C$, 4.5 mM $MgCl_2$, complete results in Table 3.

Table 2
Nucleotide positions divergent from the bovine mtDNA reference sequence

	sample	16022	16042	16057	16104	16127	16185	16228	16255	119	169	216+1	352+1	363
C24	DB-24-cell												C	
	C24-1 (RC11)		C								G			
	C24-2 (RC12)		C								G			
C52	DB-52-cell			C		T	A	G	C		G			
	C52-1 (RC13)	A			T							C		G
	C52-2 (RC14)									T	G	C		G
C92	DB-92-cell													
	C92-1 (RC15)										G	C		
	C92-2 (RC16)										G	C		

The corresponding GenBank accession numbers for samples arranged from top to bottom are: AF022922, AF022923, AF022923, U92244, U92230, U92242, V00654, AF022924, and AF022924.
+1, insertion of one nucleotide.

mtDNA in a different ratio (Fig. 2, Table 3). Two types of transmission can be differentiated (see below): neutral segregation with an input of DB mtDNA ranging between 0.4% and 18% and a marked decrease of DB mtDNA. As expected the majority of mtDNA derived from the cytoplast and represents the remaining part of the total population of mtDNA molecules.

3.1. Neutral segregation of parental mtDNA

First the percentage of DB mtDNA in the two clones which derived from early (24-cell, clone C24) and late (92-cell, clone C92) morulae will be discussed. We detected minor contributions of DB mtDNA of 13% and 18% for the clone C24 and 0.4% and 0.6% for the clone C92 (Fig. 2A,C). The possibility of mtDNA heteroplasmy [22] – which in theory might already have existed originally in the fusion partners – was excluded by confirming the non-balanced mixing in the clone C24 by the allele-specific quantification at a second mtDNA marker (position 169, compare Fig. 2C and Table 2, data not shown).

In the case of neutral segregation the ratio of parental mtDNA can be explained by a simple model. This model takes into consideration the constant amount of mtDNA during early embryogenesis (Steinborn et al., this issue) and proposes a nearly similar partitioning of mitochondria into the dividing cells. It yields theoretical percentages of 6.2%, 3.1% and 1.5% DB mtDNA for clones derived from 16-cell, 32-cell, and 64-cell donor embryos, respectively. In brief, the percentage of DB mtDNA decreases with increasing number of blastomeres in the morula stage donor embryo. We conclude that all mitochondria and mtDNA alleles contained in these two clones show neutral segregation in comparison to the nuclear background of the donor blastomere (DB) and to the initial surrounding of the RC which was expressed by the oocyte. Consequently the data indicate that the observed mixing of parental mtDNA might be dependent on the initial content of mitochondria in the blastomere used as nuclear donor.

3.2. Decrease of DB mtDNA

In the third clone which derived from the fusion between a 52-cell blastomere and two different RCs a marked decrease of about three orders of magnitude (0.006% and 0.0004%) was observed (Fig. 2B, Table 3). Several possible mechanisms could be envisaged and are discussed to account for the reduction of DB mtDNA in the nuclear background of the blastomere: (i) a replicative advantage for one parental mtDNA due to sequence differences in the CR, (ii) a difference in the turnover rate of parental mitochondria, (iii) selection at the level of the cell for respiratory chain function, (iv) randomisation of cells at the formation of inner cell mass and/or later embryonic stages or (v) active selection against DB mitochondria.

(i) Replication of mtDNA proceeds asymmetrically from two independent origins, O_H and O_L (H and L refer to the heavy and light strands of mtDNA) [23]. Leading strand synthesis is primed from O_H by a small RNA, which is generated from transcription initiation at the L-strand promoter (LSP) and processed at two conserved sequence blocks (CSBs) [24,25]. Both origins, LSP and CSB1, are completely conserved in the parental mtDNA with the exclusion of one polymorphisms located in CSB2+3 (Table 2). The stretch of consecutive cytosines at nt 216 which varies in length does not correlate with neutral or non-neutral segregation of DB mtDNA observed in the present study. Thus if the replicative advantage of the RC genome causes the observed decrease it

Table 3
Quantification of parental mtDNAs in cloned cattle by AS-TaqMan PCR

Clone	Sample	Amount of mtDNA[a]	AS-TaqMan[a]	DB mtDNA (%)
C24	DB-24 cell	20.8 ± 0.2	24.0 ± 0.0	100
	C24-1	23.1 ± 0.2	29.2 ± 0.1	13
	C24-2	20.3 ± 0.1	25.9 ± 0.2	18
	NAC1[b]	20.3 ± 0.1	30.2 ± 0.1	0.9
C52	DB-52 cell	20.9 ± 0.3	20.6 ± 0.3	100
	C52-1	21.0 ± 0.1	34.8 ± 1.1	0.006
	C52-2	21.0 ± 0.1	38.5 ± 0.6	0.0004
	NAC2	21.7 ± 0.3	40.0 ± 0.0	0
	NAC3	21.0 ± 0.1	40.0 ± 0.0	0
C92	DB-92 cell	22.7 ± 0.1	23.4 ± 0.1	100
	C92-1	16.8 ± 0.1	24.8 ± 0.2	0.6
	C92-2	18.2 ± 0.1	26.7 ± 0.2	0.4
	NAC4	23.0 ± 0.1	40.0 ± 0.0	0

The total amount of mtDNA was determined by TaqMan PCR.
[a]Threshold cycle.
[b]NAC1 represents plasmid DNA.

must be due to complexity in the mtDNA replication machinery not represented by these conserved elements. (ii) Alternatively, the turnover rate of mitochondria could be genotype-specific. Proportional replication of existing templates to restore genome copy number would then skew the genotype frequency in favour of the genotype with the slower turnover. (iii) Selection at the cell level could occur if altered respiratory chain function conferred a growth advantage on cells carrying a higher proportion of a particular mtDNA genotype. However, a fixed breed-specific type of mtDNA was not described for the population of European cattle [26]. Functional interactions are also possible between mtDNA-encoded polypeptides and other mitochondrial proteins that are not part of the respiratory chain per se. However, a model for cellular selection must accommodate a fundamental property of mitochondrial genetics, i.e. the threshold behaviour of mtDNA mutations. Because mtDNA is a high copy number genome, an altered respiratory chain phenotype is not expressed until the proportion of mtDNA carrying the mutation exceeds a particular threshold level [15]. Thus if selection results from altered respiratory chain function it must be acting at a subcellular level on organelles containing one or the other genotype. (iv) Unequal partitioning of mtDNA to the inner cell mass cells could influence the transmission pattern of DB mtDNA in the cloned calves. The amount of potentially transmitted DB mtDNA is randomised due to the fact that at the early blastocyst stage only 10–20 cells [27] give rise to the inner cell mass forming the embryo, i.e. a cytoplasmic segregation factor of about 7–20 can occur between a 1-cell embryo and the point at which embryonic tissue types are determined (gastrulation). Later developmental stages in embryogenesis may also contribute to the overall bottleneck, e.g. embryonic partitioning into the three primary embryonic tissues: endoderm, mesoderm, and ectoderm. It was shown for cattle with naturally occurring heteroplasmy [28] and for mice obtained by *intra*specific nuclear transplantation [29,30] that dramatic mitochondrial chimericism exists in tissues of different embryonic origin (brain, liver and heart). Due to the involvement of our cloned animals in breeding programmes tissue chimericism was not analysed. (v) When attempting to explain the molecular mechanism leading to a considerable decrease of DB mtDNA, it is tempting to propose that one or more DB-specific components may provide a 'label' by which the RC can identify and subsequently select against the DB mitochondria, i.e. an inhibitory effect by an unknown RC-encoded factor on DB mtDNA or DB mitochondria during early embryogenesis cannot be excluded. Alternatively, this selection process could utilise the mechanism for chloroplast inheritance of *Chlamydomonas*, where site-specific methylation protects the maternal chloroplast DNA against a restriction endonuclease [31].

Our data cannot exclude that a specific morula stage of the donor embryo leads to the observed decrease of DB mtDNA in clone C52 (DB-52 cell). This seems to be unlikely however, since recently a decrease of DB mtDNA was reported for early cloned embryos produced by fusion of nuclear donor embryos from Japanese Black cattle at the 32-cell stage and RCs from Holstein cows [32]. Using single strand conformation polymorphism of PCR fragments (PCR-SSCP) the authors did not detect DB mtDNA in born cloned calves at all. Whether a small population of DB mtDNA molecules comparable to our results is still present is unclear, since PCR-SSCP obviously did not achieve the sensitivity of AS-TaqMan PCR.

3.3. mtDNA and inheritance

During sexual inheritance the mitochondrial genome is transmitted maternally in *intra*specific crosses of mice [33] and leakage of paternal mtDNA was found only in *inter*specific crosses [33,34]. This *inter*specific backcross study in mice showed paternal transmission of mtDNA at a ratio of approximately 1:50 000, which is 50 times lower than expected if parental mitochondria had equal abilities to survive and replicate throughout development [34]. This ratio of 1:50 000 is comparable with our results concerning the clone of born calves which originated from DB-52 cell (0.006% and 0.0004%; Table 2). However, in this clone, the decrease of about three orders of magnitude is much more marked. In the case of sexual inheritance two mechanisms are currently being discussed, an active exclusion mechanism [33] and a down-regulation of mtDNA copy number during spermatogenesis [35]. In addition, genetic drift in connection with the multicopy model might also be envisaged. This model is based on the fact that the number of mitochondria in one of the parental gametes is much lower than in the gametes of the other parent. However, the model does not accord with the above-mentioned reports concerning sexual inheritance in *intra*- and *inter*specific crosses [33,34]. The fate of the sperm midpiece in the cytoplasm of hamster eggs was examined by electron microscopy [36]. During the 2-cell stage, numerous multivesicular bodies gather around the midpiece and fuse with the sperm mitochondria. At this point the mitochondria are degraded and digested by the multivesicular bodies. For further discussion concerning embryo manipulation and mtDNA heteroplasmy we refer to Steinborn et al. (this issue).

In an earlier experiment in which information about the parental mtDNA types was lacking extrachromosomal differences were found within a clone of *Bos taurus* [5], indicating that RCs were derived from individuals with different mitochondrial genomes.

Recently, the generation of cloned sheep was reported [2,3]. In these experiments an established embryonic cell line and an adult mammary gland-derived cell line were used as nucleus donors. The authors did not address the issue of parental mtDNA transmission. It is known that mature mammalian oocytes contain $1.2–2.6\times10^5$ mtDNA molecules, 50–200 times that in somatic cells [28,37,38]. Consequently, assuming similar copy numbers for sheep, comparable mtDNA amounts in somatic and embryonic cell lines as well as neutral segregation of mitochondria, theoretically a non-balanced mix of parental mtDNA in the range of about 1:100 to 1:1000 can be expected for these cloned sheep.

3.4. General conclusions

In the present study we examined the parental mtDNA in cloned cattle obtained by *intra*specific cytoplast-blastomere fusion. (i) For the first time evidence has been obtained for a low level transmission of DB mtDNA into the cloned offspring, thereby generating a heteroplasmic population of mtDNA. The current understanding of mitochondrial genetics [15,30,39] implies that heteroplasmic mtDNA produced by cloning might undergo a certain degree of segregation in the following generations, including tissue-specific segregation [30]. (ii) Neutral transmission of parental mtDNA was found

in two clones derived from different stages of donor morulae. For these cases of neutral transmission it can be proposed that depending on the developmental stage of the donor embryo, the content of DB mtDNA in the cloned calves might vary. In a third clone a marked decrease of DB mtDNA of about three orders of magnitude was observed. (iii) There is obviously no exclusion mechanism similar to that of sexual inheritance which eliminates sperm mtDNA after each *intra*-specific fertilisation. (iv) The transmission of parental mtDNA of the fusion partners which exhibit different nuclear and/or mitochondrial genomes needs further investigation. These genetic variations might also include a breed-specific origin. Further studies should confirm or falsify our finding that different developmental stages of donor blastomeres might lead to different transmission patterns of mtDNA, if parental mitochondria exhibit neutral transmission. It is also necessary to study the genetic incompatibility which leads to the observed marked decrease of DB mtDNA in its own nuclear background and a mixed cytoplasmic environment including molecular factors and time course. (v) There will also be ramifications to the animal cloning industry. Those undertaking the production of female cloned families should be particularly attentive to the source of oocytes used in the protocol, since mtDNA of the oocyte will be permanently incorporated and transmitted to all female progeny. This may be used as a tool to create 'new' breeds of livestock carrying the best of both nuclear and mitochondrial genes or, as a result of inappropriate crossing, the introduction of mutations and undesirable traits. Cytoplast-blastomere fusion is suitable for the creation of a new nuclear-mitochondrial combination with or nearly without heteroplasmy.

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