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Review

The significance of mitochondria for embryo development in cloned farm animals

Heide Schatten[a,*], Randall S. Prather[b], Qing-Yuan Sun[c]

[a]*Department of Veterinary Pathobiology, School of Veterinary Medicine, University of Missouri-Columbia, 1600 E. Rollins Street, Columbia, MO 65211, USA*
[b]*Division of Animal Science, University of Missouri-Columbia, Columbia, MO 65211-5300, USA*
[c]*State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China*

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Abstract

The role of mitochondria in remodeling of the donor cell nucleus in cloned animals has gained increased attention, as mitochondria interact in direct or indirect ways with the donor cell nuclear DNA. Mitochondria comprise 1% of the genetic material that is contributed to the developing embryo by the recipient oocyte and provide the energy that is required for embryo development. In this review we compare mitochondria distribution in various species and the importance of mitochondria distribution for embryo development. We also compare the inheritance pattern of mitochondria in cloned embryos that remains unresolved, as the donor cell nucleus is typically transferred with surrounding cytoplasm including mitochondria which become destroyed in some but not all species. We review the role of mitochondria in cloned farm animals with emphasis on nucleo-cytoplasmic interactions and consequences for embryo development.

1. Introduction

Cloning of farm animals has become attractive in recent years because of the high potential for biomedical and agricultural applications with predicted commercial significance and the potential to generate genetically modified cattle and goats producing milk or serum containing high value biopharmaceutical products and improved food or high fiber quality. Moreover, genetically modified pigs are being produced to serve as tissue and organ donors for humans because of the exceptional physiological compatibility of pigs with humans. However, the cloning efficiency is still low and ranges from 0.1–5% of cloning attempts yielding viable offspring. While the underlying causes for the low cloning efficiencies are only little understood the obvious reasons are linked to incomplete or abnormal remodeling of the donor cell nucleus following transfer, resulting in imprinting failures and abnormal gene expression throughout development. Other possible reasons for the low cloning efficiency include pathological mitochondria distributions that can result in reduced ATP generating capacity, changes

* Corresponding author. Tel.: +1 573 882 2396; fax: +1 573 884 5414.
E-mail address: schattenh@missouri.edu (H. Schatten).

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in Ca^{2+} metabolism, and an inability to support normal cell functions and subsequent development even in morphologically normal looking embryos.

The role of mitochondria in the remodeling process and the influence of the cytoplast on the donor cell nucleus has gained increased attention. The recipient cell's cytoplast is predominantly responsible for the remodeling success to correctly express the genes required for the specific cell cycle stages. Epigenetic factors include a variety of determinants within the reconstructed embryo as well as factors from the surrounding medium and/or interactions of the reconstructed egg with the surrounding cell and tissue environment. Determining and understanding the epigenetic factors that support donor cell nucleus remodeling will be a major step towards improving developmental competence and it is likely to improve cloning efficiencies with development of the reconstructed oocyte resulting in live offspring. Investigations on cell and molecular levels have begun to determine the epigenetic profile that can faithfully reprogram the donor nucleus to match that of the fertilized egg (Rideout et al., 2001), as the inability of the oocyte cytoplast to remodel the donor nucleus is thought to be among the major causes for cloning failures. As will be seen in the following chapters mitochondria are of major importance in this process, as mitochondria interact in direct and indirect ways with the donor cell nucleus.

Mitochondria have also been central to a vigorous debate as to the faithful cloning of the original animal that provided the nucleus as the genetic material because mitochondria comprise 1% of the genetic material that is contributed to the developing embryo by the recipient oocyte. Mitochondrial DNA (mtDNA) and mtDNA-nuclear DNA (nDNA) interactions may be responsible for the different phenotypes resulting from nuclear cloning. During normal fertilization the oocyte contributes all mitochondria to the developing embryo (maternal inheritance) while the sperm's mitochondria are destroyed by a process in which oocyte-driven ubiquitination takes place (Sutovsky et al., 1999; 2003a,b; Schwartz and Vissing, 2002, 2003; Sutovsky, 2004). In cloned embryos, the inheritance patterns of mitochondria are not yet clear and it appears that different species follow different modes of mtDNA inheritance (reviewed by St John et al., 2004). Offspring generated by embryo reconstruction can transmit two populations of mtDNA resulting from mixing of recipient and donor cell mitochondria (heteroplasmy), or transmit one population of mtDNA from either recipient or donor cell mitochondria (homoplasmy). In all cases there is cross-talk between the nuclear genome and the cytoplasmic genome located in the mitochondria. Interestingly, interspecies fertilization crosses are not subjected to the ubiquitin-mediated event allowing sperm mtDNA to be transmitted to the resulting offspring at low levels (Gyllensten et al., 1991) although transmission to subsequent generations does not take place (Shitara et al., 1998).

Mitochondria may also play a role in embryo abnormalities and health problems associated with nuclear cloning. Major health problems include respiratory distress, circulatory problems, heart failures, immune dysfunctions and kidney or brain malfunctions and others. Many embryos are lost due to gestational and neonatal failures. Post-natal development can be severely compromised and about half of the living offspring can die shortly after birth. Birth defects and high post-natal losses are seen in cattle, sheep and pigs. Oversized livestock at birth (Bertolini et al., 2002), cloned calf syndrome (CCS, Wells et al., 2003) or more usually the large offspring syndrome (LOS, Young et al., 1998) are frequently observed in sheep and cows for which underlying causes are still unknown. Given the recent findings that mitochondria play a role in obesity (Hesselink et al., 2003; Kelley et al., 2002; Lowell and Shulman, 2005; Ritov et al., 2005) it may well be that mitochondria abnormalities are involved in the oversized livestock associated with cloned animals.

2. Methods for cloning

All major livestock species have been cloned now including sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998a,b; Steinborn et al., 1998), goats (Baguisi et al., 1999), and pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000; Park et al., 2001) with varying cloning efficiencies. Cloning of mammalian embryos is typically achieved by transferring a somatic cell nucleus with known

genetic value into an enucleated oocyte from a different animal. Five complex multi-step processes are involved in cloning of animals that vary to some extent in different laboratories. The steps include 1. Preparation of donor cells; 2. Preparation of recipient oocytes; 3. Nuclear transfer (NT); 4. Embryo culture; and 5. Embryo transfer (ET) into a surrogate mother.

2.1. Donor cells

The *donor cells* can be generated from a variety of different tissues. Most commonly ear skin or fetal fibroblast cells are used from primary cultures after several passages. Fetal fibroblast cells are also most suited for use after genetic modifications to produce animals with the desired genetic value and trait. For synchronization of donor cells serum starvation is commonly used to obtain confluent cells at the G0/G1 stage of the cell cycle. The question has been raised whether serum starvation (reduction from 10% to less than 1% in most cases) might negatively affect mitochondria function or induce apoptotic pathways as well as irreversibly damage DNA (Kues et al., 2002). Clear answers are not available, as detailed studies have not yet been performed on individual cells that are being transferred. Clearly, for cloning success it is important to use donor cells with optimal potential to be remodeled by the recipient cell's cytoplasm. Typically, for pigs, the donor cells are maintained at 39 °C under 5% CO_2, the same temperature and atmosphere in which the recipient oocytes are cultured. Metabolic blocking agents are frequently used to synchronize cell cultures including cyclin dependent kinase inhibitors such as staurosporine to arrest cells in G1, the DNA synthesis inhibitor aphidicolin to arrest cells in G1, or more recently roscovitine to arrest cells at the G1/S boundary (Boquest et al., 2002; Wells et al., 2003).

2.2. Recipient oocyte

The *recipient oocyte* is typically recovered from ovaries taken from slaughtered livestock by aspiration of immature oocytes and then matured in vitro in the laboratory. Immature recipient oocytes are matured in vitro and enucleated by using the following procedures as example. In pigs, mature oocytes are cultured in HbT medium supplemented with 3 mg/ml BSA and 7.5 μg/ml cytochalasin B to depolymerize microfilaments and facilitate the enucleation process. Enucleation is accomplished by removing the first polar body and the metaphase II plate together with a small amount of surrounding cytoplasm using a glass pipette (outer diameter 25–30 μm).

2.3. Nuclear transfer

Nuclear transfer is performed according to the procedures reported by Tao et al. (1999, 2000) for pig oocytes. In some of our experiments, the donor cells used for nuclear transfer are fetal fibroblast cells between passages 2 and 8 of culture, which are synchronized in presumptive G0 stage by serum deprivation. Following enucleation one fetal fibroblast cell is fused to each enucleated oocyte. The cells are injected into the perivitelline space through the same slit in the zona pellucida as made during enucleation. After micromanipulation, embryos are placed in NCSU-23 medium supplemented with 4 mg/ml BSA and 0.1 mg/ml cysteine for 30–60 min. Typically the entire cell is fused although nuclear injection by using isolated nuclei has been employed successfully in mice using piezo-driven micropipettes (Wakayama and Yanagimachi, 1999a,b). It has been suggested that potentially adverse effects associated with co-transfer of mitochondria and other non-nuclear cytoplasmic constituents of the donor cells may be avoided with this technique (Cummings, 2001; St John, 2002) although detailed comparison studies have not yet been carried out to determine whether mitochondria transferred with the donor cell may contribute to genetic variations in the cloned offspring.

2.4. Oocyte activation and embryo culture

For activation of porcine oocytes, recipients are placed between 200 μm in diameter platinum electrodes in a solution of calcium-free mannitol for 2 min. Cell fusion is induced with a DC pulse of 160 V/cm lasting for 40 μs. In cases of oocytes where fusion does not occur, a second DC pulse is applied 30 min later. After the electrical pulse, oocytes are left in 0.3 M mannitol solution for 30 min. They are then activated by electrical stimulation in calcium-containing medium or by the combined thimerosal/DTT

treatment. The reconstructed embryos are cultured in vitro until embryo transfer into the surrogate mother.

2.5. Embryo transfer

For efficient embryo transfer and to provide optimal culture conditions 1-cell stage embryos are surgically transferrd to the surrogate mother's oviduct as soon as possible.

3. Mitochondria distribution and significance of the cellular microenvironment during oocyte maturation, fertilization, parthenogenesis, and nuclear cloning

To understand the underlying causes for the low cloning efficiency and developmental pathologies it is necessary to analyze the cell and molecular mechanisms that are crucial for cell division and development. A variety of factors play a role in cell cycle progression with major roles for microtubules, centrosomes (microtubule organizing centers), ionic environment, calcium gradients, and phosphorylation. *Microtubules* play essential roles throughout the cell cycle and allow active translocations of cell organelles and molecular cell components. Mitochondria are translocated along microtubules to their functional destinations to provide the energy required for specific cell cycle events. Our studies have previously shown that translocation of active mitochondria depends on microtubule activity and on the dynamic organization of microtubules during pig fertilization and early development in vitro (Sun et al., 2001d). A temporal, spatial and developmental relationship exists between microtubule organization and the progressive translocation of mitochondria allowing dynamic and precisely coordinated cell and molecular interactions. Microtubules themselves are nucleated and organized into specific patterns by centrosomes assigning centrosomes an important role in directing microtubule transport. Studies by Van Blerkom et al. (2000) and Sun et al. (2001d) revealed that translocation of mitochondria is coordinated with changes in the location of centrosomes. *Centrosomes* are the principal microtubule organizing centers that remain closely associated with the donor cell nucleus and carry out functions that are normally carried out by sperm centrosomes. During normal fertilization in most species centrosomes are essential for the organization of the sperm aster that unites maternal and paternal genomes during fertilization (Schatten et al., 1986, 1987), and for the equal distribution of DNA and cell organelles during cell division, cell differentiation, and development (Schatten et al., 1986, 1987; Sun et al., 2001a–d). It is thought that sperm centrosomes establish the axis for the first and subsequent embryonic cell divisions. By organizing microtubules, centrosomes not only play a key role in the dynamic translocation of mitochondria along microtubules but also in the resulting metabolic activity (Sun et al., 2001d). In cloned embryos centrosomes that are closely associated with the donor cell nucleus need to be remodeled by the oocyte to carry out microtubule organizing functions during cell division and embryo development (Zhong et al., 2005). It is not known whether the donor cell centrosomes play a role in the establishment of embryonic cell divisions. Abnormal microtubule organization has been implicated in the possible causes for asymmetric mitochondrial distributions (Van Blerkom et al., 1995, 2000) and it has been shown that disproportional patterns of mitochondrial inheritance (Van Blerkom et al., 2000) in 2–4-cell human embryos results in cell lysis of the blastomere that is deficient in mitochondria. The positioning of mitochondria by microtubules may be crucial for concentrating ATP and/or calcium to specific regions in oocytes, fertilized, or cloned embryos to support normal developmental processes. Thus, the distribution of active mitochondria may be indicative of the energy/ion requirement of key events during oocyte maturation, fertilization and early embryo development.

Several investigators have analyzed the *distribution of mitochondria* during oocyte maturation, fertilization, parthenogenesis, and nuclear cloning. In the mouse model, polarization of mitochondria in the unfertilized oocyte has been well described (Calarco, 1995). Our groups have studied the distribution of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro by using MitoTracker Green staining and confocal laser scanning microscopy. We also studied the regulation of mitochondria translocation by microtubules and

excluded a role of microfilaments in this process (Sun et al., 2001d). Congregation of active mitochondria in the peripheral cytoplasm and around the germinal vesicles (GV) was characteristic of fully-grown oocytes collected from large follicles. Mitochondria accumulated in the perinuclear area during meiotic progression from germinal vesicle breakdown (GVBD) through anaphase I. Interestingly, we found differences for in vitro versus in vivo matured oocytes which may correspond to the differences in developmental competence that is lower in in vitro matured oocytes (Sun et al., 2001d). Compared to the oocytes matured in vivo, in which large mitochondrial foci were distributed through the entire cytoplasm, mitochondria were not observed in the central cytoplasm in half of the oocytes matured in vitro. In fertilized eggs, active mitochondria were aggregated in the pronuclear region. Perinuclear clustering and a cortical ring were characteristic for early cleavage. Active mitochondria were distributed in both inner cell mass cells and trophectoderm cells of the blastocysts. Disassembly of microtubules with nocodazole inhibited both mitochondrial aggregations to the GV area and their inward movement to the inner cytoplasm during oocyte maturation, as well as the translocation of mitochondria to the peri-pronuclear region during fertilization. The finding that mitochondrial foci form and relocate to the central cytoplasm of mature oocytes, and the finding that mitochondrial translocation in oocytes matured in vitro is not as complete as in oocytes matured in vivo lends support for the hypothesis that mitochondria distribution may play a role in cloning success/failure. The distribution of active mitochondria in fertilized pig eggs and during early embryo development in vitro is summarized in Fig. 1.

Mitochondria distribution has also been studied in other species and it appears that distinct mitochondria distribution patterns are indicative of developmental competence. Mitochondria in mouse oocytes are asymmetrically distributed and becomes homogeneously organized during fertilization (Van Blerkom and Runner, 1984; Muggleton-Harris and Brown, 1988; Calarco, 1995). In rats, mitochondria have a perinuclear aggregation that later becomes dispersed into the cell cortex (Zernicka-Goetz et al., 1993). In hamster embryos, mitochondria undergo reorganization from a homogeneous organization in the oocyte and early pronuclear stage to a perinuclear organization during the late pronuclear stage and the 2-cell stage (Barnett et al., 1996). In bovine oocytes, various patterns of mitochondria distributions are found, including uniform distribution and cortical distributions. After fertilization, the cortical mitochondria organization is lost (Van Blerkom et al., 1990). As described earlier for oocyte maturation, in the pig the majority of mitochondria are organized in the cortical region (Luoh and Wu, 1996; Sun et al., 2001d) and show a distinct perinuclear organization in the oocytes and early embryos (Hyttel and Neimann, 1990; Sun et al., 2001d). In fertilized human zygotes pronuclear accumulation of mitochondria is seen (Noto et al., 1993; Van Blerkom et al., 2000). The pattern of mitochondria distribution has been linked to developmental potential in hamster (Barnett et al., 1997; Lane and Bavister, 1998; Squirrell et al., 2001), mice (Muggleton-Harris and Brown, 1988), pigs (Hyttel and Niemann, 1990; Luoh and Wu, 1996), and cattle (Van Blerkom et al., 1990). A recent study reports that ATP, active mitochondria, and microtubules play a role in the acquisition of developmental competence of parthenogenetically activated pig oocytes (Tiziana et al., 2005).

Many of the previous studies indicate that culture conditions and embryo quality play a significant role in achieving the mitochondria organization that promises optimal developmental potential. Mitochondria distribution patterns as well as developmental potential change under adverse conditions. Aged oocytes fail to readjust intracellular ATP at fertilization (Igarashi et al., 2005). In the hamster, embryos display a dispersed mitochondria distribution pattern under culture conditions that disrupt development (Barnett et al., 1997; Lane and Bavister, 1998; Squirrell et al., 2001) rather than a perinuclear distribution pattern that indicates normal developmental potential. The opposite is true for mouse embryos in which a homogeneous mitochondria distribution indicates a high level of developmental potential while perinuclear mitochondria organization is seen in embryos with low developmental potential (Muggleton-Harris and Brown, 1988). Homogeneous mitochondria distribution indicates high developmental potential in bovine oocytes while mitochondria surrounding vacuoles indicates low developmental potential (Stojkovic et al., 2001). Mitochondria

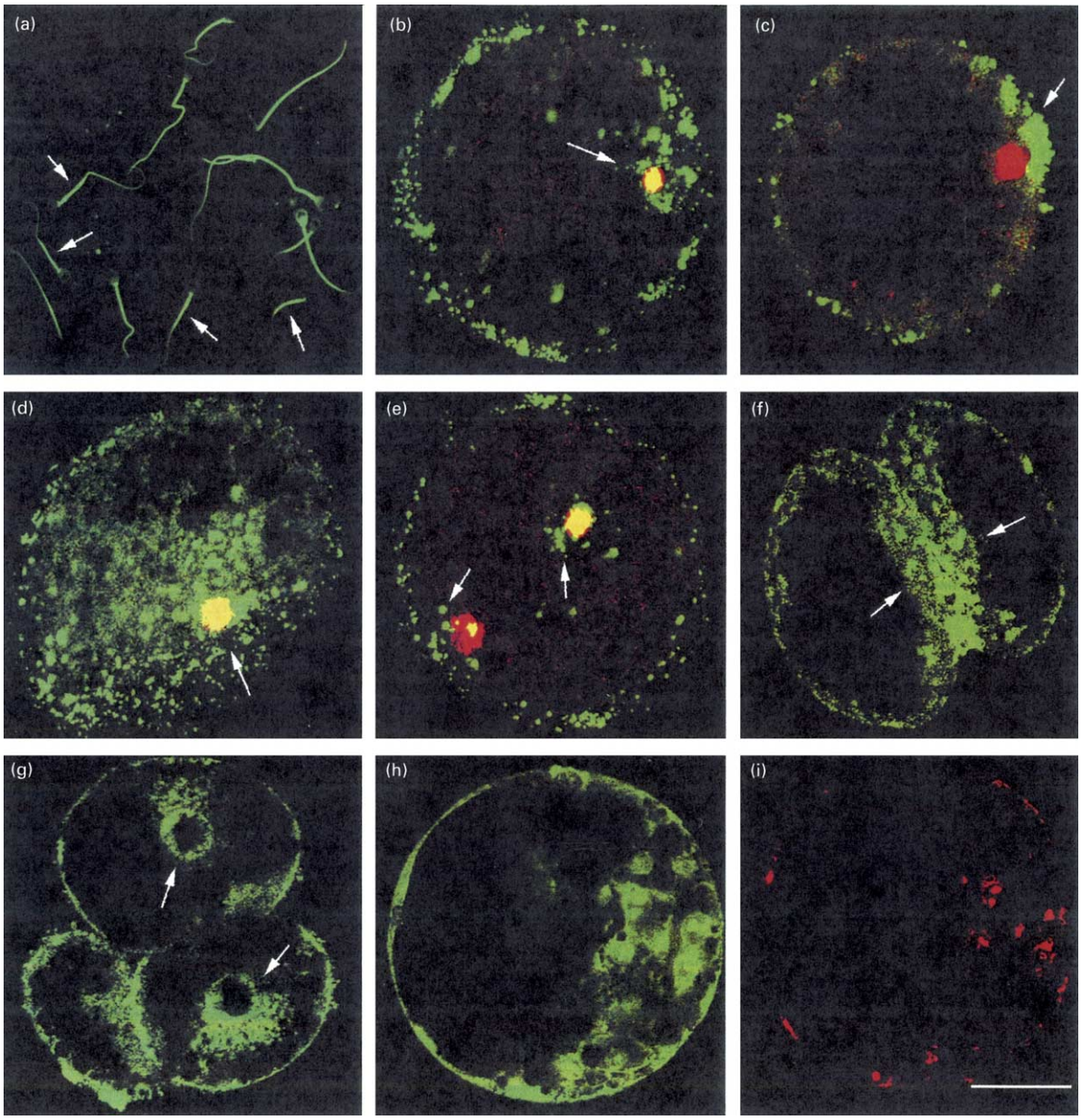

Fig. 1. Mitochondrial distribution in fertilized eggs and early embryos in pigs. (a) The tails, especially the principal segments (arrows), of spermatozoa that attach to the zona surface of fertilized eggs are stained green. (b) A fertilized egg, indicating the mitochondria distributed close to the plasma membrane and in the vicinity of a pronucleus (arrow). (c) Mitochondrial aggregation between the pronucleus and the plasma membrane (arrow). (d) In addition to the perinuclear distribution (arrow), some mitochondrial foci are observed in the inner cytoplasm of a few fertilized eggs. (e) Mitochondrial aggregation around both pronuclei (arrows). (f) Mitochondrial aggregation in the region in which the blastomeres are still in contact (arrows) during early cleavage. (g) Mitochondrial clustering around the blastomere nuclei (arrows) and in the cortex of the cell. (h, i) Mitochondria present in both inner cell mass cells and trophectoderm cells of blastocysts. Green, mitochondria; red, chromatin; yellow, overlapping of green and red. Scale bar represents 40 μm. Sun et al. (2001d). Reproduction 122, 160. © Society for Reproduction and Fertility (2005). Reproduced by permission.

clustering around vesicles indicates low developmental potential in pig embryos (Luoh and Wu, 1996). These studies reveal that mitochondria distribution is indicative of developmental potential which has implications for nuclear cloning in that it will be possible to select only those oocytes with mitochondria distribution patterns that predict the highest developmental potential. Determining the mitochondria patterns with the most promising developmental potential is possible by using multi-photon microscopy on live embryos that also allows subsequent implantation of the imaged embryos into a surrogate mother. Dual-photon microscopy uses short pulses of a lower energy laser in the infrared spectrum and photobleaching and phototoxicity that is associated with conventional confocal microscopy is substantially reduced because fluorophores outside the focal plane are not excited during image acquisition. Evaluations on live embryos and subsequent culture of only those embryos with a certain mitochondria distribution pattern and predicted developmental potential will save substantial costs and efforts resulting in increasing cloning efficiency.

Live imaging of mitochondria with multiphoton microscopy has been employed by Squirrel et al. (1999) who correlated survival of hamster eggs to the blastocyst stages with characteristic mitochondria fluorescence patterns. In these studies live imaging has been carried out to the blastocyst stages followed by subsequent implantation into surrogate mothers. The hamster eggs had been imaged without affecting development resulting in birth to live offspring by the surrogate mother. By refining this technique for farm animals it will be entirely possible to evaluate the developmental potential of cloned embryos and select only those that have excellent developmental potential.

Several methods have been used so far to determine *the fate of mitochondria after nuclear transfer* in farm animals and include MitoTracker staining on fixed cells and PCR based mtDNA amplification. The findings are still inconclusive as only mixed results have been obtained so far. As indicated in chapter 1, mitochondria are maternally inherited at normal fertilization during which sperm mitochondria become destroyed by ubiquitination which has been described in several species including the pig and the cow (Sutovsky et al., 1999, 2003a,b; Schwartz and Vissing, 2002, 2003; Sutovsky, 2004). During nuclear transfer mitochondria are derived from the enucleated oocyte as well as the donor cell. As indicated in chapter 1, three main fates for mitochondria are possible in reconstructed embryos: (a) homoplasmy in which mitochondria are inherited from the recipient oocyte while donor cell mitochondria are not inherited, resembling the mitochondria inheritance mode during normal fertilization; (b) homoplasmy for donor cells in which the recipient oocyte cell's mitochondria are destroyed and donor cell mitochondria are inherited. This mode of inheritance so far has not resulted in live offspring (as has been shown for panda-rabbit inter-species embryos) (Chen et al., 2002). Inheritance of maternal mtDNA is required for normal development of cloned embroys, as all living offspring either have homoplasmy from oocytes or heteroplasmy; and (c) heteroplasmy in which recipient and donor cell mitochondria co-exist through all stages of development as has been shown for macaca–rabbit cloned embryos (Yang et al., 2003). Homoplasmy is achieved through destruction of either recipient oocyte mtDNA or donor cell mtDNA in a process not mediated by ubiquitination. In some cases heteroplasmic mtDNA inheritance persists throughout development while in other cases heteroplasmic mtDNA contribution persists during the early stages of development but homoplasmic mtDNA is observed during later stages of development. The difficulties in obtaining clear results are oftentimes related to the techniques used so far. Some investigators suggested that MitoTracker staining techniques might not detect mitochondria accurately in later stages of development because of signal fading. Newly developed more sensitive methods have recently been employed including allele-specific PCR-amplification (AS-PCR) of mtDNA, direct DNA sequencing, and DNA chromatography. Other methods that are currently explored in our laboratory include the use of donor cells with fluorescently transfected mitochondria.

Studies in farm animals revealed that reconstructed sheep clones inherit their mitochondria entirely from the oocyte and not from the donor cell (Evans et al., 1999). For cloned bovine embryos, it was first reported that mitochondria transmission follows the maternally inherited homoplasmic pattern of inheritance (Takeda et al., 1999) but later mtDNA

heteroplasmy was reported for cloned cattle generated from fetal and adult donor cells (Steinborn et al., 2000). In this case the donor-to-recipient ratios of parental mtDNA remained the same throughout development. Using MitoTracker staining, Do et al. (2001) reported disappearance of donor mitochondria in bovine embryos before the 16-cell stage following nuclear transfer resulting in homoplasmy but, when using molecular methods, reported later that the mtDNA from donor cells was not destroyed during preimplantation development following nuclear transfer, resulting in heteroplasmic mtDNA transmission (Do et al., 2002). These differing results indicate that more research is required on a large number of samples using different techniques to analyze the contributions of recipient and donor cell mtDNA and the consequences for later development in embryos of cloned farm animals. It may also be that individual embryos display differences regarding mitochondria inheritance as mitochondria diversity is seen in other cases such as in individual unfertilized mouse oocytes (Piko and Taylor, 1987) and human oocytes (Steuerwald et al., 2000; Reynier et al., 2001).

3.1. The role of pH

Given the different requirements for culture of oocytes as compared to donor cell culture one needs to consider the effects of pH. Shifts in pH_i have been correlated with egg activation in invertebrate species (Epel, 1988), parthenogenetic activation of pig oocytes (Ruddock et al., 2000a,b; 2001) and in mammalian embryos the maintenance of its ionic balance is crucial for development. Embryos that have been removed from the oviductal environment and placed into an artificial culture medium may not represent the natural environment which may account for the variations in in vitro culture as compared to in vivo conditions of farm animal embryos. A number of different factors may be responsible for variations in mitochondria patterns and changes in pH that may account for abnormalities in mitochondria distribution. Localized small acute changes in pH_i have been implicated in cell proliferation, cell fate determination, the activity of regulatory enzymes, maintenance of the cytoskeleton (Busa, 1986), and modulation of calcium levels (Speake and Elliot, 1998). It is possible that changes in intracellular pH can be caused by clusters of mitochondria with different levels of oxidative phosphorylation (Aw, 2000) which in turn can cause alterations in cytoskeletal organization. In a recent study, Squirrell et al. (2001) have shown in hamster embryos that increase or decrease in pH_i is associated with disruption of the perinuclear organization of mitochondria followed by reduction in embryo development. Specifically, the perinuclear organization of mitochondria was disrupted which has been shown to be critical for normal development in a variety of species (Calarco, 1995; Sun et al., 2001d; Squirrell et al., 1999; Tokura et al., 1993; Barnett et al., 1996; Van Blerkom et al., 2000). These studies have not yet been extended to farm animals.

3.2. The role of phosphorylation

The different phosphorylation requirement for oocyte and donor cell cycles is an important consideration in synchronizing the two cell cycles and determining the optimal conditions for nuclear cloning. Phosphorylation of key proteins takes place at the transition from interphase to mitosis and affects a number of proteins that may be responsible for remodeling of the donor cell nucleus. Phosphorylation of nuclear and centrosome proteins plays a key role in the molecular and structural reorganizations of centrosome proteins that are crucial for microtubule organization, mitochondria distribution, and cell division. Our previous studies have correlated microtubule assembly with the mitogen-activated protein (MAP) kinase (Sun et al., 2002) in pig oocytes at different maturational stages (Sun et al., 2001b) and during fertilization (Sun et al., 2001c). Regulated phosphorylation is implicated in chromatid segregation and abnormal phosphorylation can lead to embryo fragmentation (Ma et al., 2005). Several phosphorylation events are involved in centrosome maturation from interphase to mitosis and for centrosome separation resulting in the formation of the bipolar mitotic apparatus. MAPK and Plk1 are two of the centrosome-associated kinases that are implicated in microtubule organization and separation. Abnormally phosphorylated centrosomes divide unequally which will have consequences for microtubule organization and mitochondria translocation. Plks

are important regulators of centrosome and microtubule functions in somatic cells and in mammalian oocytes (Pahlavan et al., 2000; Tong et al., 2002; Fan et al., 2003; Yao et al., 2003). During nuclear cloning somatic cell centrosomes need to be remodeled by the activated oocyte to carry out embryonic cell divisions and development. The remodeling of centrosomes into division competent organelles includes a cascade of phosphorylation events. Properly remodeled centrosomes will allow the precise organization of microtubules and equal distribution of mitochondria during cell division. Failure of the oocyte to remodel and properly phosphorylate somatic cell centrosomes after nuclear transfer may result in abnormalities of microtubule organization and mitochondria distribution.

Phosphorylation plays a critical role in the coordination of nuclear and centrosome proteins and nuclear proteins undergo significant reorganizations at the transition from interphase to mitosis. Numerous nuclear proteins are multifunctional and carry out different functions in the nucleus during interphase and after nuclear envelope breakdown during mitosis. One of the significant nuclear-centrosome proteins that becomes reorganized into centrosome proteins after nuclear envelope breakdown is the nuclear mitotic apparatus protein NuMA. NuMA is one example of a number of nuclear proteins that play multifunctional roles in somatic cells (Gobert et al., 2001; reviewed by Zeng, 2000). In unfertilized oocytes NuMA is located at the two poles of the meiotic spindle (Lee et al., 2000) where it plays a role in the organization of the meiotic apparatus. NuMA is removed with the meiotic spindle during enucleation before nuclear transfer. In reconstructed embryos, NuMA is derived from the donor cell nucleus. In interphase, NuMA is not associated with the interphase centrosomes. It is located in the nucleus and needs to be remodeled after nuclear transfer. NuMA needs to be regulated by the oocyte's cytoplasm in order to function as mitotic centrosome protein during cell division. To become a functional mitotic protein NuMA must be translocated from the nucleus into the cytoplasm and from the cytoplasm to the mitotic centrosomes where it forms an insoluble crescent around the centrosomes that tethers microtubules into the bipolar mitotic apparatus (Saredi et al., 1997; Merdes and Cleveland, 1998). In somatic cells, NuMA is regulated by several steps of phosphorylation throughout the cell cycle in which cdk1/cyclin B plays a critical role. In reconstructed eggs, cyclin B needs to be supplied by the oocyte's cytoplast to regulate translocation from the donor cell nucleus into the oocyte's cytoplasm and its subsequent association with the mitotic apparatus during mitosis. NuMA translocates along mitotic microtubules by using a dynein-dynactin complex to assemble around centrosomes and form a tight crescent-shaped association with the centrosome complex during mitosis. Phosphorylation abnormalities prevent the accurate association of NuMA with centrosomes resulting in abnormal spindle microtubule formation and abnormalities in cell division with consequences for genomic instability, as chromosomes become distributed unequally to the resulting daughter cells (Saunders et al., 2000; Gehmlich et al., 2004). Abnormal association of NuMA with mitotic centrosomes will also have consequences for mitochondria distribution, as mitochondria will be distributed unequally during subsequent cell cycles.

One of the problems associated with phosphorylation requirements in reconstructed embryos is the significant differences between embryonic and somatic cell cycles. Embryonic cells do not have a G1 phase (please see Alberts, 4th edition, pp988, 1003). Moreover, embryonic cell cycles employ different regulatory mechanisms as compared to somatic cells and may not have the accurate set of factors that are necessary to precisely regulate somatic donor cell nuclear components. For example, in embryonic cells, the synthesis of cyclin is constant throughout the cell cycle and cyclin B accumulation results from a decrease in its degradation. In most other cell types, however, cyclin B synthesis increases during G2 and M, primarily as a result of an increase in cyclin B gene transcription. This increase in cyclin B leads to gradual accumulation of cdk1/cyclin B as the cell approaches mitosis. These regulatory mechanisms may play a significant role in remodeling nuclear components such as NuMA. Supplementation of factors in the culture medium may be needed to induce altered regulatory processes in the oocyte that are required for modulation of gene expression in the donor cell nucleus.

3.3. The role of calcium

The role of mitochondria in calcium regulation has recently been reviewed by Van Blerkom (2004). Calcium regulation is critical for normal fertilization and it is clear that the calcium oscillations normally triggered by the fertilizing sperm play a significant role in the activation program and remodeling of the donor nucleus and its associated cytoplasmic components, including mitochondria, for subsequent cell divisions and development.

Invertebrate species have provided excellent information on sperm triggering Ca^{2+}- induced Ca^{2+} release (CICR) that in turn triggers cortical granule exocytosis through interaction with the smooth endoplasmic reticulum (SER) (reviewed in Schatten and Chakrabarti, 2000). In somatic cells (Pozzan et al., 2000) mitochondria are involved in the regulation of intracellular free Ca^{2+} through mechanisms involving electrical fluxes (Ichas et al., 1997), CICR pathways (Duchen, 2000), and apoptotic pathway signals (Berridge et al., 1998). In MII oocytes and fertilized eggs mitochondria are also involved in the regulation of intracellular free Ca^{2+} (Liu et al., 2001) and, through signaling association with SER, mediate the Ca^{2+} oscillations during activation. Dumollard et al. (2003, 2004) showed in MII oocytes and freshly fertilized eggs that sperm-triggered Ca^{2+} oscillations are signaled to mitochondria and stimulate mitochondria respiration. It is not known whether these events are faithfully mimicked in reconstructed embryos that are activated by electrical stimulation. Because calcium regulates a number of cellular events that are interdependent, altered calcium gradients may also affect microtubule polymerization (formation) and in turn alter mitochondria distribution.

Aberrant intracellular changes in calcium or pH in the reconstructed egg will have consequences for later development. It has been demonstrated by Ozil and Huneau (2001) that experimentally induced changes in intracellular free Ca^{2+} resulted in subsequent lethal consequences during organogenesis after implantation while no morphological abnormalities were detected in earlier stages of development. It has also been proposed that the high frequencies of post-implantation failures of thawed human oocytes may be related to mitochondria abnormalities in the oocyte (Jones et al., 2004). These factors may play a role in the developmental failures associated with animal cloning as well as the oftentimes delayed embryo development.

4. Correlation of mitochondria structure and function

Thin section transmission electron microscopy has provided most of the information on the structure of mitochondria in the oocyte and early development (Sotelo and Porter, 1959; Baca and Zamboni, 1967). Mitochondria are immature in the unfertilized oocyte and display spherical/ovoid rather than elongated morphology with a diameter of less than 0.5 μm as studied in human oocytes (Dvorak et al., 1987). A dense matrix and few cristae are seen initially but as mitochondria mature matrix density decreases while cristae formation increases to the blastocoel stage in human embryos (reviewed in Van Blerkom and Motta, 1979; Makabe and Van Blerkom, 2004). In bovine embryos, three types of mitochondria are described: (a) mature mitochondria with well-developed, evenly stacked cristae; (b) immature (embryonic) mitochondria with few peripheral cristae of hooded appearance; c) vacuolated mitochondria with membrane-bound vesicle (Crosier et al., 2000; Kölle et al., 2004). The immature morphology of oocyte and early embryo mitochondria may limit the possibility of oxidative phosphorylation as studied in the mouse (Dummolard et al., 2004) and reduce the potential for generating reactive oxygen species (ROS), that would initiate oxidative stress followed by apoptosis (Liu and Keefe, 2000; Liu et al., 2000). On the other hand, immature (embryonic) mitochondria may have special functions related to energy metabolism that can not be fulfilled by mature mitochondria (Kölle et al., 2004). Mitochondria actively engaged in ATP production display an increased number of lamellar cristae in the inner mitochondrial matrix. This pattern of mitochondria maturation has been observed in the mouse (Hillman and Tasca, 1983), sheep (Calarco and McLaren, 1976), pig (Hyttel and Niemann, 1990), bovine (Plante and King, 1994), and in non-human primates (Panigel et al., 1975). However, very few studies have addressed the structure/function relationships. It is not known whether different culture conditions affect mitochondria structure and function and how

the structure of mitochondria differs and changes in the developing embryo after reconstructions through nuclear transfer.

Changes in mitochondrial membrane potential are considered an important aspect regarding the developmental potential in fertilized and reconstructed eggs (Acton et al., 2004; Jones et al., 2004; Van Blerkom, 2004). Mitochondria membrane potential reflects pumping of hydrogen ions across the inner mitochondrial membrane during the process of electron transport (ET) and oxidative phosphorylation (Mitchell and Moyle, 1967) and is a key indicator of cellular viability. Aberrant shifts in mitochondria membrane potential, as measured with the mitochondria membrane potential-sensitive dye JC-1 (Reers et al., 1991; Smiley et al., 1991), have been associated with decreased developmental potential (Wilding et al., 2001, 2002, 2003; Van Blerkom et al., 2002, 2003; Acton et al., 2004; Jones et al., 2004). Differences have been reported for in vivo fertilized 2-cell stage mouse embryos when compared to in vitro fertilized 2-cell stage mouse embryos. Changes in pericortical mitochondrial membrane potential have also been observed in cryopreserved human oocytes (Jones et al., 2004) which significantly affects intracellular calcium metabolism.

5. Mitochondria genetics, pathologies, and disease: implications for nuclear cloning and the significance of mitochondria for later development, implantation and signaling between embryo and placenta

Mitochondria are semi-autonomous key organelles for the production of cellular ATP. The primary pathway for ATP production is OXPHOS via the electron transfer chain (ETC) which is encoded by nDNA and mtDNA genomes. Mammalian mtDNA consists of 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) and close interactions with the nucleus are required for transcription and replication (Clayton, 2000; Trounce, 2000). Mitochondria contain double stranded DNA that encodes thirteen of the polypeptides that are part of the ETC (Anderson et al., 1981).

The mature human oocyte (MII stage) contains between 120,000–350,000 mitochondria that arise during oogenesis (Marchington et al., 1997; Smith and Alcivar, 1993) from as few as 10 mitochondria present in each primary oocyte in a primordial follicle cell (Jansen and deBoer, 1998) as determined by TEM studies. Mouse oocytes contain 90,000 mitochondria as studied by TEM. Detailed studies are not available for farm animals. These numbers differ when different methods are used. When mtDNA copy numbers are determined in human MII oocytes using PCR methodology (Van Blerkom, 2004) the number of human MII mitochondrial genomes ranges between 20,000–800,000 (Chen et al., 1995; Steuerwald et al., 2000; Reynier et al., 2001; Barrit et al., 2002; Van Blerkom, 2004) that are capable of maintaining embryonic development after fertilization (Jansen, 2000; Reynier et al., 2001). The reduction in maternal germline mtDNA copy number in the primordial germ cells is called the ‘genetic bottleneck’ (Hauswirth and Laipis, 1982; Poulton, 1995; Bergstrom and Pritchard, 1998) and results in homoplasmic transmission of mtDNA. The number of mitochondria for MII oocytes differs even in the same cohorts. The need for oxygen increases from the one-cell to the blastocyst stages (Trimarchi et al., 2000), coinciding with the time during which mitochondria are not replicating. Structural changes take place in mitochondria that correlate with increased mitochondria function. Replication of mtDNA does not occur prior to the blastocyst stage (Piko and Taylor, 1987) and only occurs after implantation. Therefore, replication defects may only be seen during embryogenesis and play a role in postimplantation developmental defects.

There are numerous mitochondrial diseases some of which are inherited maternally while others are acquired through mutations. Wild type DNA and mutations constitute a heterogeneous population within the same oocyte and the phenotypic onset of disease often depends on the ratio of mutant to wild type mitochondria population. Some of the mutations arise from mutations in nuclear encoded components that are vital to mtDNA transcription and replication (for review please see St John et al., 2004). The mitochondrial population introduced with the somatic cell during nuclear transfer may have adverse effects on the reconstructed egg although this question has not yet been fully addressed. Critical nuclear and cytoplasmic interactions may be determined by mitochondria. As indicated in chapter 1, mitochondria dysfunctions might occur after nuclear transfer due to

failure in nucleo-cytoplasmic interactions related to failure of nuclear remodeling that needs to take place to initiate mitochondria differentiation. It is possible that adverse developmental consequences may have originated within the oocyte's cytoplasm followed by disproportionate inheritance during early cleavage stages. Recent work by Hiendleder et al. (2004) reports negative consequences of nuclear-cytoplasmic interactions after bovine nuclear transfer for fetal development, indicating complex oocyte cytoplasm-dependent epigenetic modifications and/or nuclear DNA-mitochondrial interactions. It is also possible that mitochondrial dysfunctions may contribute to the activation of the apoptosis program resulting in developmental defects and abnormalities.

To understand mitochondrial–nuclear interactions in reconstructed embryos more detailed studies are needed. Recently, the correlation and fate of mtDNA in cells with different nuclear backgrounds have been analyzed (Dunbar et al., 1995; Barrientos et al., 1998, 2000; Moraes et al., 1999; Dey et al., 2000; McKenzie and Trounce, 2000; McKenzie et al., 2003) and cytoplasmic hybrids (cybrids) with donor and recipient mtDNA have been created to study nucleo-cytoplasmic interactions (King and Attardi, 1988, 1989). Studies on same-species and inter-species cybrid generations show the extent of variation in mtDNA replication and transcription that can result from these experimentally created constructs (reviewed in St John et al., 2004). We know little about the ATP-generating capacity of mitochondria in such cybrids but one can envision that phenotypes with mtDNA depletion syndromes (Larsson et al., 1994; Poulton et al., 1994) might be among the result. Further studies are needed to investigate mitochondria genetics and pathologies in reconstructed embryos.

In reconstructed embryos, donor cell mitochondria do not undergo the ubiquitination process after nuclear transfer to deconstruct donor cell mitochondria as seen for sperm mitochondria after fertilization (Sutovsky et al., 1999; Schwartz and Vissing, 2002, 2003; Johns, 2003; Sutovsky, 2004). In many species, heteroplasmic mitochondria distribution to many tissue types is seen in various stages of development and in subsequent generations (Hiendleder et al., 2003). It is not clear whether the increased number of mitochondria in reconstructed eggs affects mitochondria metabolism. Overproduction of ATP may adversely affect the developing embryo as toxicity could develop as a result of increased levels of oxidative free radical production leading to nuclear and mitochondrial DNA damage. In addition, as the mitochondria of the donor cells are not eliminated by ubiquitination, apoptosis could be induced in which mitochondria may undergo self-destruction rather than ubiquitination. Alternatively, disproportionate mitochondria segregation could occur during cleavage resulting in death of individual blastomeres and fragmentation of the embryo. The consequences for disproportionate patterns of inheritance resulting in lysis of the blastomere that is deficient in mitochondria has been reported by Van Blerkom et al. (2000). Unequal mitochondria distribution could well result from nuclear transfer by which the nucleus is typically fused at the peripheral area of the recipient cell. It has been shown in mouse studies that foreign mtDNA injected into the cytoplast segregated preferentially to one of the daughter cells and is not equally distributed to the cleaving embryos (Meirelles and Smith, 1998). These studies also proposed that the position of mitochondria within an oocyte plays an important role in their distribution within the developing embryo and offspring (Meirelles and Smith, 1998). The positioning of mitochondria might be among the factors explaining the variations in developmental consequences for cloned embryos. It is also important to consider a fourth possibility in that during maturation a pattern is established for cortical and cytoplasmic mitochondria in the MII oocyte as has been shown by Calarco (1995) for the mouse that may result in blastomeres with different mitochondria distribution. This possibility is supported by recent work of Piotrowska-Nitsche et al. (2005) that suggests different potentials of early mouse blastomeres dependent on the oritentation of their cleavage plane. The consequences for cloning is that mitochondrial distribution patterns may be altered during nuclear cloning when cytochalasin B is used in the enucleation procedures which may affect embryo development and cloning efficiency. Selecting suitably oocytes and modifying the cloning procedure may lead to increases in cloning efficiency. Such studies have not yet been performed on farm animals.

6. Epigenetic effects and culture conditions: manipulations and future directions

As indicated in the previous chapters, nuclear remodeling requires complex interactions between the transplanted donor cell nucleus and the ooplasm that are only poorly understood. Cell-specific gene expression and a cell's identity is related to function and morphology (Shi et al., 2003). Donor cell nuclei need to be remodeled to carry out the characteristics of a fertilized egg in all stages of development. Epigenetic factors play an important role in the development of a fertilized egg (Reik and Dean, 2003) and imprinting disorders can be lethal (Lee et al., 2002). Even in normally looking cloned animals subtle epigenetic defects may exist that are below the threshold affecting viability (Jaenisch and Wilmut, 2001). More research is needed to investigate the effects of epigenetic factors on mitochondria function. Changes in culture medium (Boquest et al., 2002) and changes in nuclear transfer procedures (Walker et al., 2002) may prove valuable in increasing cloning efficiency and the production of healthy offspring. In addition to nuclear remodeling, the oocyte's regulatory machinery needs to be remodeled to activate the nuclear DNA as well as other components in the nucleus such as nuclear matrix components and proteins that are translocated into the cytoplasm for cell division and subsequent development.

The analysis of mechanisms underlying cloning failures is essential for the development of strategies aimed toward increasing cloning efficiency. Several changes in the donor nucleus are required for successful nuclear cloning and include epigenetic changes in the genome and subsequent changes in gene expression. So far several reports have shown that cloned embryos exhibit defects in the expression and regulation of key genes (Boiani et al., 2002) and abnormalities in DNA methylation (Humphreys et al., 2001; Ohgane et al., 2001; Kang et al., 2001a,b,c; Reik et al., 2001; Dean et al., 2001; Chen et al., 2004; Chen et al., 2005; Zhang et al., 2005). The pluripotent gene products that are not expected to be present in donor cell nuclei such as Oct-4 are needed for embryo development (Boiani et al., 2002; Bortvin et al., 2003). Furthermore, the functional interactions between donor cell nDNA and recipient cell mtDNA need to be established as they may be changed by epigenetic factors. The two populations of mtDNA from recipient and donor cells may require different epigenetic regulation. The nucleus that regulates mtDNA transcription and replication may result in different cells adopting the phenotype of either nuclear donor cell type mtDNA or recipient cell mtDNA (reviewed by St John et al., 2004), possibly resulting in differences in ATP generating capacities for cells in different organs that may not be representative of the mtDNA copy number that are characteristic for each cell type (Moyes et al., 1998). Taken together, it is clear that determining the interactions between nDNA and mtDNA is important for successful animal cloning. Inefficient cytoplasmic-nuclear protein trafficking, inefficient changes in chromatin structure, inaccurate posttranslational regulation of key proteins (Chung et al., 2002, 2003) and other factors have been suggested to cause abnormalities of nuclear-ooplasmic interactions. Modification in culture conditions have been proposed to alter and induce characteristics in the donor nuclei (Gao et al., 2003) which calls for further improvements in culture conditions to improve nuclear remodeling.

It is conceivable that mitochondria in successfully cloned embryos resulting in live offspring may have undergone changes to adapt to the newly created genetically modified offspring. It is not clear how much influence the nucleus exerts over cytoplasmic remodeling which is still an under-researched area. Very little is known about the influence of the nuclear genome to possibly induce changes in mitochondria. Among the many different factors that are involved leading to successful or unsuccessful nuclear cloning, perhaps the live offspring represent a natural selection of nucleo-cytoplasmic compatibility that is only seen in a small percent of reconstructed embryos. The offspring of cloned farm animals appear normal in all studies conducted so far indicating that the initial difficulties between nucleo-cytoplasmic compatibilities are not a problem in the offspring of cloned farm animals.

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