

Somatic cell nuclear transfer: failures, successes and the challenges ahead

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ABSTRACT Somatic cell nuclear transfer (SCNT) has a broad spectrum of potential applications, including rescue of endangered species, production of transgenic animals, drug production, and regenerative medicine. Unfortunately, the efficiency of SCNT is still disappointingly low. Many factors affecting cloning procedures have been described in several previous reviews; here we review the most effective improvements in SCNT, with a special emphasis on the effect of mitochondrial defects on SCNT embryo/ foetus development, an issue never touched upon before.

KEY WORDS: somatic cell nuclear transfer, SCNT, nuclear reprogramming, mitochondria

Introduction

The latest Red List of the International Union for Conservation of Nature (IUCN) shows that 21% of all mammals are threatened. The worst news is that the global number is likely underestimated due to lack of recent data from more than 58% of breeds, therefore classified as unknown risk (FAO, 2018).

To keep up with this alarming situation, it has been suggested that genetic banks, mainly in the form of cell lines (preferably fibroblast), should be established for critically threatened species. Scientists believe that those cells may be used to re-establish or expand the threatened population by somatic cell nuclear transfer (SCNT), a unique utilisation of preserved genetic material (Loi *et al.*, 2008; Saragusty *et al.*, 2016; Hildebrandt TB *et al.*, 2018).

SCNT has tremendous potential, not only for the rescue of endangered breeds and species (Loi *et al.*, 2001), but also as a reproductive technology for genetically valuable farm animals or for generation of transgenic animals (Rodríguez-Osorio *et al.*, 2009).

Theoretically, SCNT is a simple technique, involving removal of nuclear DNA from an oocyte and its replacement with a somatic cell nucleus. Yet, despite its simplicity, the efficiency is basically the same since the first cloned animal was delivered (Wilmut *et al.*, 1997). For this reason, many attempts to modify/improve SCNT efficiency have been reported (Iuso *et al.*, 2013; Czernik *et al.*, 2016). These improvements concerned the technical aspects (Wakayama *et al.*, 2010; Czernik *et al.*, 2016) as well as attempts as bulk or targeted modification of donor nucleus, before or af-

ter embryo reconstruction (Wakayama, 2007; Wakayama and Wakayama, 2010; Iuso *et al.*, 2015). This review will address the main improvements in the SCNT technique, as well as the role of mitochondria in cloned embryos and foetuses.

Technical improvements in SCNT

Improvement of the technique

The traditional method of cloning (TDC) used in the majority of mammalian nuclear transfer laboratories involves the enucleation of matured (MII) oocytes, and subsequently replacing it (by different approaches) with a donor somatic nucleus (known as Nuclear Transfer) (Fig. 1A). The cytoplasm of human oocytes as well as laboratory animals like mouse and rat is clear and “transparent” under the microscope, which facilitates identification of MII chromosomes, hence their easy removal. On the other hand, the cytoplasm of large domestic animal’s oocyte is much darker, due to high lipid content, making it necessary to use fluorescence staining Hoechst 33342, ultraviolet (UV) exposure and cytochalasin B treatment during enucleation. Exposure to UV, however, has harmful effects on embryonic development (Gil *et al.*, 2012). To avoid UV-related damages, Iuso and colleagues developed a new enucleation method in a large animal model, the sheep (Iuso

Abbreviations used in this paper: IVF, *in vitro* fertilization; IVP, *in vitro* produced; Mfn2, mitofusin 2; MII, metaphase II; mtDNA, mitochondrial DNA; NR, nuclear reprogramming; SCNT, somatic cell nuclear transfer; TSA, trichostatin A; UV, ultraviolet.

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Submitted: 27 September, 2018; Accepted: 11 October, 2018.

ISSN: Online 1696-3547, Print 0214-6282

et al., 2013). Those authors showed significantly higher blastocyst rates when SCNT was performed without exposure of the oocytes to UV, as compared to the traditional method.

Traditional method of nuclear transfer needs expensive equipment, as well as highly skilled personnel. To avoid that, several laboratories adopted a more economic procedure called Hand Made Cloning (HMC) (Vajta, 2007). The first successful HMC was done by Peura and colleagues (Peura et al., 1998). The technique then spread to the field of large domestic animal: buffalo (George et al., 2001), sheep (Zhang et al., 2013), horse (Lagutina et al., 2005), and pig (Du et al., 2007). In a further modification, researchers have successfully enucleated zona-free oocytes using sharp microblade, and attached the enucleated oocytes and donor cells using phytohemagglutinin, without any sophisticated equipment (Fig. 1B).

Reconstruction of the enucleated oocytes can be performed by direct injection or by electrofusion of the somatic cell to the cytoplasm of the oocyte. There are two major methods for direct injection of donor cells' nuclei. In the traditional way, membrane of the donor cell is lysed by repeated aspiration and ejection with a micro-capillary. The alternative way of reconstruction uses the Piezo device. The Piezo-driven technique, also known as the "Honolulu technique", was first used by Wakayama, then at the University of Hawaii, to produce the first cloned mouse (Wakayama et al., 1998).

The source of the donor nucleus is a crucial aspect when aiming to increase nuclear reprogramming efficiency. Most of the information available is on mouse model (Wakayama, 2007). The most commonly used donor cells, for ease of retrieval, are the cumulus cells (Wakayama et al., 1998). In addition to cumulus cells, tail tip fibroblasts (Wakayama and Yanagimachi, 1999), Sertoli cell (Ogura et al., 2000), foetal fibroblasts (Wakayama and Yanagimachi, 2001a), embryonic stem cells (Wakayama et al., 1999), natural killer T cells (Inoue et al., 2005), and primordial germ cells (Miki et al., 2005) have all successfully been used. In large mammals, adult and foetal fibroblasts, cumulus cells, and embryo-derived cell lines were used (Kato and Tsunoda, 2010; Akagi et al., 2014). However, even though development to the blastocyst stage was sometimes reported to have been improved in nuclear transfer embryos, especially in those reconstructed with embryonic cells, the percentage of clones developing to term is still disappointingly low.

Without deeply touching the issue, which was thoroughly discussed in authoritative reviews, cloned embryos display a wide array of epigenetic alternation, including DNA methylation, histone acetylation, methylation, and non-coding RNA transcripts expression (Matoba and Zhang, 2018). Correcting these shortcomings will certainly improve mammalian cloning efficiency. In the next section, we critically describe the solutions thus far adopted to minimise epigenetic alterations typically found in cloned embryos/conceptuses, and discuss the challenges ahead.

Treatment with histone deacetylase inhibitors

One of the biggest problems of cloned embryos is deacetylation of histones in the transferred cell nucleus. Acetylation of histones is generally associated with activation of gene transcription stemming from a more "open" chromatin structure. Zygotic gene activation, a crucial event at the beginning of zygotic genes transcription, occurs during early embryo development. Therefore, histone acetylation on transferred cell chromatin is important for proper zygotic gene transcription and embryo development that follows. However, it was reported that the level and state of several histone acetyla-

tion marks in SCNT embryos chromatin were different from those in IVF embryos (Wang et al., 2007; Yamanaka et al., 2009). To improve cloning efficiency, several histone deacetylase inhibitors (HDACis) have been used (Fig. 1C). So far, one of the most effective and commonly used inhibitors is Trichostatin A (TSA) that inhibits class I and II HDACs. Treatment of cloned embryos with TSA represses deacetylation on histones in embryo chromatin (Wang et al., 2007), improves the transcriptional activities at the 2-cell stage, and increases the efficiency of full-term development in mice (Rybouchkin et al., 2006; Kishigami et al., 2006, 2007). TSA treatment of porcine cloned embryos also improved both pre-implantation and full development (Li et al., 2008).

However, while the benefits arising from HDACis are incontestable in mouse, its effects on large animal remains controversial. In fact, TSA treated bovine SCNT embryos showed improved pre-implantation development (Akagi et al., 2014), but no improvement in offspring rate (Sawai et al., 2012). Conversely, treatment of donor cells with TSA improved preimplantation development of embryos reconstructed with treated cells in buffalo (Luo et al., 2013). While in pigs, TSA treatment improves both pre-implantation development and offspring rate, in bovine and buffalo it improves only pre-implantation rate. Therefore, this method cannot be applied in large animal species.

Impeding Xist expression

One of the well-investigated epigenetic events in embryo development is X Chromosome Inactivation (XCI), in female mammalian cells. Since in mammals' female cells have two X chromosomes, one copy is silenced so as to have the same proportion of gene products as that of males (dosage compensation). *Xist*, a non-coding RNA that is transcribed from the silenced X chromosome, is one of the better indicators when investigating the condition of XCI. In fertilized pre-implantation embryos, *Xist* shows monoallelic expression from the maternal X chromosomes; however, biallelic expression is observed in both inner cell mass and trophectoderm in cloned mice blastocysts (Nolen et al., 2005). Using *Xist*-deleted donor cells, to prevent this ectopic *Xist* expression, improved full-term development more than 7-8 times as compared to the normal procedure in mice (Matoba et al., 2011; Fig. 1D). Surprisingly, correction of *Xist* expression improves not only transcripts from X chromosome but also that from autosomes (Inoue et al., 2010). Moreover, injection of short interference RNA (RNAi) of *Xist* into reconstructed embryos, represses *Xist* expression transiently and improves full-term development of male (Matoba et al., 2011) but not female (Oikawa et al., 2013) cloned embryos. Ectopic expression of *Xist* is also observed in bovine (Inoue et al., 2010) and pig (Yuan et al., 2014, Zeng et al., 2016) cloned embryos and RNAi-mediated knockdown of *Xist* increases full-term development in male porcine cloned embryos (Zeng et al., 2016). Therefore, the partial conclusion is that *Xist* repression enhances cloned embryo development in males, and not only in the laboratory mouse.

Histone lysine demethylase family member mRNA injection into embryos

Methylation on histones is generally associated with repression of most of the genes transcription. Therefore, repressive marks on transferred donor cell chromatin, such as H3K9me3, are another epigenetic barrier in cloned embryos that prevents proper gene transcription after zygotic gene activation. Removal of histone

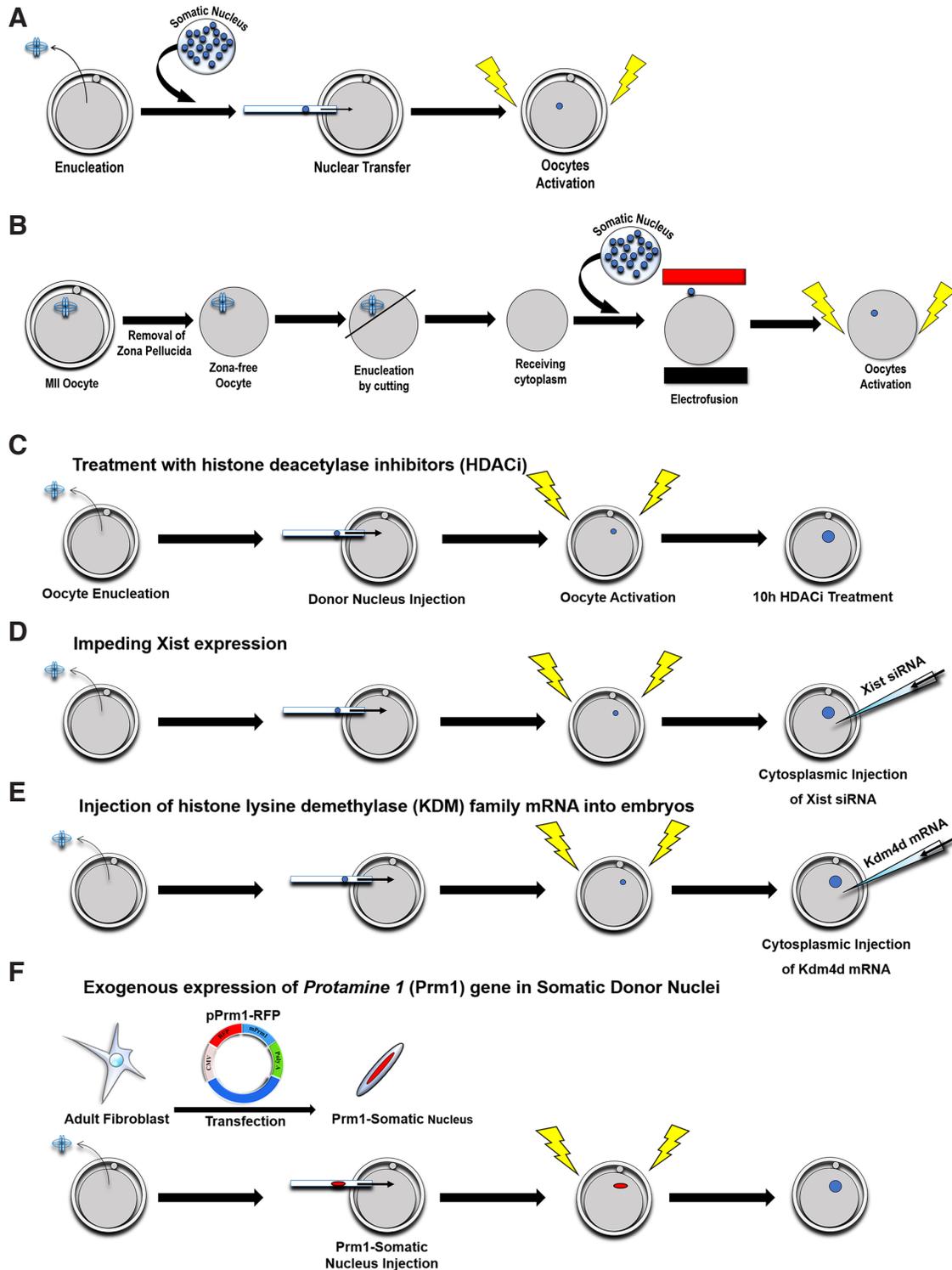


Fig. 1. Improvements in somatic cell nuclear transfer (SCNT) techniques. (A) Traditional cloning (TDM). The MII plate is removed from the oocyte by micromanipulation, the enucleated oocyte is directly injected with the somatic donor nucleus and subsequently artificially activated. **(B)** Handmade cloning (HMC). The matured oocyte is exposed to Pronase to remove the zona pellucida; zona-free oocytes are enucleated by cutting with a micro sharp-blade, the somatic donor nucleus is aggregate with phytohemagglutinin and subsequently electro-fused with the ooplasm. Finally, the reconstructed embryo is artificially activated. **(C)** SCNT improvement by exposure of histone deacetylase inhibitors (HDACi) after reconstructed oocyte activation. **(D)** SCNT improvement by downregulation of Xist expression with siRNA. **(E)** SCNT improvement by ablation of the repressive histone mark, H3K9me3, through Kdm4d mRNA injection. **(F)** SCNT improvement by NT with protaminized somatic nucleus, through exogenous expression of human Protamine 1 gene (pPrm1-RFP).

methylation has been achieved by injection of histone lysine demethylase into reconstructed embryos (Matoba *et al.*, 2014; Fig. 1E). This treatment significantly improves full-term development of cloned mice (Matoba *et al.*, 2014) and macaque monkeys (Liu *et al.*, 2018) of both sexes. This application has even been used in human SCNT (Chung *et al.*, 2015). It is expected that this method will be applied to other mammals in the near future.

Treatment of reconstructed embryos with ascorbic acid

It is reported that ascorbic acid treatment improves efficiency of reprogramming somatic cells to induced pluripotent cells in mouse and human (Esteban *et al.*, 2010). This report prompted cloning scientists to test the possibility of improving nuclear reprogramming in SCNT embryos. Actually, vitamin C treatment of cloned embryos improves both preimplantation development and pregnancy rate in pigs (Huang *et al.*, 2011). Moreover, combinations of the three components: deionized BSA, TSA and ascorbic acid treatment significantly improved cloning efficiency in mice (Miyamoto *et al.*, 2017; Azuma *et al.*, 2018). It is believed that the mechanism of this improvement is through repression of ROS and/or production and reduction of methylation on histone H3K9.

Exogenous expression of the protamine 1 (*Prm1*) gene in somatic donor nuclei

The male gamete is the perfect nuclear transfer device. Its DNA, tightly packed around protamines, confers the sperm nucleus a hydrodynamic shape to easily reach and fertilise the female gamete (Samans *et al.*, 2014). Upon entering the oocyte, the sperm genome “springs out,” revealing its intrinsic totipotency. This may suggest that any successful NR strategy must mimic the nuclear reorganisation of the spermatozoon. In fact, this is the only nuclear formation the oocyte has evolved to deal with. Twenty years of experiments following the birth of “Dolly”, resulting in thousands of SCNT-derived embryos, showed invariably that the nuclear organisation of a somatic cells is rarely reset by the oocyte.

Nuclear remodelling during spermatid maturation occurs through a time-regulated translation of mRNAs for histone variants that have accumulated earlier, in spermatogonia (Govine *et al.*, 2013). Incorporation of such testis-specific histone variants into the chromatin leads to destabilisation of nucleosomes (Rathke *et al.*, 2014). Subsequently, post-translation modifications of the histone variants further prepare the ground for the incorporation of transition proteins first, and then protamines, that compact the nucleus (Shabazianet *et al.*, 2007). At present, it is impossible to repeat the stepwise spermatid nuclear remodeling in a somatic cell. Surprisingly, it has been recently shown that expression of protamine 1 alone is sufficient to compact the nucleus in a shape reminiscent of those of spermatids (Iuso *et al.*, 2015; Czernik *et al.*, 2016; Palazzese *et al.*, 2018) (Fig. 1F). Furthermore, these authors observed that the protamine, when binding to the DNA, replaces the somatic histones, including H3K9me3, a critical epigenetic barrier of SCNT reprogramming (Iuso *et al.*, 2015). Additionally, protaminised nuclei injected into enucleated, sheep oocytes resulted in an increase in blastocyst formation rate compared to traditional nuclear transfer (14% vs 4%, respectively). Those unique results, that demonstrate a radical reorganisation of somatic chromatin from histone to protamine, provide a promising approach for improving mammalian cloning efficiency (Iuso *et al.*, 2015).

Embryo aggregation

As we have described above, developmental outcomes of cloned embryos have improved by several kinds of treatments. However, the defects in the trophoblast cell lineage, such as structural abnormalities and hyperplasia (Miki *et al.*, 2009), have not been eliminated in any of these methods. Tetraploid embryo complementation is a widely-used application to prevent embryo lethality caused by placental dysfunction (Okada *et al.*, 2007). Aggregation of inner cell mass (ICM) of diploid cloned embryos with trophectoderm (TE) derived from two or more tetraploid fertilised embryos can improve both full-term development ratio and placentomegaly in mice (Lin *et al.*, 2011). Importantly, this improvement is not observed in case of whole embryo aggregation (Miki *et al.*, 2009). Gestation period in large mammals is longer than in mice so this application might be more helpful for them.

Mitochondria might affect the development of clones

In this paragraph, we will address the limited success of the SCNT procedure from the mitochondrial perspective, an unexplored topic thus far. Investigations on mitochondria in SCNT procedures are limited to mtDNA hetero/homoplasmy in different tissues of cloned offspring (Lee *et al.*, 2010). No data is available for an eventual role of mitochondrial dysfunction in developmental failure of the clones. Moreover, it is important to point that mitochondrial activity is strictly controlled by nuclear signals, suggesting that incomplete nuclear reprogramming in cloned nuclei might be responsible also for impaired mitochondrial function in cloned embryos/foetuses. Compared with nuclear reprogramming, which has been a leading research topic over the last ten years, very few, if any, studies have focused on problems related to the association between mitochondria and nuclear reprogramming.

In the next sub-sections, we will try to evaluate whether mitochondrial dysfunction affect the embryo proper, the placenta, and foetal development.

Mitochondria in SCNT embryos

Following fertilisation and up to implantation, the embryo depends on the function of existing mitochondria, present in the oocyte at ovulation (Spikings *et al.*, 2006). As cell division begins, the total number of mitochondria within each blastomere decreases due to dilution with no new mitochondrial biosynthesis (John *et al.*, 2010). Early stage embryos do not express the nuclear-encoded replication factors required to multiply mtDNA. Mitochondrial DNA and mtDNA-nuclear DNA (nDNA) interactions might be responsible for the different phenotypes resulting from nuclear cloning. Lack of nuclear-mitochondrial interaction at the molecular lever can explain the potentially high development rate of early embryos [(mouse: 52,8% (Wakayama and Yanagimachi, 2001); sheep: 27,3% (Wilmut *et al.*, 1997); bovine: 69,4% (Wells *et al.*, 1999)], even in distant inter-species nuclear transfer (mouflon: 30,4% (Loi *et al.*, 2001). Replication defects of mitochondria may only be seen during embryogenesis, and play a role in post-implantation developmental defects. Consequently, any adverse influence on mitochondrial dysfunction (i.e., accumulation of mutational load to the mtDNA) may negatively impact the development of pre- and post-implantation cloned embryos.

Mitochondria exhibit an interesting quality maintenance function. They have numerous periods of fusion and fission. Active mitochondria are able to fuse with other mitochondria to transfer

components and maintain or improve the function of damaged or poorly performing members (Mouli *et al.*, 2009).

In mammals, three proteins are required for the fusion process. Two mitofusins are responsible for the fusion of the outer membrane, mitofusin 1 (MFN1) and mitofusin 2 (MFN2), while a single dynamic family member, OPA1, is required for inner membrane fusion. It is known that MFN2 plays a central role not only in the fusion process but is also responsible for key cellular functions such as oxidative metabolism, cell cycle maintenance, cell death, and mitochondrial axonal transport.

The importance of mitochondrial fusion in early embryo development was reported by Mishra and Chan (2014). It was later demonstrated that MFN2 plays a crucial role in mitochondrial fusion, which is essential for mouse blastocyst formation (Jiang *et al.*, 2015). Inappropriate mitochondrial activity at the pronuclear stage is associated with early developmental arrest (Blerkom *et al.*, 2000) and such embryos show decreased expression of mitochondrial genes (Duran *et al.*, 2011).

Preliminary analysis of early mouse embryos stained with MitoTracker Green dye and evaluated by time-lapse microscopy showed minimal fusion process in SCNT blastocysts as compare to control groups (Czernik *et al.*, unpublished data). Moreover, sheep and mouse early SCNT embryos show drastic differences in mitochondrial structure between SCNT and *in vitro* produced (IVP) blastocysts. Additionally, decreased density of mature mitochondria, very high degree of cytoplasmic vacuolisation, numerous cytoplasmic vesicles and autophagosomes, as well as significantly lower expression of major mitochondrial, autophagic and apoptotic proteins were all observed in SCNT embryos (Czernik *et al.*, unpublished data).

Mitochondria in SCNT placenta

High frequency of first trimester losses, as well as late gestation and post-natal losses, are observed with SCNT pregnancies as compared to *in vitro* fertilised and *in vivo* control pregnancies (Heyman *et al.*, 2002). In most SCNT pregnancies, high rate of foetal/embryonic loss is associated with placental malformation (Loi *et al.*, 2006). Placental insufficiency resulting from abnormal cotyledon formation, decreased numbers of cotyledons, placental degeneration and reduced placental vascularisation are considered to be the cause for diseases typically documented in SCNT foetuses and neonates, including respiratory distress, malnutrition, and cardiopulmonary disease (Loi *et al.*, 2006). The placenta has a crucial role in maternal control of foetal development and it represents an interface between the maternal environment and the foetus. Furthermore, many of the genes that regulate placental development also regulate foetal brain development (Murphy *et al.*, 2006).

As was mentioned before, fusion of mitochondria plays crucial role in their proper function. It has been shown that mice, deficient in MFN2 protein, die in utero at mid-gestation of placental deficiency due to placental abnormalities, particularly disruption of the trophoblast giant cell layer (Chen *et al.*, 2003). Additionally, it has been shown that low expression of MFN2 in human placentas is associated with mitochondrial damage in placental cells and unexplained miscarriage (Pang *et al.*, 2013). Recently, Czernik *et al.*, (2017) reported that deregulated expression of mitochondrial proteins (MFN2 and BCNL3L) cause abnormalities in early pregnancy placenta in sheep. Abnormalities were mainly presented as damaged and malformed mitochondria, as well as swollen endo-

plasmic reticula (Czernik *et al.*, 2017). Similar findings were also reported by Wakisaka and colleagues in mouse cloned placentas (Wakisaka *et al.*, 2008). These observations clearly suggest that mitochondria in SCNT placentas do not work properly and this may negatively affect SCNT embryo development.

Mitochondria in SCNT foetuses

Many SCNT embryos are lost due to gestation and neonatal failures. Birth defects and high post-natal losses are seen in cloned cattle, sheep and pigs as well as in laboratory animals. Oversized livestock at birth (Bertolini *et al.*, 2002), cloned calf syndrome (Wells *et al.*, 2004) or more usually the large offspring syndrome (Young *et al.*, 1998) are frequently observed in sheep and cow. Young and co-workers reported that oversized livestock at birth is related to imprinting dysregulation of IGF2R (Young *et al.*, 2001) but full explanation of the underlying causes is still missing. Given the finding that mitochondria play a role in obesity (Ritov *et al.*, 2005), it may well be that mitochondria abnormalities are involved in oversized live-stock neonates associated with cloned animals.

Major health problems include respiratory distress, circulatory problems, immune dysfunctions and kidney and heart failure. Moreover, problems with movement and balance have been observed in cloned animals, displaying uncoordinated limb movements, and therefore movement primarily by writhing on their abdomens (Wells *et al.*, 2004).

In mouse mutants, homozygosity for both *Mfn2* or *Mfn1* is lethal at early stages of development (Chen *et al.*, 2007). Cerebellum-specific inactivation of *Mfn1* or *Mfn2* has been achieved by crossing *Mfn1*^{loxP} or *Mfn2*^{loxP} mice. These mice express a cerebellum-specific Cre recombinase-driven promoter (pMeox2-cre). *Mfn1* inactivation in the cerebellum resulted in mice with normal growth, development, and fertility. However, cerebellum-specific inactivation of the *Mfn2* gene resulted in one-third of the mice dying within one day after birth and the surviving animals showing severe defects in movement and balance. The cerebellum of the *Mfn2*-deficient mice was only 25% the size of control mice at post-natal days 15-17; this disparity was associated with reduced and deteriorating Purkinje cells and increased apoptosis of granule cells (Chen *et al.*, 2007). The mouse *Mfn2* pMeox2-cre mutants-associate abnormalities overlap with those observed in neonatal clones.

Mitochondria and nuclear reprogramming

The mitochondrial genome transcribes only 13 proteins, while the remainder mitochondrial proteins (1500-2000) are encoded by the nucleus, where the crucial mitochondrial genes have been transferred to benefit from more accurate (error-free) DNA replication. Critical nuclear and cytoplasmic interactions may be determined by the mitochondria. There is a great deal of communication between the nuclear and mitochondrial genomes, and this communication strictly controls mitochondrial function (Chappel, 2013). It is important to mention, once again, that the major reason for the low efficiency and abnormalities observed in SCNT embryos/foetuses is incomplete somatic cell Nuclear Reprogramming (NR). It might be that mitochondrial genome is not activated properly due to incorrect NR and this causes malfunction of the mitochondria, and hence abnormalities in cloned placentas and foetuses.

These findings suggest that mitochondria dysfunction might occur following nuclear transfer due to failure in nucleus-cytoplasmic interaction leading to failed nuclear remodelling. Proper nuclear

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