Cloning of Livestock by Somatic Cell Nuclear Transfer

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Abstract

Since the cloning of "Dolly" by somatic cell nuclear transfer in 1996, numerous articles have been published concerning the application of this technology to a large variety of mammalian species including all the major livestock species. While live births have been obtained for many species, the efficiency of cellular reprogramming essential for success has not been significantly improved. This chapter will attempt to address the inputs utilized for this procedure and the major manipulation steps with the objective of identifying the major factors which might affect this efficiency of reprogramming and some of the studies addressing these factors. Finally, the challenging task of setting optimum end-points for experiments involving domestic species will be discussed.

The first nuclear transfer experiments were conducted in amphibians (Briggs and King 1952) and involved the transfer of a nucleus from a differentiated cell into an enucleated mature oocyte. The essence of nuclear transfer is to reprogram the genome of a differentiated cell, and development from the one-cell zygote through to term is recapitulated. The first somatic cell nuclear transfer (SCNT) in a mammal was achieved in sheep with the birth of "Dolly" (Wilmut et al. 1997). This achievement was remarkable because a fully differentiated somatic cell from an adult animal was used as the donor nucleus. Complete reprogramming of a differentiated cell by nuclear transfer had previously been opinioned to be "biologically impossible" (McGrath and Solter 1984). Except for this dramatic difference involving the donor cell nucleus, the techniques used in this experiment were very similar to those previously used to clone livestock utilizing embryonic cells (Willadsen 1986; Prather et al. 1987; Bondioli

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H. Niemann, C. Wrenzycki (eds.), *Animal Biotechnology 2*,
https://doi.org/10.1007/978-3-319-92348-2_1

et al. 1990). Since the birth of "Dolly," SCNT has resulted in live birth in a large number of mammalian species (Edwards and Schrick 2015). It appears that SCNT can be successful in terms of resulting in live birth in all mammalian species. While SCNT is very successful in this respect, the overall efficiency of the procedure remains low in all species and generally is less than 5% when calculated as proportion of cells fused to oocytes resulting in live birth surviving more than a few days. This efficiency is in comparison to the more than 40% birth rate obtained with embryos for in vitro fertilization using the same in vitro procedures of in vitro oocyte maturation and in vitro embryo culture. In addition, offspring, especially those from livestock species, have a high incidence of abnormalities including large offspring syndrome (LOS), severe placental abnormalities, respiratory problems, prolonged gestation, and dystocia (Young et al. 1998; Yang et al. 2007; Edwards and Schrick 2015). This overall low efficiency and incidence of abnormal development are likely due to incomplete and/or incorrect nuclear epigenetic reprogramming.

In all livestock species, the procedures are basically the same, consisting of enucleation of a mature metaphase II oocyte, fusion of an intact somatic cell by electroporation followed by some sort of activation treatment, and in vitro culture prior to transfer into a recipient for development to term. Exact protocols for SCNT in pigs (Giraldo et al. 2012) and cattle (Ross and Cibelli 2010) are published and will not be repeated here. This chapter will discuss the key inputs and major steps, highlighting different approaches (between and within species), and how each of these inputs and steps may contribute to the overall low efficiency observed for this procedure.

1.1 Oocytes Used for SCNT

The oocytes used in nuclear transfer are a key biological component for the procedure. Largely unknown components in the cytoplasm of the oocyte are responsible for the genomic reprogramming allowing development to be directed by the genome of a differentiated somatic cell. The reprogramming events in SCNT have a lot in common with those occurring after fertilization. All evidence points to the fact that the same characteristics of an oocyte important for developmental competence after SCNT are the same as those required for developmental competence following fertilization. The oocytes used in the nuclear transfer procedure represent a major input that usually accounts for the greatest cost associated with application of the technology. The oocytes also contribute one of the largest sources of variability. Since the procedure has a low overall efficiency, a relatively large number of oocytes are required to produce live offspring or conduct meaningful experiments. This requirement for large numbers and a general lack of knowledge concerning what is a "good oocyte" makes acquiring this input problematic and variable. In contrast to the situation in rodents, essentially all SCNT in livestock use abattoir-derived in vitro matured oocytes. Completely in vivo matured oocytes are not a particularly viable option for livestock. Recovery of mature oocytes with expanded cumulus cells is very difficult in livestock species, and attempts to recover in vivo matured oocytes by ultrasound guided aspiration in cattle (Sarmiento 2014) resulted in low

efficiency. A few studies in pigs (Bondioli et al. 2001) and goats (Reggio et al. 2001) have utilized oocytes recovered from midsize to large follicles following gonadotropin stimulation. Oocytes recovered in this manner are still immature (germinal vesicle stage) and require in vitro maturation but are from larger more mature follicles than typically recovered from abattoir-sourced ovaries. There are too few studies of this nature to determine if this partial in vivo maturation yields a higher efficiency for SCNT, and the vast majority of procedures both commercial and research have relied upon the abattoir-derived in vitro matured oocytes.

Both abattoir sourcing and in vitro maturation can contribute to the variability and overall low efficiency of SCNT. When ovaries obtained from an abattoir are used for follicular aspiration, little to nothing is known about the animals they came from. This can lead to extreme variability due to seasonal differences, nutritional status, animal age, and management practices (culling rate). An example of the latter might be that when milk or calf prices are high, producers cull fewer animals, and those that are culled may be older or reproductively unfit. The majority of pigs slaughtered in the USA and in Europe are prepubertal at the time of slaughter. While oocytes from prepubertal or peripubertal pigs have been used for in vitro fertilization and pronuclear injection, they are not very suitable for the type of manipulation required for SCNT. Oocytes for porcine SCNT are preferably recovered from older sows, which significantly complicates the task of abattoir sourcing. The small ruminant livestock industries are not well developed in the USA; thus slaughter facilities for these species are generally small and not uniformly distributed regionally with the USA. Slaughter of horses for food is no longer allowed in the USA, so abattoir-sourced equine oocytes are not an option in the USA. All of these factors combine to make abattoir-sourced oocytes of sufficient quantity for SCNT problematic and a source of significant variability. The ability to cryopreserve oocytes could alleviate many of the difficulties. While cryopreservation of human oocytes by vitrification and subsequent fertilization or intracytoplasmic sperm injection has become a common clinical procedure, far less research has been conducted with cryopreservation of oocytes from domestic species. Oocytes cryopreserved by vitrification have been used for SCNT in cattle (Hou et al. 2005; Yang et al. 2008) and sheep (Moawad et al. 2011). Use of vitrified bovine oocytes resulted in live birth (Hou et al. 2005) and late-term pregnancy (Yang et al. 2008), and the experiments in sheep produced blastocysts. In each case in vitro and in vivo development rates were lower with vitrified oocytes compared to non-cryopreserved oocytes.

The difference in developmental competence between in vitro matured oocytes and in vivo matured oocytes has been established, with in vivo matured oocytes reaching higher rates of embryo development following fertilization than their in vitro matured counterparts. (Labrecque and Sirard 2014). While embryo development following fertilization is not the same as embryo development following nuclear transfer, there are many similarities. Both situations require extensive genome reprogramming, and nonnuclear cytoplasmic organelles are crucial for both. The sub-optimum developmental competence of in vitro matured oocytes compared to in vivo matured oocytes can certainly contribute to the overall low efficiency of SCNT. Factors affecting the developmental competence of in vitro

matured oocytes have been a major area of investigation because they also contribute to poor developmental potential of in vitro fertilized oocytes in domestic animal breeding. These factors and their optimization particularly for bovine oocytes are dealt with in other parts of this volume and will not be repeated here. It is likely that improvements made for in vitro maturation of immature oocytes will enhance developmental potential for SCNT embryos as well as in vitro fertilized embryos.

In addition to the genomic reprogramming function of the oocyte cytoplasm, the oocyte utilized in SCNT is also the source of numerous cytoplasmic organelles crucial for embryo development. Of particular significance in this regard are the mitochondria contributed by the oocyte in a nuclear transfer procedure. The special role of mitochondria in this discussion results from two factors: (1) the important role of mitochondria in controlling cellular metabolism and the proposed link between metabolism, pluripotency, and reprogramming following nuclear transfer (Folmes et al. 2011; Esteves et al. 2012) and (2) the fact that mitochondrial function requires coordinated activity between mitochondria factors and nuclear-encoded proteins. Abnormal mitochondria function has been proposed to directly impact reprogramming in SCNT (Hiendleder et al. 2005), and aberrant mitochondria-nucleus cross-talk is a contributing aspect of this disturbed function (Lloyd et al. 2006). In the case of SCNT, not only is there a possibility of aberrant cross-talk because of the difference between somatic cell mitochondria function and embryonic mitochondria function but also a very real possibility of aberrant cross-talk due to genetic distance between the oocyte donor and the nuclear donor. Some degree of genetic distance is essentially guaranteed in the case of outbred domestic animals and accentuated by the likelihood of there being dramatic breed differences between oocyte and nuclear donors. When bovine oocytes are abattoir sourced for SCNT, they frequently are collected from the ovaries of dairy (primarily Holstein in the USA) breeds. This is simply because production dairy cattle are most commonly culled because of milk production, rather than reproductive failure making oocyte recovery more effecient. Production beef cattle on the other hand are frequently culled because of sub-optimum reproductive performance and are generally older, making oocyte recovery less effecient. Many beef breeds that may be used for nuclear donor cells are hybrids of *Bos taurus* and *Bos indicus* breeds and thus would represent considerable genetic distance if these donor cells are fused into an oocyte from a Holstein cow. An example of extreme genetic distance between the donor nucleus and the recipient oocyte could exist for porcine SCNT as well. In some cases, it is preferable to create porcine biomedical models in one of the breeds of miniature pigs that have been established (Cho et al. 2007). When these cells from the miniature pigs are used for SCNT following genetic manipulation, they would likely be fused into oocytes recovered for domestic pigs. This would most likely represent considerable genetic difference between the donor nucleus and the recipient oocyte and may lead to aberrant cytoplasmic-nuclear cross-talk. The possible effect of genetic distance between nuclear donor cells and recipient oocytes on cytoplasmic-nuclear cross-talk is an understudied potential complication of SCNT in domestic species.

The nuclear transfer procedure most commonly used for livestock involves the fusion of an intact cell with the enucleated oocyte. This procedure creates the

possibility of mitochondria heteroplasmy or a mosaic mitochondria population in nuclear transfer-derived embryos and offspring. The fate of the somatic mitochondria from the donor cell in the oocyte cytoplasm has been studied in livestock species (Meirelles et al. 2001). While some degree of mitochondrial heterplasmy has been detected in nuclear transfer embryos and offspring, in the majority of cases, the mitochondria population of the oocyte predominates, and the mitochondria from the donor cell do not replicate.

1.2 Donor Cells Used for SCNT

The first example of SCNT in a mammal, the birth of "Dolly," utilized a mammary epithelial cell as a donor cell. This choice of cell type was driven more by a commercial interest than a biological choice of what cell type would most likely be successful. There are more than 200 cell types distinguishable by morphology in mammals, and less than 5% of these have been tested as nuclear donors. Of those tested all support development to blastocysts, but some repeatedly failed to generate viable offspring (Kato et al. 2000; Wakayama and Yanagimachi 2001; Oback and Wells 2002). The large majority of SCNT experiments in livestock have been conducted with skin fibroblasts, and the majority of these experiments have used skin fibroblasts recovered from early pregnancy fetuses. Very little specific information is available concerning what makes the ideal donor cell. The decision of what cell type to use is generally made from three considerations: (1) the objective of the SCNT procedure, 2) the ease of collecting the tissue from the donor animal, and 3) the ability to culture various cell types in a particular laboratory environment.

To date the majority of SCNT procedures with domestic livestock have been experimental in nature aimed at investigating factors that may affect the efficiency of the procedure. In these cases, the objective does not greatly influence the choice of donor cell type and cells that have been selected on an assumption of which cells will be most successful. In these cases, the choice has generally been skin fibroblasts from early pregnancy fetuses. A second objective, primarily for porcine (Polejaeva et al. 2016) but in a few cases for bovine (Kuroiwa et al. 2002), has been to use SCNT to support genetic manipulation particularly for gene knockouts in order to create biomedical models. This is similar to the situation for purely experimental objectives, and fetal fibroblasts have generally been used. A very different situation exists if the objective is to use SCNT in animal breeding to duplicate a specific genome demonstrated to be of value. This situation virtually dictates the use of cells from an adult animal and perhaps from an aged or even deceased animal. There have been reports of using skin fibroblast cells from adult animals for SCNT (Li et al. 2013), but most of the information we have about the procedure has been derived from experiments utilizing fetal fibroblasts. Factors to consider which could be very different in cells from aged animals include incidence of genetic mutations, altered epigenetic profiles, and lack of maintenance of parental imprinting patterns. If the goal of using SCNT in livestock breeding programs is to be realized, these factors will need to be investigated in the context of which cell types should be used as donor cells.

Ease of collecting the tissue has certainly been a factor in the choice of skin fibroblasts particularly if tissue from adult animals is collected. Skin biopsies such as a simple "ear notch" are readily collected from livestock species and seldom require any sort of anesthesia. The initial difficulty of collecting fetal tissue is understood, but once a fetus is recovered, the collection of skin fibroblasts from that fetus is simple and straightforward. Related to the ease of collection is the relative ease and success of establishing cultures from skin tissue. Viable cell cultures can be established from skin by either enzymatic digestion or simple outgrowth for tissue pieces. There does not seem to be any difference in the viability of cultures established by these two methods in cattle (Giraldo et al. 2007a). Once established skin fibroblast cultures are easily maintained with the use of standard tissue culture media (such as DMEM or TCM 199), supplemented with 10–15% bovine serum and passage by trypsinization. These cells also survive cryopreservation by routine methods very well. What is not assured by these routine cell culture methods is long-term culture without development of chromosomal abnormalities. It is not entirely clear what the average incidence of chromosomal abnormalities is in cultured fibroblasts, but the incidence of chromosomal abnormalities in the form of aneuploidy can be high in cell populations after repeated passage (Giraldo et al. 2007a). This can be a very important factor if genetic manipulation in the donor cell population is conducted prior to SCNT because these procedures often require long-term culture. If a donor cell with a gross chromosome abnormality such as aneuploidy is used for SCNT, the resulting embryo would have little or no chance of development. It is not clear how much this contributes to the inefficiency of the procedure.

Related to in vitro culture conditions are the dynamics of the cell cycle. When skin fibroblasts are cultured with the conditions described above, they display a long cell cycle (2–3 days) with up to 70% of the cells at any time being in the G1 phase of the cell cycle (Giraldo et al. 2007b). This is advantageous for their use as donor cells for SCNT because G1/G0 is the preferred cell cycle stage. The work of Keith Campbell and associates (Campbell et al. 1996a) established the importance of cell cycle synchrony between the donor cell and the oocyte in nuclear transfer procedures with embryonic and somatic cells. Cellular reprograming is enhanced if the donor nucleus is exposed to the reprogramming factors of the oocyte cytoplasm immediately after transfer. This is accomplished by fusion of donor cells into oocytes when MPF is high which leads to nuclear envelope breakdown and premature chromosome condensation (Campbell et al. 1993). A decrease in MPF consistent with oocyte activation will lead to DNA replication in preparation for the first mitotic division. If the transferred nucleus has begun (S phase) or completed (G2 phase) DNA replication, this replication will be reinitiated (Johnson and Rao 1970) which leads to a chromosome content inconsistent with a normal mitotic division. When a donor cell is fused with an oocyte with high MPF levels, it is optimum for that cell to be in G1 or the quiescent G0 stage of its cell cycle to ensure a normal DNA replication producing a 4C nucleus consistent with a normal first mitotic division in the nuclear transfer embryo. The skin fibroblast cultured under normal conditions with approximately 70% of cells in G1 at any time produces this condition with minimal manipulation.

Of particular interest as donor cells for SCNT are stem cells. It has been proposed that stem cells would be more reprogrammable and thus lead to greater efficiency when used as donor cells. At least one study in mice (Rideout et al. 2000) resulted in higher cloning efficiency with embryonic stem cells. Attempts to isolate embryonic stem cells comparable to those used in the mice experiments (capable of generating germline chimeras) have not been successful in livestock. Recent descriptions of induced pluripotent stem cells which are essentially the same as embryonic stem cells in mice have likewise led to the hypothesis that these cells would more reprogrammable when used at donor cells. These cells have not been thoroughly described for livestock species, and this hypothesis has yet to be tested. Somatic stem cells for many tissues from various species including livestock have been described. While these cells display some cell-type plasticity in culture, "lineage plasticity" of these cells remains controversial, and the use of these cells as nuclear donors has not led to any increase in cloning efficiency (Oback 2008).

1.3 Treatment of Donor Cells Prior to Fusion

A number of treatments have been applied to donor cells prior to fusion for nuclear transfer. The first such treatment for SCNT in livestock was inducing cells to exit the growth cycle and arrest in the G0 quiescence state by culture in low serum conditions, "serum starvation" as described by Keith Campbell (Campbell et al. 1996b; Wilmut et al. 1997). The value of this treatment is unclear (Kasinathan et al. 2001), and one study (Kues et al. 2000) has shown that serum starvation can induce DNA fragmentation in bovine fibroblasts. As discussed above, fibroblasts in culture have an elongated G1 phase, and culturing these cells to confluence can create a population with a high incidence of G1 without serum starvation. A recent report has shown that induction of quiescence by serum starvation results in hypomethylation of DNA and lysines 4, 9, and 27 of histone H3 resulting in a more relaxed chromatin structure and enhanced reprogramming following nuclear transfer (Kallingappa et al. 2016).

The process of cellular reprogramming is an epigenetic event, and the incomplete and/or incorrect reprogramming as the root of low efficiency with SCNT involves incomplete and/or incorrect epigenetic remodeling. Epigenetic marks, being a posttranslational modification, are created and altered by enzymatic reactions. A variety of molecules and procedures have been developed which affect the activity of these enzymes and are referred to as epigenetic modulating agents. A variety of these agents has been used to treat somatic cells prior to fusion with oocytes in an attempt to correct epigenetic marks. One of the most prevalent examples of these is trichostatin A (TSA), a potent inhibitor of the histone deacetylase enzymes (HDACs). Inhibition of the deacetylase enzyme would be expected to increase the level of histone acetylation which is an epigenetic mark which increases gene expression. A second prevalent epigenetic modulator is 5-aza-2′-deoxycytidine (5azadC) which is a DNA methyltransferase inhibitor. Inhibition of DNA methyltransferase (DNMT) especially the "maintenance enzyme" DNMT1 will decrease DNA methylation, which is also an epigenetic mark usually consistent with

increased gene expression. If cellular differentiation is characterized as sequential inhibition of gene expression, particularly those genes necessary for early embryo development, removal of those epigenetic marks to allow those genes to be expressed would be fundamental for the reprogramming during SCNT. These agents alone or in combination have been used to treat bovine cells (Enright et al. 2003, 2005; Giraldo et al. 2007b; Ding et al. 2008; Wang et al. 2011; Fig. 1.1).

Fibroblasts cultured for an extended period of time have increased levels of acetylated histones and decreased levels of methylated DNA (Enright et al. 2003; Wilson and Jones 1983). Giraldo et al. (2008) compared bovine fibroblasts after 5 and 35 population doublings (PD). Cells at 35 PD had reduced levels of transcripts for DNMT 1 and 3A with constant levels of transcript for DNMT 3B. Fibroblasts at 35 PD also had lower levels of methylated DNA than at 5 PD. A higher proportion of SCNT embryos from PD35 donor cells developed beyond the 8–16-cell stage. When day 7 SCNT embryos were transferred to recipients and recovered at day 13, a higher proportion of those reconstituted with PD35 donor cells showed subsequent development with larger conceptuses.

In addition to extended culture or chemical inhibitors of epigenetic-modifying enzymes, small interference RNA (siRNA) can be used to reduce transcript levels encoding these enzymes and thus reduce the levels of the modifying enzymes (Giraldo and Bondioli 2011). This approach utilizing siRNA directed against DNMT1 in bovine fibroblasts resulted in a reduction of the transcript encoding this enzyme and hypomethylation in the treated cells (Giraldo et al. 2009). Use of these cells as donor nuclei in nuclear transfer resulted in reduced methylation levels in early SCNT embryos (Fig. 1.2).

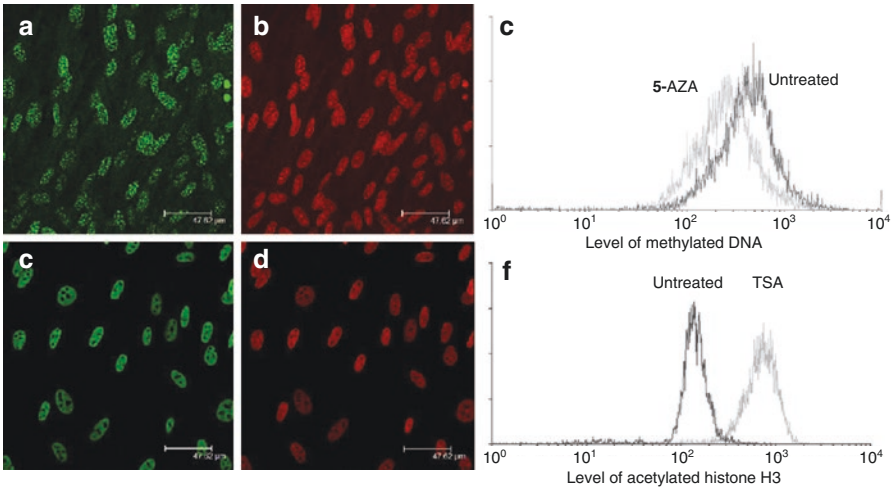

Fig. 1.1 Bovine cells incubated with anti-methylcytidine antibody, labeled with Alexa Fluor 488 (**a**) and counterstained with propidium iodide (**b**). Level of methylated DNA after incubation with 1 μM of 5-azacitidine for 48 h (**c**). Cells incubated with anti-acetyl-histone H3, labeled with Alexa Fluor 488 (**d**) and counter stained with propidium iodide (**e**). Level of acetylated histone after incubation with 1 μM of trichostatin (TSA) for 12 h (**f**). From Giraldo et al. (2007b)

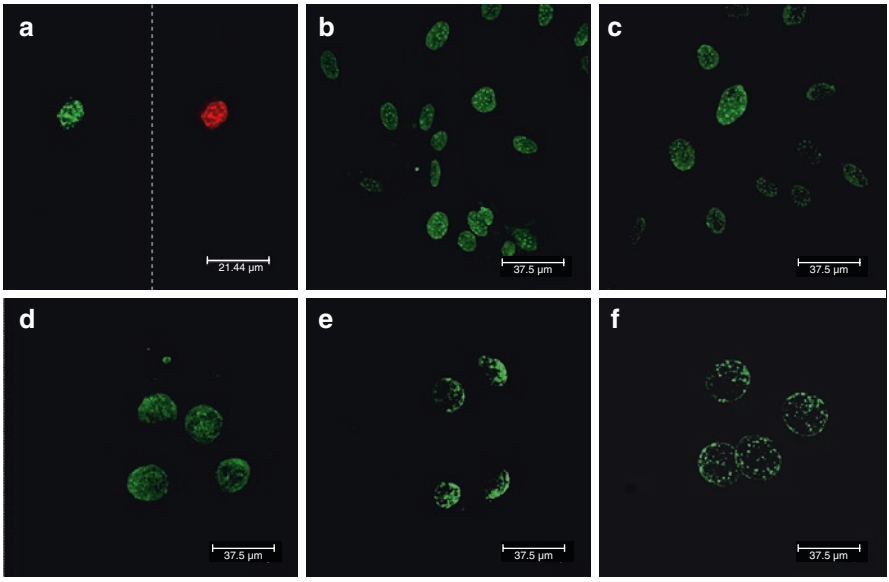

Fig. 1.2 (**a**) Bovine cells incubated with anti-methylcytidine antibody and (left) labeled with Alexa Fluor 488 and (right) counterstained with propidium iodide. Methylation patterns of fibroblast cells treated with (**b**) non-silencing and (**c**) DNMT1-specific siRNA 24 h post-transfection. Level of DNA methylation of 4-cell-stage embryos produced by (**d**) IVF and nuclear transfer using donor cells treated with (**e**) non-silencing or (**f**) DNMT1-specific siRNA. From Giraldo et al. (2009)

The use of chemical agents such as TSA for inhibition of HDACs in cultured cells was discussed above. These chemical agents are inhibitors of the entire family of 18 different HDACS which have been shown to regulate many cellular functions including cell proliferation, differentiation, and development (Yang and Seto 2008). Staszkiewicz et al. (2013) used siRNA targeting specific HDACs in bovine fibroblasts and studied the effect on expression of pluripotency genes. Their data suggests that reduction in the activity of SIRT3, one of five members of the sirtuin family of HDACs could play a role in upregulation of the Oct4-Sox2-Nanog transcriptional network. SIRT3 is preferentially localized to mitochondria and is associated with energy metabolism (Ahn et al. 2008). Energy metabolism and promotion of glycolysis have been linked to establishment of pluripotency and cellular reprogramming (Folmes et al. 2011; Esteves et al. 2012).

1.4 Oocyte Enucleation

For the majority of nuclear transfer procedures, oocytes are enucleated as mature metaphase II oocytes, with the manipulation setup depicted in Fig. 1.3 using the approach depicted in Fig. 1.4. The process involves location of the extruded first polar body, puncturing the zona pellucida with a beveled pipet and aspiration of the

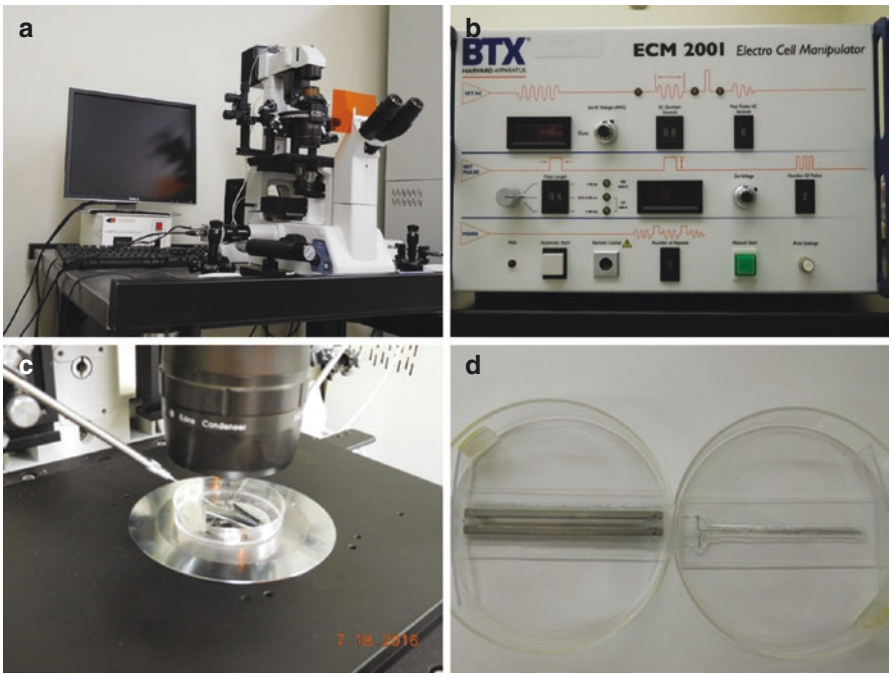

Fig. 1.3 Manipulation equipment used for somatic cell nuclear transfer. (**a**) Inverted microscope equipped with hydraulic-controlled micromanipulators. (**b**) Orientation of micromanipulator-controlled micropipettes used for oocyte enucleation and insertion of donor cells. (**c**) Square wave pulse generator used for fusion of enucleated oocytes and donor cells. (**d**) Two examples of fusion chambers used with pulse generator shown in **c**

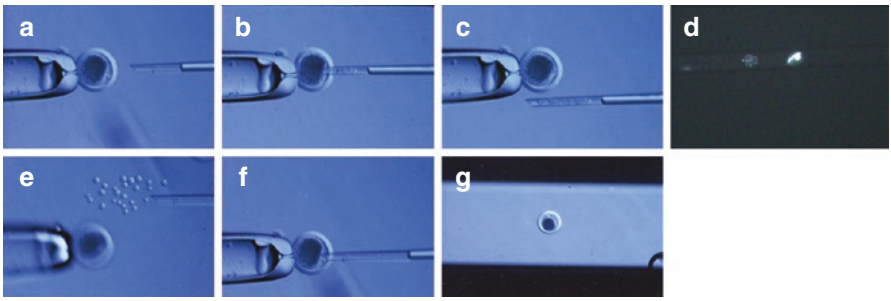

Fig. 1.4 Porcine somatic cell nuclear transfer. (**a**) Mature unfertilized porcine oocyte on holding pipette with the polar body in the "3 o'clock position" and beveled micropipette used for enucleation. (**b**) Enucleation of Hoechst 33342-stained oocyte by withdrawing the polar body and adjacent cytoplasm. Polar body and adjacent cytoplasm in enucleation pipette. (**c**) Illumination with visible light. (**d**) Illuminated with UV light. (**e**) Porcine fetal fibroblasts. (**f**) Injection of fibroblast donor cell between the enucleated oocyte and the zona pellucida. (**g**) Enucleated oocyte and donor cell in fusion chamber (see Fig. 1.1d) with oocyte and donor cell oriented for fusion

polar body and a small amount of adjacent oocyte cytoplasm. The process is complicated for oocytes of domestic species due to the inability to visualize the metaphase spindle. Enucleation in this manner relies upon the assumption that the metaphase spindle will be adjacent to the first polar body. This assumption is true for a period after extrusion of the polar body but becomes less likely as oocytes age post-maturation. Prior to enucleation oocytes are stained with a membrane-permeable fluorescent DNA stain such as bisbenzimide (Hoechst 33342) by incubation at a low concentration for 10–15 min. It is possible to visualize the metaphase chromosomes directly by excitation with UV, but this is usually avoided because of concern for damaging effects to the oocyte from the energy released in the form of heat with UV excitation (Li et al. 2004b). Alternatively, "blind" enucleation is attempted without UV excitation and then confirmed by visualization of the spindle in the pipet as depicted in Fig. 1.2, and UV excitation of the oocyte is avoided. If the spindle is not observed, a second attempt can be made, or the oocyte is rejected. If oocytes are enucleated immediately after extrusion of the first polar body, this approach will be highly successful. A limited number of studies have been conducted to determine the effect of staining and UV illumination on the viability of oocytes with varying results dependent upon species and length of UV irradiation. In a study conducted with porcine mature oocytes, the combination of exposure to Hoechst 33342 and UV irradiation decreased subsequent development following in vitro fertilization and was more pronounced with increased exposure to the UV illumination (Maside et al. 2011). The in vitro fertilization model is clearly different from the SCNT procedure because of the likely effect on the oocyte nuclear component, which is essential for development after fertilization but not essential for nuclear transfer. In a study of bovine nuclear transfer utilizing embryonic cells, Westhusin and colleagues (Westhusin et al. 1992) found that development to term was not affected by either exposure to the DNA stain or irradiation with UV.

An alternative method of enucleation is utilized in the zona-free and manipulator-free system of cloning referred to as "handmade cloning" (Vajta et al. 2005). In this system the oocyte zona pellucida is removed, and a small segment of the oocyte adjacent to the polar body is cut with a handheld razor blade. One of the possible consequences of this approach to oocyte enucleations is a decrease in oocyte cytoplasmic volume. This could affect the ability of the oocyte to reprogramming the donor nucleus and affect the nuclear/cytoplasmic volume ratio of blastomeres in the developing embryo. Attempts at chemical enucleation and PolScope microscopy enucleation have been described but have not been adapted to a repeatable method of livestock SCNT (Li et al. 2004a).

1.5 Oocyte and Donor Cell Fusion and Activation

For livestock species, fusion of the oocyte and donor cell is accomplished by electroporation of the two adjacent cells. A typical electrofusion instrument and fusion chambers are shown in Fig. 1.3. Electroporation instruments used for cell fusion are square wave generators that produce a square-shaped pulse as opposed to the

exponential decay-type curves normally used for cell electroporation. In most cases, the donor cell is placed between the oocyte and the zona pellucida, and the zona pellucida is important to ensure contact. The electrofusion instrument shown in Fig. 1.3 has an alternating current (AC) cell alignment function, but this function is of questionable use for nuclear transfer because of the large difference in cell size. For SCNT, alignment of the oocyte and donor cell fusion plane perpendicular to the electrical field is essential but is accomplished manually. The AC alignment function may serve to enhance contact between the oocyte and donor cell once proper alignment is accomplished manually. In the case of the zona-free "handmade cloning" described earlier, contact is enhanced by the use of the lectin phytohemagglutinin (PHA). No advantage has been reported for the use of PHA for fusion in the presence of a zona pellucida. An alternative cell fusion system is available and is generally referred to as the "Chop Sticks" approach. With this system, fusion is conducted with the aid of micromanipulators, and the electrodes are placed next to the oocyte/cell combination that is held in proper alignment on a holding pipet. This system can be highly successful but requires that fusion be conducted one couplet at a time as opposed to fusion in a chamber where multiple oocyte/cell couplets can be fused simultaneously. Fusion is accomplished in a nonionic solution consisting primarily of a sugar such as mannitol, sucrose, or sorbitol. These solutions typically have a low concentration of calcium. If ionic strength of the fusion medium is too high, it will generate heat which can be detrimental to the embryos. The strength of the electrical field, which induces electroporation, is expressed in volts per centimeter where volts are the voltage applied and centimeter is the distance between the electrodes. Numerous fusion protocols have been reported for livestock species, which vary between laboratories and between species. In general, electrical fields of 1.2–1.5 kV/Cm are applied in single or multiple pulses of less than 100 microsecond duration.

It is possible at least for some species to induce oocyte activation with the electroporation pulse used for fusion if the calcium concentration in the fusion medium is high enough. In most cases fusion is induced with conditions (low calcium concentration) that do not induce activation. This allows for MPF levels in the oocyte to induce nuclear envelope breakdown and exposure of the donor chromatin to the oocyte cytoplasm. Oocyte activation is then induced several hours later. For porcine SCNT, this activation is induced with the same electroporation pulse used for fusion but with a higher concentration of calcium in the medium. For ruminant species, especially cattle, some sort of chemical activation is required. This normally involves a two-step process consisting of brief exposure to a calcium transporter such as ionomycin followed by an extended exposure to the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) (Susko-Parrish et al. 1994) or the protein synthesis inhibitor cycloheximide (Presicce and Yang 1994). The effect of these chemical activation procedures on subsequent embryo development is not known. One study utilized cRNA encoding the sperm activation factor phospholipase C zeta (Ross et al. 2009) for bovine SCNT. Further studies utilizing alternative activation procedures for bovine SCNT are warranted.

1.6 Treatment of Nuclear Transfer Embryos Post Fusion and Activation

There have been a number of investigations involving treating nuclear transfer-derived embryos after fusion and activation with epigenetic modulating agents. The majority of these treatments have involved inclusion of histone deacetylase inhibitors in culture medium after fusion and activation during the first cell cycle of the reconstructed nuclear transfer embryo. Histone acetylation appears to be a key epigenetic modification for reprogramming. Hyperacetylation of histones brought on by inhibition of the deacetylase enzymes corresponds to chromatin relaxation creating a transcriptionally permissive state (Rybouchkin et al. 2006; Zhao et al. 2010). While creation of the transcriptionally permissive state is not sufficient for reprogramming, it is likely that it facilitates and may be necessary for reprogramming to occur. A number of histone deacetylase inhibitors (HDACi) such as TSA (Cervera et al. 2009; Ding et al. 2008; Enright et al. 2003; Wee et al. 2007), valproic acid (VPA) (Costa-Borges et al. 2010; Huang et al. 2011), Scriptaid (Wang et al. 2011; Zhao et al. 2010), sodium butyrate (Das et al. 2010; Shi et al. 2003), suberoylanilide hydroxamic acid (Ono et al. 2010), m-carboxycinnamic acid bishydroxamide (Dai et al. 2010), and oxamflatin (Hou et al. 2014; Mao et al. 2015) have been applied to nuclear transfer-derived embryos post fusion and activation to modify the epigenetic pattern of the donor chromatin and enhance in vitro and/or in vivo development. The majority of these studies have investigated in vitro development to the blastocyst stage, but some (Zhao et al. 2010; Mao et al. 2015) have shown modest improvements in in vivo development of porcine SCNT embryos. The optimum duration of treatment has been reported to be between 14 and 16 h for VPA and Scriptaid (Huang et al. 2011; Whitworth et al. 2011), and exposure to TSA for longer than 14 h has been detrimental to cloning efficiency (Kishigami et al. 2006).

1.7 Culture and Transfer of Cloned Embryos

Post fusion in vitro culture and transfer of cloned embryos are related topics for domestic animal species because the type of transfer for each species determines the length of in vitro culture. For those species which nonsurgical embryo transfers are efficient (cattle and horses), SCNT embryos are cultured in vitro to the late morula or blastocyst stage and transferred to the uterus of recipients. Gestation and delivery of twins frequently result in life-threatening results for mother and/or offspring in both cattle and horses; thus the best procedure is to transfer only one embryo per recipient. In vitro culture to the blastocyst stage allows for some selection of SCNT embryos prior to transfer which is an important efficiency consideration. Despite the fact that cloned embryos maintain some metabolic features of somatic cells rather than early-stage embryos, embryos from SCNT are cultured to the blastocyst stage using media shown to be most efficient for culture of mammalian embryos produced by in vitro fertilization (Ross and Cibelli 2010; Arias et al. 2013).

In domestic species for which nonsurgical embryo transfer is not an option such as the pig, SCNT embryos are transferred to recipients shortly after fusion. In pigs it is common to transfer large numbers (over 100) of SCNT embryos to a single recipient to produce litters of approximately eight piglets (Giraldo et al. 2012).

1.8 Evaluation of Cloning Efficiency

Probably the most difficult aspect of research concerning SCNT is how to evaluate efficiency and determine if specific treatments or conditions enhance reprogramming and subsequent embryo development. Clearly, the result of a successful SCNT procedure is the birth of full-term offspring with normal survival and lacking any abnormalities. Studies with full-term birth and survival as endpoints are limited even with laboratory animals and extremely limited with livestock species. Most of the studies with domestic animal SCNT that include full-term development result in less than five surviving offspring spread over multiple treatments. While such a result does demonstrate that full-term development is possible, this is not a statistically meaningful result concerning the treatments and involves a significant expenditure of time and resources.

A large number of SCNT experiments have in vitro development to the blastocyst stage as an endpoint for evaluation of reprogramming efficiency. In vitro development to blastocysts following parthenogenic activation alone at rates similar to those for SCNT has been reported for cattle (Wang et al. 2008) and pigs (Cheng et al. 2007). Since these blastocysts have no chance of producing full-term offspring upon transfer, it is highly questionable if this endpoint accurately reflects developmental potential for SCNT embryos. It is conceivable that some cases of incomplete reprogramming might enhance development to blastocysts yet decrease the efficiency of full-term development. Many of the studies that have used in vitro development as an endpoint have included total blastocyst cell numbers and/or the inner cell mass to trophectoderm cell ratio. While this added data has some value, there is no data showing that a statistically significant increase in cell number at the blastocyst stage will lead to a statistically higher rate of development to term. In the bovine commercial embryo transfer industry, extensive data have shown essentially normal pregnancy rates and full-term development for half embryos created by embryo splitting (Gray et al. 1991). In addition to cell number data, many studies ending at the blastocyst stage have included gene expression data at the blastocyst stage. Most of these studies have measured transcript levels for the major pluripotency genes, Oct 4, Nanog, and Sox 2. This is potentially valuable information but requires careful interpretation. In addition to the reservation that transcript levels do not necessarily equate to protein levels, particularly during early embryo development, there is no clear picture of what are "normal" expression levels for these genes in embryos, and it is not clear if statistically significant increase reflects higher development potential (Radzisheuskaya et al. 2013).

One of the characteristics of SCNT procedures is a high rate of loss between the blastocyst stage and early fetal development at or around the time of implantation

(Edwards and Schrick 2015). The long gestation period for livestock species and the cost of transfer of embryos to be carried to term make any methods that extend development beyond the blastocyst stage valuable tools for determining SCNT efficiency. Important to this aim is the ability to evaluate a large enough sample to achieve statistical significance. This is crucial for bovine nuclear transfer because single embryos are transferred to each recipient if the embryos are to be carried to term. Giraldo and colleagues (Giraldo et al. 2008) transferred up to 25 SCNT embryos to single synchronous recipients at the blastocyst stage and recovered elongated and ovoid embryos (Fig. 1.5) at day 13 or 14 by nonsurgical flushing. Survival of the SCNT embryos was estimated from the number of advanced-stage embryos collected and the presence of an embryonic disc. These embryos can also be used for gene expression analysis, and the embryonic disc can be separated from the trophectoderm and analyzed separately. There is clearly extensive embryo loss beyond the elongation stage for SCNT embryos, but for cattle, this system can be an efficient method to extend the development period beyond the blastocyst stage without requiring a large number of live animals. In pigs where nonsurgical embryo transfers and recovery are not an option, large numbers of SCNT embryos can be

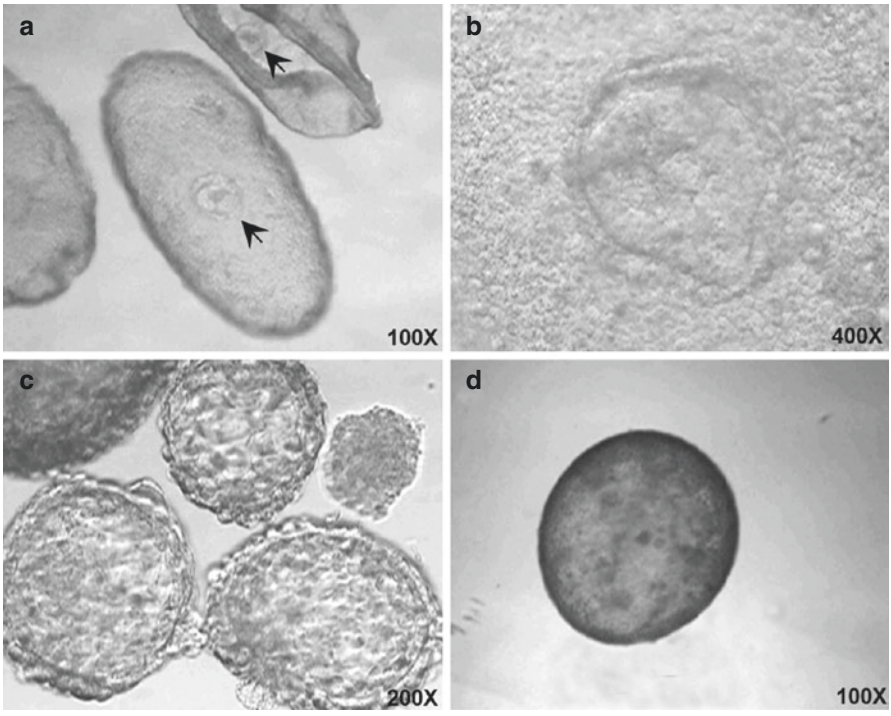

Fig. 1.5 Bovine embryos collected on day 13 postestrus. Elongating in vivo embryos (**a**) with an identifiable embryonic disc (**b**). Spherical and ovoid cloned embryos reconstructed using cells with high and low levels of DNMT1 mRNA, respectively (**c**, **d**). Arrows indicate the presence of an embryonic disc in in vivo embryos. From Giraldo et al. (2008)

transferred and conceptuses recovered by euthanasia at approximately day 25. At this stage of development, the number of surviving conceptuses and the presence or absence of a normal amount of fetal tissue within the conceptus can be evaluated.

1.9 Future Directions

Application of SCNT in domestic livestock is limited by the low efficiency and the high incidence of developmental abnormalities leading to low survival. It is very likely that these two factors are closely related, and as efficiency increases, the incidence of developmental abnormalities will decrease. It is clear that the low efficiency and developmental abnormalities result from incomplete and/or incorrect epigenetic reprogramming of the donor cell genome. Improvements in reprogramming of the donor cell genome can be achieved in the donor cell prior to fusion and in the oocyte after fusion. The ability to improve reprogramming after fusion is limited by the low number of cell divisions before the embryonic genome is activated. Major improvements in reprogramming will come from reprogramming in the donor cell prior to fusion. The success with induced pluripotency of somatic cells in some species suggests that this will be a fruitful approach for SCNT in livestock. Increased expression of key pluripotency genes, facilitated by epigenetic modifiers, is a promising approach. These epigenetic modifiers can also facilitate reprogramming in the oocyte after fusion of an induced pluripotent cell. It is important to consider that as somatic cells are induced into pluripotency, it can be expected that the cell cycle will be more like that of embryonic stem cells and cell cycle synchrony between the donor cell and the oocyte will become more critical and challenging.

Improvement in the developmental competence of in vitro matured oocytes also has the potential of significantly improve the efficiency of SCNT. Developmental competency of oocytes and the ability to reprogram an incoming genome probably have multiple pathways in common. Controlling the genetic distance between the donor cell and the recipient oocyte is an important consideration. Refinement in procedures for oocyte enucleation, fusion, activation, and culture can impact the efficiency of SCNT, but they are secondary to the central issue of reprogramming the somatic cell nucleus.

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