MINIREVIEW

Advances in Livestock Nuclear Transfer

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Abstract. **Cloning and transgenic animal production have been greatly enhanced by the development of nuclear transfer technology. In the past, genetic modification in domestic animals was not tightly controlled. With the nuclear transfer technology one can now create some domestic animals with specific genetic modifications. An ever-expanding variety of cell types have been successfully used as donors to create the clones. Both cell fusion and microinjection are successfully being used to create these animals. However, it is still not clear which stage(s) of the cell cycle for donor and recipient cells yield the greatest degree of development. While for the most part gene expression is reprogrammed in nuclear transfer embryos, all structural changes may not be corrected as evidenced by the length of the telomeres in sheep resulting from nuclear transfer. Even after these animals are created the question of "are they really clones?" arises due to mitochondrial inheritance from the donor cell versus the recipient oocyte. This review discusses these issues as they relate to livestock.**

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The procedure of nuclear transfer in livestock was developed in the late 1980s and was focused on the production of identical offspring derived from blastomeres. When serial cloning was first used (1), there was a limit to which this number could be increased. This review is designed to be an update of a paper recently published in this journal (2). We will therefore focus on recent advances in livestock nuclear transfer and try to avoid repeating the history of animal cloning and data that were discussed earlier in a variety of other reviews (3–6).

A milestone in the field of nuclear transfer (NT) was the production of offspring derived from a cultured, differentiated embryonic cell line in sheep (7). Since then a dramatic change in the perspective of NT occurred because an "unlimited" source of donor-karyoplasts was potentially available, thus providing the potential for the generation of identical transgenic offspring. Genetic modifications are the basis for the potential application of NT-animals for the production of pharmaceutical proteins (gene-farming), xenotransplantation, or increasing agricultural productivity. The application of NT for the generation of transgenic animals in comparison with other transgenic strategies is reviewed by Chan (8) and Piedrahita *et al.* (9) and will not be discussed in this article.

The possibility of producing offspring from cultured adult cell lines enables the generation of offspring from donors of high genetic value with proven performance. Additionally, it might provide a powerful tool in saving en-

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dangered species or populations and protecting biodiversity (10).

In this paper, we will discuss the use of different cell types (embryonic, fetal, adult) and subsequent changes in NT. We will focus on the cell cycle and activation and will also discuss new findings concerning the role of the recipient cytoplasm. Possible reasons for the high losses and abnormal development in connection with NT and future aspects will conclude this review.

Source of Donor Karyoplasts

In the previous review (2), one of the future aspects was the production of embryonic stem (ES-) cells in livestock that might open the possibility of having an "unlimited" source of identical donor-karyoplasts, which also have the potential of being genetically transformed. Several experiments were reported concerning the production of morphologically undifferentiated, ES-cell-like cell lines as reviewed by Prelle *et al.* (11) and Stice *et al.* (12). Although pregnancies were achieved after NT in cattle, no development beyond Day 60 was reported (12). When these stem-cell-like cells were used for aggregation with control embryos, chimeras developed up to Day 85, but pregnancies were lost, and this loss was thought to be a result of a deficiency or absence of cotyledons and a hemorrhagic response in the caruncles.

Another way to produce pluripotent cells is the culture of primordial germ cells (PGCs) to establish embryonic germ (EG) cells, which show similar characteristics to ES-cells. When freshly isolated PGCs were used for NT in cattle, one healthy calf was produced after re-cloning of the developing blastocyst (13), and another calf died soon after delivery because of immature lung development (14). To date, no offspring have been produced when cultured EG-cells were used as donors. Surani (cited in 15) found that murine chimeras derived from EG-cell injection into control embryos showed skeletal abnormalities. When NT was performed using EG-cells, resulting embryos were small and had abnormal placentas. When the expression of imprinted genes was analyzed, either both parental copies were completely repressed or both were active, indicating that lack of imprints was at least part of the problem (15).

Campbell *et al.* (7) produced lambs derived from a differentiated cultured embryonic cell line after *in vivo* culture in agar cylinders for 6 days. After this report, many used cell donors for NT that were suspected of being further differentiated. While the use of *in vitro* matured (IVM) oocytes as recipient cytoplasts is established in cattle, in other species (sheep (16) and goat (17)), *in vitro* systems still result in significantly lower development and need further improvements. In Campbell's report, *in vivo* matured oocytes were used as recipient-cytoplasts, whereas Wells *et al.* (16) compared the production of NT-lambs using *in vivo* versus *in vitro* matured oocytes. Blastocysts were transferred into recipients after 6 days *in vitro* and, offspring were obtained from both oocyte sources. However, the lamb derived from an *in vitro* oocyte died within 10 min after birth. Reports of the production of calves after NT with fetal fibroblasts followed soon from various groups (16,18–20). Finally, NT-goats were also produced using cultured fetal cell lines and *in vivo* (17) or *in vitro* matured oocytes (21).

Much effort has been made in NT in pigs; still efficiencies are far below those in other species. One of the many reasons for this could be a higher sensitivity of porcine oocytes to the routinely used flourochrome bisbenzimide (Hoechst 33342, Sigma, St. Louis, MO). Tao *et al.* (22) showed a negative effect of Hoechst-staining on further *in vitro* development of oocytes after parthenogenetic activation (even without exposure to UV light). High lipid content of the porcine oocyte and the early maternal-zygotic transition have been discussed as being among the reasons for the lack of success in porcine NT. However, recent results are promising. Blastocyst development was achieved when fetal fibroblasts were used (23–25), and later confirmed by PCR or the expression of the green fluorescent protein (GFP: Kühholzer *et al.*, unpublished data). Elongated embryos were recovered from two recipients 12 days after NT; one of the recipients received embryos derived from transfected fetal fibroblasts, and their origin was confirmed by PCR (Tao *et al.*, unpublished data).

A further step in the use of differentiated cells was first achieved in sheep. The production of a lamb derived from cultured adult mammary cells was a major breakthrough (26). For some time the results of this experiment were questioned by others, but shortly afterwards, when the production of live offspring from adult donor cells was repeated by several other groups in cattle, there was no question about the possibility of reprogramming adult, differentiated cells. The variety of cultured adult cells successfully used in NT is many-fold, reaching from cumulus cells (27) and mural granulosa-cells (28) to skin fibroblasts and mammary cells (29, 30).

Although the overall efficiency of producing offspring is still low in the reports mentioned above (≈ 1%), Wells *et al.* (28) was able to increase the efficiency for the production of calves derived from adult mural granulosa cells. Ten percent of the transferred NT-blastocysts developed to term and the overall efficiency was 2.8%.

Technical Changes

Differentiated, cultured cells are smaller in diameter when compared with blastomeres. This has caused difficulties in the fusion between donor cell and recipient cytoplast (16, 31, 32). Different parameters for the cell-to-cell fusion were necessary because of the smaller contact between the two counterparts. In addition, previous electrical alignment of the cells became impossible due to the different size of both cells. Many authors are using manual alignment of the donor and recipient cells and different parameters for the DC-pulse (16, 28, 31). Lavoir *et al.* (31) added phytohemagglutinin-P to the manipulation medium to improve contact between the donor cell and cytoplast. Further, they cen-

trifuged the reconstructed embryos immediately after the electrical pulse to improve fusion rates. Although no data were presented, a positive effect of this protocol on the fusion rate was mentioned. Dehydration of the cytoplast during insertion of the donor cell might also facilitate a closer contact between the cell and cytoplast when cells are rehydrated before fusion (16, 28).

Some groups have tried to use an intracytoplasmic injection of the donor cell, which succeeded in the production of offspring in mice (33) and cattle (34). A study using intracytoplasmic injection of fetal fibroblasts for NT in pig (25) resulted in *in vitro* development comparable to an earlier study done in the same group using cell-to-cell fusion (24). The use of a piezo-driven injection improved the survival rate of mouse oocytes (33) and may also be advantageous for the survival rate after intracytoplasmic injection in other species. For a schematic of the process of NT, see Figure 1.

Cell Cycle

Investigations on the cell cycle for NT using blastomeres have concentrated on the basic question about the DNA content (G_1, S, or G_2) of the donor cell to maintain correct ploidy after NT and to avoid pulverization of the chromatin. Campbell *et al.* (35) suggested the use of preactivated cytoplasts (low maturation promotion factor (MPF)) as universal recipients for NT (80% of donor blastomeres are in S-phase). Use of meiotic metaphase II (MII)-oocytes

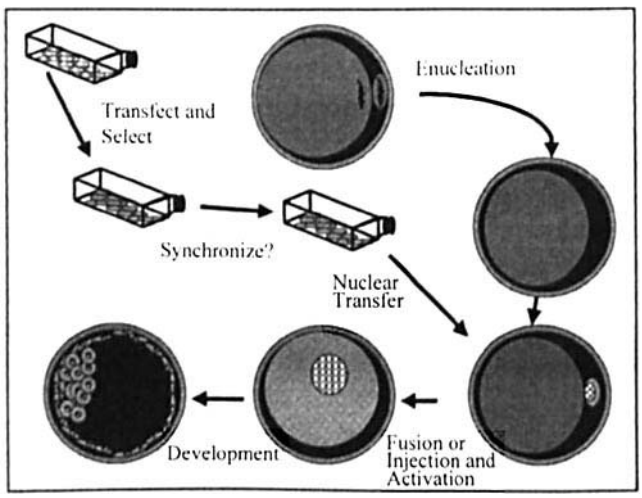

Figure 1. Schematic of the process of nuclear transfer. A cell line is transfected, and transgenic cells are selected. The transgenic cells may then be synchronized prior to nuclear transfer. Oocytes are "enucleated" by removing the metaphase chromosomes as well as the first polar body. The donor cells are then either transferred under the zona pellucida of the recipient oocyte as depicted here, or the nucleus of the donor cell can be microinjected into the cytoplasm of the oocyte. If the cell is transferred, then the two must be fused, and at some point the oocyte must be activated to initiate development of the nuclear transfer embryo. Upon transfer of the donor nucleus into the cytoplasm of the oocyte, the nucleus is remodeled (depicted by a different nuclear pattern), and the developmental clock is reset. The transgenic embryo then grows to the blastocyst stage.

with high maturation promotion factor would only result in development when donor cells in the G_1 stage were used. The previous use of cultured cells offers the possibility of using cells that are in a quiescent stage of the cell cycle. This G_0-stage includes cells that exit the cell cycle (mostly reversible) before entering the S-phase and stay in a transcriptionally inactive stage with a reduced protein-content. Although some cell types are physiologically arrested in the G_0-stage (cumulus cells, Sertoli cells, neuronal cells), cultured cells can be artificially arrested by deprivation of growth factors. A common method is to culture cells in a medium with reduced serum (0.1% or 0.5%) or in the absence of other growth factors (36).

The use of G_0-arrested donor cells was first suggested by Campbell *et al.* (35), who claimed that the altered nuclear structure and reduced transcriptional activity of these cells facilitates reprogramming of differentiated cells after fusion with enucleated meiotic metaphase II oocytes (36). Unfortunately there is no synchronization protocol that enables 100% synchronization in one stage of the cell cycle. Serum starvation increases the percentage of cells in G_0 significantly (37, 38) and therefore the probability that NT-embryos are derived from the G_0 population. However, it cannot be proven that these embryos and offspring are not derived from a subpopulation in a stage other than G_0. Comparisons of the use of serum starved versus cells that are in a presumptive G_1-stage have provided evidence for the theory that cells in G_0 result in higher rates of development when compared with cells in the G_1-stage (20, 39, 40). Although in most of the recent experiments, donor cells that were pre-S phase were used, Lai *et al.* (41) showed that donor cells in presumptive G_2/M phase extrude a polar body when injected into enucleated porcine MII-oocytes. Although a similar experiment in mice (42) resulted in the production of multiple offspring, in pigs the developmental rate was lower when presumptive G_2/M stage cells were compared with serum-starved donor cells (presumptive G_0 (41). However, it was shown that due to the extrusion of a polar body, the correct ploidy could be maintained.

Activation, Structural Changes, and Reprogramming

Different mechanisms of artificial activation by different stimuli have been described in detail in a review by Macháty *et al.* (43). Here we will only discuss the timing of activation for NT and subsequent changes in the reconstructed embryo. In earlier studies using blastomeres for NT, simultaneous fusion and activation or fusion of donor cells with preactivated or aged cytoplasts resulted in offspring. After simultaneous fusion and activation, the nucleus was completely remodeled (similar to a zygotic pronucleus), and this remodeling was mediated by the exchange of proteins between donor cell and cytoplast (44). In preactivated or aged oocytes, with low MPF levels, nuclear envelope breakdown (NEBD) does not or only partially occurs (45). When differentiated cells are used for NT, a

higher level of reprogramming (facilitated by high levels of MPF) is required when compared with blastomeres of early-stage embryos. Although offspring have been produced from preactivated oocytes and simultaneous fusion and activation, it seems to be advantageous that the donor cells are synchronized prior to S-phase (6, 28, 36).

Inheritance of Mitochondrial DNA (mtDNA)

For several years animals cloned by NT were assumed to be transmitochondrial individuals (meaning they had the same nuclear genome but different mtDNA) and, therefore, were not truly genetically identical individuals. Recently, several studies investigated the inheritance of mtDNA in bovine embryos and offspring derived by NT using blastomeres. The mtDNA carries genes for oxidative phosphorylation and electron transfer, and mutations in the mtDNA are also associated with certain diseases in humans.

During normal fertilization, mixing of maternal and paternal mtDNA occurs; whereas the oocyte has about 2×10^5 molecules, the sperm only includes 50–75 mtDNA molecules. After a few cleavages, the sperm mtDNA is generally eliminated, and only maternal mtDNA is found in offspring. In contrast to fertilization, in early NT-embryos, mixing and coexistence of mtDNA derived from donor blastomere and oocyte are found (46). In early-stage embryos, mtDNA replication is absent (47), and a predominant contribution of maternal (oocyte) mtDNA is expected due to neutral segregation in early embryos.

Controversial results were published when mtDNA was investigated in offspring derived by NT. Steinborn *et al.* (46) found that 13% and 9% of mtDNA was derived from the donor blastomere in morulae and blastocysts, respectively. Takeda *et al.* (48) used PCR-SSCP (single strand confirmation polymorphism) with a sensitivity of 3%–4% and found that only 1 out of 27 analyzed cloned calves showed mtDNA heteroplasmy (including the donor cell and recipient oocyte mtDNA genotype). When Evans *et al.* (49) analyzed the inheritance of mtDNA of Dolly and 10 other sheep derived from fetal cells, mtDNA exclusively derived from the oocyte was detected. Steinborn *et al.* (50) used the more sensitive allele-specific TaqMan-PCR and found levels of bovine donor blastomere derived mtDNA ranging from 0.0004% to 18%. Higher levels are explained by neutral segregation in early embryos, whereas the reasons for lower levels could be the following: (i) replication advantage for one parental mtDNA due to sequence differences in the mitochondrial control region; (ii) different turnover rates; (iii) growth advantage for cells with particular mtDNA genotype; (iv) randomization of cells at the formation of the inner cell mass (ICM) or later stage embryos; or (v) active selection against blastomere mitochondria (50). Also the possibility of preferential replication of perinuclear mitochondrial genotypes during morula and blastocyst formation has been discussed (47). Hiendleder *et al.* (51) found different mtDNA-types in all but one of seven investigated clones using restriction fragment length polymorphism (RFLP) analysis of mtDNA, with an unexpectedly large number of different mtDNA-haplotypes (12 out of 29 individuals). In this study, extensive heteroplasmy was also found in all investigated individuals within two different clones, ranging from 21:79 to 57:43 (which is much higher than values expected from blastomere contribution) whereas in two other clones, only a mild heteroplasmy was detected.

Further studies are required to investigate the observed deviations from expected blastomere mtDNA contribution. Also, inheritance of mtDNA following NT with differentiated cells as donor karyoplasts should be analyzed.

Gene Expression

Previously, developmental reprogramming in livestock mainly was investigated indirectly as a result of structural changes of the nucleus as well as the ability to produce offspring, as reviewed by Prather (2, 52). In a recent study, gene expression patterns were analyzed in bovine blastocysts of different origins (53). Appropriate expression of genes is thought to be important for embyonic and fetal development; therefore, reprogramming events should alter expression patterns of the donor cells. DeSousa *et al.* (53) used differential display (DD-) PCR to analyze the conservation of mRNA expression in fibroblast cells in comparison with blastocysts of different origins. The DD-PCR profile from all blastocyst types (blastomere- and fibroblast-derived NT-, *in vivo*–produced blastocysts) was highly conserved (95%) when compared with *in vitro*–produced blastocysts. However, there was a significant difference in the banding in fibroblasts and MII-oocytes. It was concluded that the blastocyst pattern must have resulted from activation of the embryonic genome. Identification of putative mRNAs that are uniquely associated with specific embryo groups is currently in progress (53).

Analysis of Telomere Lengths

Telomeres, structures found at the ends of eukaryotic chromosomes, play an important role in the maintenance of chromosome structure and integrity. In humans, telomeres shorten during aging. Telomerase-mediated telomeric DNA synthesis occurs only in the germ line, not in somatic tissues (54, 55). In recent reports, the telomere length of sheep produced by NT using fetal or adult donor cells was investigated (56, 57). The mean terminal restriction fragment (TRF) length in control animals was found to shorten about 0.59 kilobases per year from 1 to 6 years of age, but surprisingly an increase in TRF length during the first year of life was observed. When the TRF lengths of NT-offspring were analyzed, they were comparable to the donor cells and age-matched control DNA, considering the decrease in TRF length during cell culture (0.157 kilobases per population doubling). These findings indicate that NT does not repair telomere erosion. The extent of TRF-shortening might be mitigated by minimizing the culture time for donor cells (56, 57). Further investigation on the effects of this telomere shortening on NT-offspring is necessary.

Future Aspects

The techniques of NT have improved dramatically over the last 5 years, but still are far from using a perfect protocol. Problems with fetal and neonatal mortality cannot be denied and have to be the subject of future research. The phenomenon of the large-calf-syndrome was already observed in offspring derived from *in vitro*–produced embryos, as reviewed by Walker *et al.* (58) and Young *et al.* (59), and efforts are necessary to overcome problems such as those by developing defined culture systems (60). However, the incidence of placental dysfunction (absence of deficiency of cotyledons), early and late fetal losses, neonatal abnormalities like edema, defects in the urogenital tract (hydronephrosis, testicular hypoplasia), the respiratory and cardiovascular systems, or lymphoid hypoplasia have increased significantly since somatic cells are used for NT (6, 16, 28, 61–63). These abnormalities seem to be an indication of incomplete reprogramming, and much effort will be put into investigating this issue. One important step to enlighten the exact mechanisms of reprogramming is analyzing differences in gene-expression patterns of early embryos derived *ex vivo, in vitro,* and by NT using different sources of donor cells as well as species-specific differences.

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