MINIREVIEW

Nuclear Transplantation as a Method for Cloning Embryos (43112)

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Historical Perspective

The concept of transferring nuclei from multicell stage embryos to enucleated oocytes was first proposed by Spemann in 1938 (1). Spemann was a developmental biologist whose concern at the time was that of nuclear equivalence. The idea of the basic composition of the nuclei changing during early embryo development gave rise to the theory that nuclei from different tissues were not equal. To answer this question, Spemann proposed an experiment to transfer nuclei from progressively more advanced embryos to enucleated, activated oocytes. The conclusion of the experiment would be determined when inequivalence was achieved and the transferred nucleus would not support development to a mature adult. Results from Spemann's experiment were first reported in 1952 by Briggs and King (2) in the amphibian *Rana pipiens*. They (2) showed that nuclei from preblastula stage embryos could promote development to the blastula stage. In addition, they alluded to the observation that nuclei from beyond the blastula stage had a lesser probability of promoting development after nuclear transfer.

The spin-off of such a project is the possibility of cloning. Since all of the nuclei of the early embryo are presumed to be identical (all containing the same complement of genetic material), subsequent transfer to enucleated, activated oocytes with development to term should result in genetically identical individuals (the reality of identical individuals will be discussed later). This procedure, in combination with serial nuclear transfer (i.e., growing the first nuclear transfer embryos to the donor cell stage and then repeating the procedures) could, theoretically, result in an unlimited number of identical individuals. The possibility of this in mammals became much more likely after the successes of such procedures were reported in 1986 by Willadsen (3) and Prather *et al.* (4) in sheep and cattle, respectively.

In this review I hope to provide information on the procedures for nuclear transfer, present the concept of nuclear remodeling and reprogramming, list the factors to consider regarding the similarity of nuclear transfer embryos, and what will be necessary for commercialization of such technology.

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Nuclear Transfer Procedures

The procedures for nuclear transfer are basically the same as those described by Briggs and King (2) for amphibians and adapted to mammals by McGrath and Solter (5). First, a group of recipient unfertilized oocytes is enucleated. This is accomplished after treating the oocytes with microfilament- and microtubule-inhibiting drugs such as cytochalasin B and colchicine. With the disruption of the cytoskeleton, the plasma membrane is much less likely to rupture. The recipient oocyte is held in place by aspiration with a micropipette (Fig. 1A) and another micropipette is punctured through the zona pellucida. The polar body and metaphase chromosomes are aspirated into the pipette. When the pipette is removed, the plasma membrane pinches off, forming two membrane-enclosed vesicles (one inside the pipette and one inside the zona pellucida). Originally, the aspiration was done blind, but visualization under ultraviolet light with a DNA stain permits successful enucleation every time (6). Next, the donor embryo is aspirated against the holding pipette and a single blastomere, or karyoplast, is aspirated into the transfer pipette (Fig. 1B). The transfer pipette is then inserted into the perivitelline space of the enucleated oocyte and the karyoplast is deposited (Fig. 1C). The two cells (donor karyoplast and recipient oocyte) are then allowed to regain their spherical shape (Fig. 1D). The resulting nuclear transfer embryo is placed

between two electrodes and an electrical pulse that causes a transient breakdown of the plasma membranes is applied. When the membranes reform, small channels are created that, due to their thermodynamic instability, enlarge forming a single cell (7, 8). The electrical pulse required for cell fusion also results in the activation of the oocyte, i.e., simulation of fertilization, thus setting into motion the events necessary for early development.

Nuclear Remodeling

After the successful transfer of a karyoplast to an enucleated, activated oocyte, the transferred nucleus undergoes remodeling such that it morphologically and metabolically resembles a pronucleus. In amphibians, this is observed by an increase in diameter of the transferred nucleus (9) and the disappearance of nucleoli within the transferred nucleus (10). In mammals, where the nucleoli only undergo ultrastructural modifications during early development and not the complete disappearance as in amphibians, the nucleoli revert to their early cleavage stage morphology (11). In addition, there is significant swelling of the transferred nuclei in mice (11, 12), rabbits (13), and pigs (14). There appears to be a 1½-hr window around the time of activation of the oocyte in which chromatin can be remodeled to be similar to pronuclei (11, 12). If the nuclei are transferred outside this window, then the nuclei either condense or fail to swell. In the rabbit, the timing criteria are even more confining since optimum activation rates are achieved only during a narrow window after ovulation (15). Interestingly, when zygotes, which are outside this window, are used as recipients development does not continue to term in cattle (16) or mice (17).

Theoretically, for optimum reprogramming, the nuclei should swell to a size similar to an endogenous pronucleus. Nuclear swelling is a result of the exchange of proteins between the cytoplasm and the nucleoplasm (18, 19). This exchange of proteins is thought to be the inducer of the swelling, not a consequence of nuclear swelling (20). Since there are components in the cytoplasm that affect nuclear volume, it is important to note that the early mammalian nuclear transfer experiments used recipient oocytes from which half of the cytoplasm was removed. This likely removed half of the components within the cytoplasm that are responsible for nuclear volume and subsequent reprogramming. A 2 × 2 factorial experiment was designed to evaluate the affect of cytoplasmic volume on nuclear swelling. Nuclear size was measured in embryos resulting from the transfer of an intact or half blastomere to either an intact or half oocyte. Neither the removal of half of the cytoplasm from the recipient oocyte, nor the transfer of half of a blastomere decreased the degree of swelling observed of the transferred nucleus (14). However, it is not yet known if these parameters are related to subsequent development.

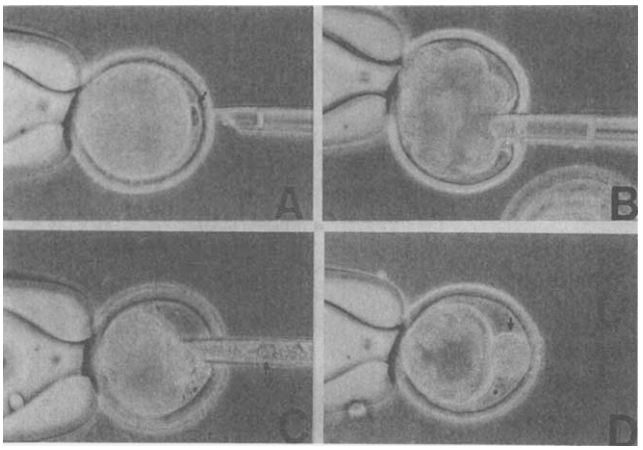

Figure 1. Nuclear transfer in cattle embryos. (A) Mature unfertilized oocyte (first polar body, a result of the completion of meiotic metaphase I, is denoted by the arrow). (B) Aspiration of blastomere containing donor nucleus. (C) Transfer of blastomere to enucleated oocyte. Arrow indicates the donor nucleus within the transfer pipette. (D) After transfer of donor blastomere, arrow indicates donor blastomere (original magnification ×400; diameter of the zona pellucida is about 150 μm). Reprinted with permission from Prather *et al.* (40).

In amphibians, after nuclear transfer, there is a shift of protein from the oocyte cytoplasm into the transferred nucleus and a shift of protein from the nucleus into the cytoplasm. This selective exchange of proteins between the nucleus and cytoplasm does not appear to be limited by the nuclear envelope, but by selective binding sites within the nucleus (21). In *R. pipiens*; nonhistone [^{3}H]tryptophan-containing proteins leave transferred endodermal nuclei, but [^{3}H]-lysine-containing proteins remain in the nucleus (18, 19). Synchronous with the release of labeled proteins is the acquisition of both acid and basic proteins by the nucleus (20).

In mammals, there is at least one suggestion of a similar exchange of nuclear lamins between transferred nuclei and cytoplasm. Nuclear lamins are a class of intermediate filaments that line the inner nuclear envelope and polymerize and depolymerize with the cell cycle. In the mouse, cow, and pig the A/C nuclear lamin epitope (antibody J9) becomes undetectable after the transition to zygotic control of development. In both the mouse and pig, if nuclei beyond the transition to zygotic control of development are transferred to enucleated activated oocytes, then the transferred nucleus acquires the lamin A/C epitope (Fig. 2) (22, 23). This is suggestive of acquisition of the lamin A/C from the cytoplasm of the oocyte. However, in the mouse, if nuclei are transferred to either an intact or enucleated zygote, then the transferred nuclei do not acquire the epitope (23). The inability of nuclear lamins to exchange between interphase cells has also been demonstrated in tissue culture (24). I hypothesize that the

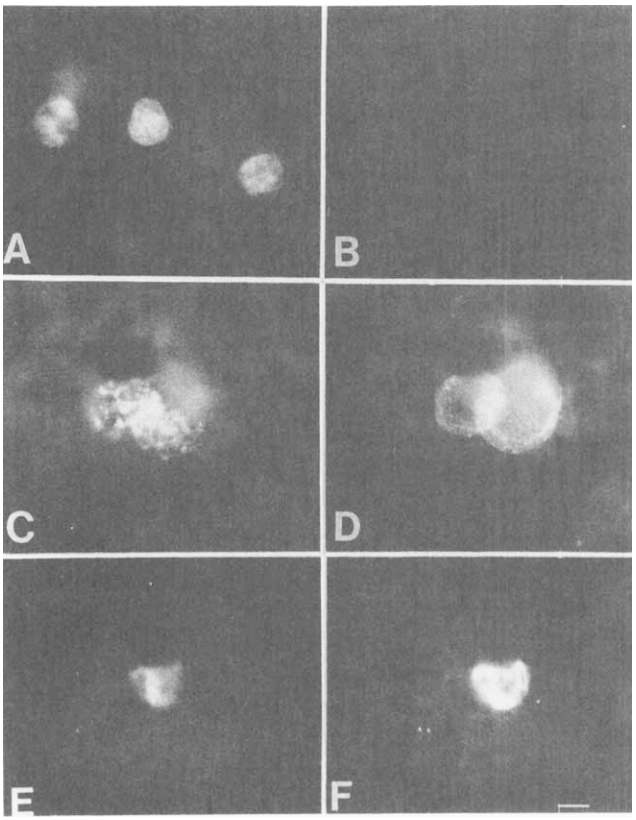

Figure 2. Nuclear lamin epitope after transfer of a 16-cell stage porcine nucleus to an activated, enucleated meiotic metaphase II arrested oocyte. DNA (Hoechst 33258) in cells from a 16-cell stage embryo (A), pronuclear stage egg (B), and activated egg after nuclear transfer (nucleus from 16-cell embryo in A; E), and corresponding lamin A/C (antibody J9) reactivity (B, D, and F, respectively). Note the absence of lamin A/C reactivity in the 16-cell stage blastomeres, whereas after transfer to an activated enucleated meiotic metaphase II oocyte and 2 hr of culture, the nucleus acquires the antigen. B, D, and E were photographed and developed under identical conditions. Cells in A, B, E, and F were mounted and stained on the same coverslip (bar = 10 μm). Reprinted with permission from Prather *et al.* (22).

inability to acquire the lamin A/C epitope is a result of the sequestering of cytoplasmic lamin A/C by the endogenous pronuclei. Thus, when an interphase cell is used as a recipient, the nuclei do not have access to the A/C lamin proteins within the cell. However, when a metaphase cell is the recipient, then the nuclear lamin proteins are dispersed in the cytoplasm and available to be incorporated into the nuclear envelope of the transferred nucleus.

Additional remodeling that is necessary before development can continue is a synchronization of the DNA synthetic phase of the donor and recipient cells. Nuclei that are in G_1 when transferred to an activated, enucleated oocyte need to complete DNA synthesis and progress to G_2 before the recipient cell attempts to divide. It is also important that nuclei in G_2 do not undergo DNA synthesis before mitosis, as the resulting embryo would be polyploid. The inability to complete DNA synthesis may be one of the most important factors affecting the resulting development. Most of the chromosomal abnormalities in amphibian nuclear transfer embryos can be traced to events that occur in the first cell cycle (25). The major problem in achieving synchrony between the donor and recipient cells is the fact that as a tissue differentiates the length of the cell cycle increases. These nuclei with long cell cycles, when transferred to an activated, enucleated oocyte, are required to complete DNA synthesis and prepare for another mitosis within an hour. Thus, relatively more differentiated nuclei are more likely to result in chromosomal abnormalities (26, 27), but any conclusions about differentiated cells versus less differentiated cells is confounded with the length of the cell cycle of the donor embryo.

Nuclear Reprogramming

The only indication, published to date, of reprogramming in mammals is the reprogramming of morphologic events. For example, if a cell from a 32-cell stage bovine embryo is transferred to an enucleated activated metaphase II oocyte and is not reprogrammed, the resulting embryo should attempt to form a blastocoele after one cleavage (at the 2-cell stage). If successful this would result in a trophoblastic vesicle that contains no inner cell mass, and hence no fetus. In addition, the absence of reprogramming would require that the nuclear transfer embryo be transferred to a surrogate mother than was synchronous with the donor 32-cell stage embryo. This would be true since domestic animals require close synchrony between the stage of the estrus cycle and stage of the developing embryo. If, instead, the 32-cell stage nucleus was reprogrammed to behave as a zygote, then the resulting embryo would begin compaction at the 32-cell stage, form a blastocoele at the 64-cell stage, and be transferred to a surrogate that ovulated synchronously with the nuclear transfer. Thus, a reprogrammed nucleus would retraverse the early cleavage stages followed by compaction and blastocoele formation, whereas control nonmanipulated embryos would attempt to continue their developmental sequence of events.

For a full appreciation of the biochemical reprogramming that presumably occurs after nuclear transfer in mammals, it should be understood that the early mammalian embryo does not begin producing RNA until a species-specific cell stage. Transcription can first be detected in the mouse embryo at the 2-cell stage (28), at the 4-cell stage in the pig (29) and rat (30), and at the 8- to 16-cell stage in the cow, rabbit, and sheep (31–33). Prior to this time the embryo relied upon RNA stored in the oocyte during oogenesis. Thus, for detectable biochemical reprogramming, nuclei need to be transferred at or after the transition to zygotic control of development. The most advanced nucleus to result in development to term by species is listed in Table I.

Table I. Relative Degree of Differentiation and Development after Nuclear Transfer to an Enucleated Activated Oocyte

Species	Stage of major transition to zygotic of development	Most advanced cell reported to result in term development
Pig (37)	4-Cell	4-Cell
Rabbit (13)	8- to 16-Cell	8-Cell
Cow (38–40)	8-Cell	32-Cell
Sheep (34)	8- to 16-Cell	Inner cell mass

The listing in Table I should not be interpreted to be maximum stage of development that will result in complete reprogramming, as few studies have attempted determine this parameter. The sheep should result in the most dramatic biochemical reprogramming since an inner cell-mass stage nucleus has resulted in term development (34).

In amphibians, the reprogramming is much more specifically described. Not only is there obvious morphologic reprogramming, similar to that described above, but there is also very specific biochemical reprogramming that occurs. Two of the most specific examples include the muscle-specific actin gene and the *5S*^{ooc} gene. Muscle-specific actin is produced by new transcription and translation in the differentiating myotome cells of the gastrula stage embryo. After the transfer of postgastrula stage myotome cell nuclei to enucleated activated oocytes, the production of muscle-specific actin RNA ceases. Muscle-specific actin RNA synthesis is not reinitiated until the embryo develops to the gastrula stage, and then only in the differentiating myotome cells (35). Similarly, the *5S*^{ooc} gene is translated and transcribed for a short period of time at the late blastula stage. Nuclei from beyond the blastula stage that are transferred to an enucleated activated oocyte transcribe the *5S*^{ooc} gene for a short period as the resulting embryo passes through the blastula stage (36). Thus, the biochemical reprogramming that occurs in amphibians is very precise.

Identical Individuals

Genetic Variation. Since all of the nuclei of early embryos are presumed to be identical, the offspring resulting from nuclear transfer should all have identical nuclear genetics. However, if prior to nuclear transfer some chromosomal rearrangement occurs in a single blastomere of the donor embryo, then this genetic defect would be passed to all subsequent embryos. Some possibilities of chromosomal rearrangement include DNA rearrangements (as happens in the normal differentiation of immunoglobulins) (41), gene amplification, translocations, and diminution.

A second factor to consider is the non-nuclear genetics. Cytoplasmic inheritance would be important when the source of the recipient oocytes are not known, such as when using *in vitro* matured oocytes derived from slaughterhouse animals. The main source of cytoplasmic inheritance is likely from the mitochondria. Even if the recipient eggs are derived from the same breed of livestock, it is disturbing that within a single maternal line of cattle the mitochondrial genome has been observed to change (42). Since the nucleus directs the synthesis of proteins used in the mitochondria (43), the interaction between the two may be very important.

There may be other organelles within the cytoplasm that have their own genome. One example may be the centriole. Recent evidence suggests that basal bodies have their own genome (44). Basal bodies are derived from the centriole, and thus the conclusion that centrioles may have their own DNA. It is very interesting to note that centrioles are absent in mammals during the first few cleavages after fertilization (45, 46). Since the presence or absence of the centrioles is related to the shape of the mitotic spindle (46), it is interesting to speculate what would happen to the centrioles and shape of the mitotic spindle after the transfer of an embryonic cell which had centrioles to an oocyte which has no centrioles!

Whether differences in cytoplasmic inheritance as described above are important in determining subsequent development remains to be determined. The possibility of such factors affecting development does exist and should be investigated.

Phenotypic Variation. The factors that affect phenotypic variation include environment and genetics. Since the possible genetic variation has already been discussed, this section will focus on environmental affects on development.

Phenotypic variation exists even in monozygotic twins. Since both cytoplasmic and nuclear genetics are presumed to be identical in monozygotic twins, all variation observed is due to environmental factors. Interestingly, cattle embryos split at the morula stage result in calves that are considered monozygotic twins, but they do not always look alike. Differential migration of the melanocytes results in twins that have the same basic color pattern, but are not identical. For example, one of the twins may have a red patch over its eye, while the other has a similar patch below its eye (47). Embryos that result from nuclear transfer are in different environments from the moment of transfer. Thus, the differences in epigenetic phenomena, *in vitro* environment, uterine environment, neonatal environment, and postnatal environment all have effects upon the resulting phenotype (48).

The first requirement for nuclear transplant embryos to be identical is identical genetics, and the second requirement is identical environments. It is likely that identical genetics (both cytoplasmic and nuclear) can

be evaluated and controlled; however, an identical environment is much more difficult to control.

Lest one is left with the impression that nuclear transfer embryos, or for that matter twins, are not phenotypically similar, it is necessary to end this with additional information. Above the impression has been given that nuclear transfer embryos or twins are not phenotypically identical. Using a strict definition this is true; however, for most researchers an increase in genetic uniformity would be very advantageous for reducing the number of animals needed for statistically valid results. Genetically similar animals not only have more similar growth characteristics than unrelated controls, but also have more similar behavior patterns (reviewed by Biggers (49)). Thus, from a research standpoint genetically uniform animals would be very useful. There would also be advantages for commercial livestock production as described below.

Commercialization

For the commercial livestock application of cloning by nuclear transfer, additional technologies must be developed. *In vitro* matured oocytes must be used as recipient oocytes, it must be possible to develop the resulting embryo *in vitro* to a stage that can be nonsurgically transferred to a recipient, and it must be possible to preserve the embryos by freezing. The pig is at a disadvantage as compared with the sheep and cow, since pig embryos are less tolerant to freeze preservation (50). In addition, only recently have methods been presented that result in normal *in vitro* development (51, 52) and offspring following nonsurgical embryo transfer (53).

An additional requirement for commercial application is a market. This could be developed after the uniformity of an individual clonal line is established. A company or producer could market a clonal line of embryos that would have a guaranteed birthing ease, neonatal growth rate, postweaning growth rate, behavioral characteristics, disease resistance, marbling characteristics, and size and shape of various meat cuts. As the phenotype of the clonal line is established and tested, these parameters would be defined as well as the ration and environment to achieve these parameters.

Future Direction

Future directions should focus mainly upon making the procedures more efficient, defining the impact of the genetic contributions, and defining the biochemical reprogramming that occurs to transferred nuclei. The current source of donor nuclei is from early stage embryos; however, a possible additional source of donor nuclei may be from embryonic stem cells. These cells have been isolated from mouse embryos, they can be cultured *in vitro* to large numbers, and, most important, they can be chimerized with the inner cell mass of blastocyst stage embryos. The embryonic stem cells that are incorporated into the fetus are stabile and can contribute to the germ line (reviewed by Prather *et al.* (54)). Since these are a relatively undifferentiated cell type, they are good candidates for a source of nuclei for the nuclear transfer. Since these cells can be transformed *in vitro*, lines of transgenic animals are a distinct possibility.

The clonal technology has a very bright future. Currently, the various steps in the procedure need not only to be refined, but in some cases defined. The procedures are inefficient, but they do work and it is now our opportunity to evaluate the parameters that affect development after nuclear transfer to make the overall procedure more efficient.

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