MINIREVIEW

Progress in Cloning Embryos from Domesticated Livestock (43989)

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The techniques necessary to successfully clone embryos by nuclear transfer have been developed in the last half of this century and applied to domestic animals in the last decade. The concept of transferring nuclei from early embryos to oocytes to coerce the nuclei to recapitulate the early developmental events is credited to Spemann (1). Spemann's interest was a question of nuclear equivalence or commitment. The techniques initially developed in amphibians (2) have also allowed the researcher to "clone" an embryo. Nowadays, individuals often clone genes or portions of a DNA molecule from a nucleus(i); in contrast, this nuclear transfer technique permits cloning of the entire genome and the creation of animals that have identical nuclear genomes.

Because many excellent reviews have been written on the subject of cloning by nuclear transfer (3, 4) and a similar review was presented in this journal earlier (5), the focus here will be an update of our understanding since that time and will highlight one of my interests: nuclear reprogramming. The review will begin with a general review of the current status of the techniques and a discussion of nuclear reprogramming and oocyte activation. This section will be followed by examples of some general problems of having embryos *in vitro*, and the review will finish with a discussion of the development of embryonic stem cells and the direction in which this leads.

General Procedures and Theory

The techniques for cloning by nuclear transfer were initially developed by Briggs and King in 1952 (2) for use in amphibians. In general, the procedures use unfertilized oocytes and donor nuclei. Chromosomes from unfertilized recipient oocytes are removed or destroyed with a micropipette or by exposure to ultraviolet irradiation, respectively. Next, the cells from a donor embryo or cell line are dispersed, and individual cells are aspirated into a micropipette. The pipette has a diameter small enough to rupture the plasma membrane of the donor cell. The cell lysate is then injected deep into the cytoplasm of the oocyte (6). The penetration also activates the oocyte (for this review the term activation will refer to the molecular events associated with the resumption of meiosis). If the biochemical status of the cytoplasm and nucleus can reach a compromise, development will continue.

Due to the small size of mammalian cells, these techniques were modified by a number individuals over a number of years (7–9). Generally, these modified techniques involve treating the cells with drugs that depolymerize the microtubules and microfilaments, which provides an elasticity to the plasma membrane such that it does not rupture. Micropipettes can then be used to pierce the zona pellucida, aspirate, or bleb-off the metaphase chromosomes from the recipient oocyte. Donor cells, or portions thereof (karyoplasts), are placed adjacent to the recipient oocyte and then fused to the recipient oocyte. This cell-to-cell fusion is mediated either electrically, virally or chemically. Upon fusion, the transferred chromosomes may or may not condense, depending upon such factors as the level of maturation-promoting factor (MPF) in the oocyte. Then a two-way exchange of protein between the cytoplasm and the chromatin occurs and the developmental status of the nucleus is modified. Theoretically, for the greatest developmental potential, the

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nucleus should now behave as though it were an early zygotic pronucleus. This description will serve as a definition of complete reprogramming. Successful application of such a technique to mammals was first described in 1986 by Willadsen (10) and Prather *et al.* (11). Unlike many techniques, which were first developed in mice, these were successfully applied to mice only after they had been sufficiently perfected in the embryos of domesticated animals (12). While in some cases it is difficult to separate oocyte activation, nuclear remodeling, and nuclear reprogramming, an attempt will be made below.

Nuclear Remodeling Results in Nuclear Reprogramming

In the previous reviews (5, 13, 14, 15), the subjects of nuclear remodeling and nuclear reprogramming were discussed separately; however, since the former likely leads to the latter, here they will be discussed together. This portion of the review will start with modifications to the nucleus when the cell-to-cell fusion is synchronous with activation of the oocyte and the oocyte is fresh with high levels of active MPF.

The first descriptions of remodeling of the nucleus and reprogramming of transcription of specific genes after nuclear transfer was in amphibians (16–19). These studies illustrate the precision by which the cytoplasm of the unfertilized oocyte can regulate nuclear morphology and transcription. These studies evaluating genes such as the $5S^{ooc}$ and muscle-specific actin gene served as the basis for many of the mammalian studies listed below.

In contrast to the methods in amphibia, nuclear transfer in mammals is facilitated by induced cell-to-cell fusion. This cell-to-cell fusion brings the cytoplasmic components of the two cells together: nucleus and cytoplasm from the donor cell and cytoplasm from the recipient oocyte.

Nuclear reprogramming can be evaluated based on the timing of morphological events. For example: in rabbits, blastocoele formation follows the 8-cell stage by 72 hr, but after nuclear transfer with a blastomere from an 8-cell stage embryo it is 120 hr until blastocoele formation. This is similar to the time it takes for a zygote to develop a blastocoele (20). Thus, morphologically it appears that the nuclei are reprogrammed. This is further illustrated by the ability of nuclei from inner cell mass cells to direct term development after nuclear transfer (21, 22). Development would not continue if the embryos derived from the transfer of an inner cell mass (ICM) cell were transferred to a surrogate dam that was developmentally equivalent to the donor cell. This inability to develop would arise because a 1-cell stage embryo would be transferred to the uterus of an animal 7 days after the onset of estrus. This developmental asynchrony is lethal. Below is a discussion of the molecular events that transpire after nuclear transfer that may explain this morphological reprogramming.

At cell-to-cell fusion of the donor cell with the recipient cytoplasm the nuclear envelope transiently breaks down. An epitope recognized by a monoclonal antibody (I1) is released into the cytoplasm or masked (23). The nucleus begins to swell to the size of a pronucleus (24). Coincident with the swelling is an acquisition by the nucleus of nuclear lamins A/C (epitopes) that are specific to the very early cleavage stages (25–27). This extensive remodeling presumably results in a modification of the developmentally regulated pattern of RNA synthesis. The early embryo of many species has a unique period of very little RNA synthesis. In the mouse, pig, human, cow, and sheep, the major onset of RNA synthesis begins at a species-specific cell stage: 2- (28), 4- (29), 4- (30), 8- (31), and 8-cell stage (32), respectively. Prior to this stage the embryo relies primarily upon maternal transcripts.

A specific morphological indicator of active rRNA synthesis is the morphology of the nucleolus. When viewed with electron microscopy, inactive nucleoli have a very tight, compact, and agranular appearance, while nucleoli that are actively synthesizing rRNA have a granular and reticulated appearance. Nucleoli from pig zygotes, parthenogenetically activated oocytes as well as from 8-cell stage nuclei (post-onset of rRNA synthesis) that are transferred to a recipient oocyte are tight, compact, and agranular (33). Similar observations were reported (34) using rabbit embryos. These modifications of the nucleoli suggest that rRNA synthesis is shut off after nuclear transfer (however, see Relevance of Cell Cycle Synchrony below).

An indirect indicator of mRNA synthesis is mRNA processing. This can be evaluated by using antibodies that recognize proteins involved in the splicing of pre-mRNA prior to exit from the nucleus. The monoclonal antibody Y12 recognizes the B/D protein of the core small nuclear ribonuclear proteins (snRNPs). In the pig, these proteins are absent prior to the 4-cell stage. After the transfer of a 16-cell stage nucleus to an oocyte the antibody no longer recognizes an epitope (35). The absence of the snRNP proteins suggests that mRNA processing is no longer occurring and thus there may no longer be any mRNA synthesis.

Downstream from the regulation of mRNA synthesis is protein synthesis. This has been evaluated in at least two studies assessing the production of stage-specific proteins. While both studies concluded that the majority of proteins produced were translated from oocyte-derived mRNA, there still were some proteins produced by mRNA derived from the transferred blastomere (bovine embryos [36]; pig embryos [37]). The detection of these late stage–specific proteins even in the presence of α-amanitin confirms that they are a

result of the carry-over of mRNA in the cytoplasm of the transferred blastomere. It should be noted that the bovine study used aged oocytes as recipients (see the significance of this below). To overcome this potential problem of transferring significant amount of RNA and protein with the karyoplast, Collas and Barnes (38) modified the procedures to be more similar to the amphibian procedure; that is, they injected just the nucleus into the recipient oocyte, rather than use cell-to-cell fusion to facilitate the nuclear transfer. Unfortunately, they observed no benefit to using the modification.

An additional indicator of reprogramming in mammals is provided by Van Stekelenburg-Hamers (39). In the bovine embryo an antigen defined by the TEC-03 monoclonal antibody appears at the 32-cell stage. Morulae stage donors were used for nuclear transfer and the resulting embryos were analyzed at various developmental stages. The TEC-03 antigen did not appear in the nuclear transfer embryos until compaction (39). This suggests a tight developmental regulation of the transcriptional (translational) control of this gene.

In contrast to the reprogramming described above, Pinto-Correai *et al.* (40) describe end points in rabbit nuclear transfer and suggest that the nuclear modifications that occur upon nuclear transfer are not consistent with full reprogramming. An antibody to fibrillarin, a nucleolar-associated protein, does not recognize an antigen in the early cleavage stage embryo; only beyond the onset of significant amounts of transcription. After nuclear transfer and development to the 8- to 16-cell stage, some, but not all, of the nuclei from the resulting embryo cross-react with the antibody. Phosphorylation status of the mitotic spindle was also evaluated in nuclear transfer embryos; the pattern of expression was not identical to normal embryos. Thus, nuclear modifications that result in a recapitulation of early developmental events do not always occur.

The above studies suggest that as measured by some end points nuclear reprogramming occurs, but not when other end points are evaluated. In addition, as we shall see below, the degree of nuclear modifications will also depend upon the techniques and timing of some of the procedures of nuclear transfer.

Relevance of Cell Cycle Synchrony

The transfer of a donor nucleus into the cytoplasm of a meiotic metaphase II oocyte often results in incorrect DNA synthesis in the resulting embryo. Without cell cycle synchronization the donor cell is likely in G_1, S or G_2 of interphase while the oocyte is arrested in meiotic metaphase II. The levels of MPF are high in a cell in metaphase and low in an interphase cell. When the interphase nucleus is exposed to high levels of active MPF, the nuclear envelope begins to break down and the chromosomes condense. This is generally the case when a fresh oocyte is used as a recipient for the nuclear transfer. The integrity of the nuclear envelope is very important for the regulation of DNA synthesis in the donor nucleus (41). If the nuclear envelope loses its integrity, a DNA synthesis licensing factor will be allowed to enter and additional DNA replication should occur. In rabbits, sheep, and pigs, additional DNA synthesis has been shown to occur after the transfer of nuclei in G_2 to fresh oocytes (40, 42, 43). Furthermore, nuclei in G_2 not only undergo additional DNA synthesis, but complete a full round of DNA synthesis and become tetraploid (42). Such outcomes can have a catastrophic effect on the development of the resulting embryo.

To circumvent the problems associated with DNA synthesis and eventual polyploidy, many investigators have begun using either aged oocytes or pre-activated oocytes as recipients. Both of these techniques accomplish the same goal: reducing active MPF levels. If active MPF is lowered by parthenogenetic activation or by senescence, then when a nucleus is transferred to the cytoplasm of the oocyte it will be less likely to undergo nuclear envelope breakdown. Thus, there will be less of a chance that the DNA synthesis licensing factor will be able to enter. Generally, the rate of development is enhanced when either aged or pre-activated oocytes are used as recipients and the donor nuclei are derived from early cleavage stage embryos (12, 44, 45). The lack of nuclear envelope breakdown, however, creates other problems with the remodeling of the transferred nucleus, specifically with the nucleoli and nuclear lamins as discussed below.

Earlier in this review, it was suggested that the nucleoli are completely remodeled to be similar to those in a zygotic pronucleus. While complete remodeling appears to be true for nuclear transfer that is a result of activation synchronous with nuclear transfer it may not be true when the oocytes are aged and/or pre-activated (46). Also, if advanced nuclei are transferred into either nucleated or enucleated zygotes, the transferred nucleus does not acquire the A/C type of nuclear lamins (25, 26). Additional studies in the mouse suggest that there is a window within which remodeling is optimal (47, 48). Thus, for the early-stage nuclei used in our current nuclear transfer systems fidelity of DNA synthesis is much more important than complete remodeling and reprogramming of the nucleus. As investigators advance the differentiative state of the donor nucleus remodeling will likely become just as important as the integrity of DNA synthesis.

Another review could be easily written describing the procedures for artificial activation of the oocyte. The goal is to stimulate the recipient oocyte such that it behaves as though it were fertilized. Such an acti-

vation stimulus would result in all of the early and late events that occur normally at fertilization. Excellent reviews of the mechanisms involved at fertilization are provided by Whitaker and Swann (49) and Swann and Ozil (50).

Potential Problems Associated With the Development of Nuclear Transfer Embryos

One intriguing problem has arisen as a result of the production of calves after nuclear transfer, namely large calves at birth. This has been reported by a number of individuals (51–54), although in retrospect large calves and lambs are also reported as a result of being *in vitro* during a portion of the first week of development (55–57) and thus are not a symptom of nuclear transfer. The observation of increased birth weight several months after a short-term exposure to *in vitro* conditions presents a significant challenge to biologists. Some environmental (*in vitro*) factors are having a significant effect on the transcriptional/translational machinery of the embryo.

Multiple Copies

If the final goal is to create large numbers of genetically identical animals, then a large number of nuclei must be available (Fig. 1). This can be accomplished by either (i) serial nuclear transfer or (ii) the establishment of stem cells. Serial nuclear transfer would entail performing a first generation of nuclear transfer with one donor embryo. Subsequent nuclear transfers would be performed using donor nuclei derived from the embryos that resulted from the first generation of nuclear transfer. Such possibilities were briefly reported by Willadsen (58) and by Westhusin *et al.* (59). Two additional reports provide more details on the possibilities, but one (60) concludes that development is reduced after multiple generations of nuclear transfer, while the other does not see such a drop in development (61). Third-generation calves have been produced (60).

The second possibility is that of the production of embryonic stem (ES) cells that could be used as donor nuclei. The terms "ES cell" and "totipotent" have been used very loosely in the literature (62). Dr. Marie DiBerardino has provided some very specific definitions to which I would like to refer (Table I, personal communication). While there have been many claims of "putative" or "ES-like" cells, four groups claim to have produced domestic animal offspring (63–68); examples of nuclear transfer using the embryonically derived cells as donors and examples by chimera production. The development and establishment of ES cells for domestic animals is in progress by a number of laboratories, and an adequate review of the literature would be a significant undertaking. The development of ES cells for livestock would be a boon to the production of transgenic animals by incorporating site-specific homologous recombination. Addition or deletion of genes would have immediate impact. To date there are no reports of totipotent cell lines for domestic animals as defined in Table I. Once offspring are reported from the above or other examples, then, and only then, can the cell lines be defined as totipotent.

Future Developments

While it is difficult to predict future developments, it is apparent that progress in the development of ES cell lines from domestic animals will lead the way. Potentially, many of the techniques initially developed in mice, cattle, sheep, and pigs will have significant agricultural and biomedical applications. This author maintains that the best development will result from

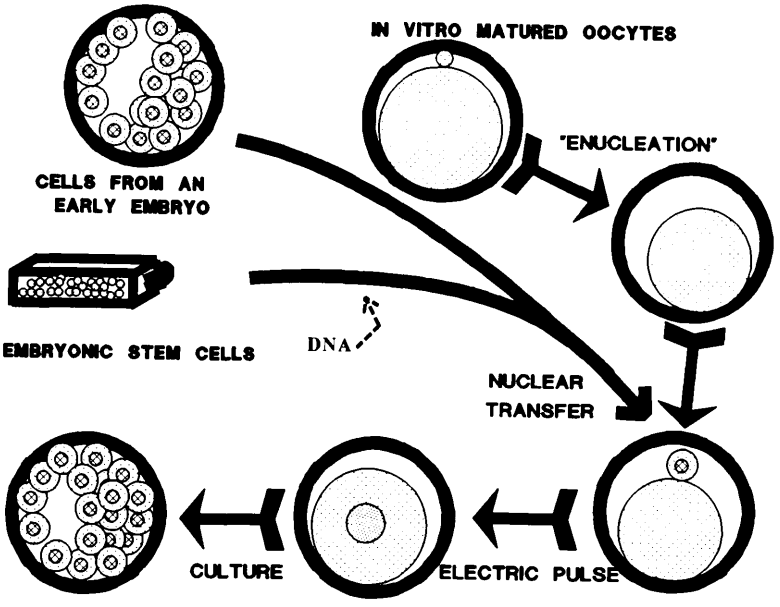

Figure 1. Current goals toward defining ES cells for livestock. Cells from either an early embryo or tissue culture are transferred to oocytes from which the metaphase II chromosomes were removed. The cell-to-cell induced fusion and activation are synchronous. Subsequently the embryos would develop to the blastocyst stage and be suitable for transfer to a surrogate dam or recloning.

Table I. Definitions of Totipotency, Pluripotency, Multipotency, and ES Cells

Totipotency	The nucleus (genome) can direct the formation of fertile organisms that participate in the production of normal progeny, i.e., TOTAL.
Pluripotency and multipotency	(The dictionary defines these terms equally, i.e., as more than one; many.) The nucleus (genome) can direct the formation of infertile adults and anything less, down to post-neurula embryos with primitive organ systems. Obviously, this denotes a wide spectrum of potentialities and requires description of the extent of development
ES Cells	Only embryonically derived cells transferred to blastocysts that become normal gametes and participate in the formation of normal progeny are totipotent. Anything less is multipotent.

nuclear transfer embryos when nuclei in G_1 are used as donors and the nuclear transfer is performed synchronous with the activation stimuli. These procedures may entail removing many of the proteins from the transferred nucleus such that they do not participate in the differentiation process. After the initial breakthroughs in the late 1980s, significant developments have occurred and have made the cloning process more efficient. It is anticipated that more-efficient procedures will be developed and transgenic cloned individuals will be available in the foreseeable future.

I would like to dedicate this review to my father (Elvin K. Prather, DVM; 1923–1995) who passed away during the preparation of this manuscript. He taught me a work ethic and responsibility, and with his great interest in the control of reproduction in farm animals inspired me to study the development of early embryos.

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