Nuclear reprogramming and stem cell creation

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The transplantation of a somatic cell nucleus to an enucleated egg results in a major reprogramming of gene expression and switch in cell fate. We review the efficiency of nuclear reprogramming by nuclear transfer. The serial transplantation of nuclei from defective first-transfer embryos and the grafting of cells from such embryos to normal host embryos greatly increases the proportion of nuclei that can be seen to have been reprogrammed. We discuss possible reasons for the early failure of most nuclear transfers from differentiated cells and describe the potential value of growing oocytes, rather than unfertilized eggs, as a source of nuclear reprogramming molecules and for the eventual identification of these molecules. Nuclear transfer provides a possible route for the creation of stem cells from adult somatic cells.

Nuclear reprogramming is a term used to describe changes in gene activity that are induced experimentally by introducing nuclei into a new cytoplasmic environment. When nuclei from partially or fully differentiated cells are transplanted to enucleated eggs of Amphibia or mammals in second meiotic metaphase, blastula or blastocyst embryos can be obtained, and these can form a wide range of tissues and cell types. The multipotency of these nuclear transplant embryos means that they share some characteristics of early stem cells. Indeed those nuclear transplant embryos that undergo growth (after feeding in Amphibia, and after implantation in mammals) to become adults must contain stem cells for renewing tissues. To this extent nuclear transplantation can achieve the creation of stem cells or stem-like cells from somatic cells of very restricted developmental potential.

In contrast, the identification and isolation of natural stem cells from normal tissues is a difficult process and has not yet been successful for most vertebrate tissues (1). Furthermore the differentiated state of cells is very stable, and it is hard to induce cells that have embarked on one pathway of differentiation to switch to another. Therefore nuclear transplantation is at present the most reliable way of deriving multipotential cells from a tissue of any kind. For this reason, nuclear reprogramming is of interest as a means of creating a range of replacement cells of the same genetic type as the donor source, thereby avoiding the need for immunosuppression as is required with most genetically nonhomologous grafts or implants. The aim of this article is to summarize the efficiency of nuclear reprogramming by nuclear transfer and hence to comment on its potential as a source of stem cells.

Nuclear Transplant Embryo Development

How efficiently and effectively can somatic cell nuclei be reprogrammed to an embryonic state? In the case of Amphibia, these questions have been addressed by the early nuclear transfer experiments carried out with *Rana* and *Xenopus*. For reasons that are still not clear, nuclear transfer success declines rapidly with increasing donor age in *Rana* (2), and nuclear transplant development is much more successful in *Xenopus*. In the endoderm lineage, which has been analyzed in greatest detail (3, 4), it is now known that cells express the endoderm-lineage marker endodermin (5) from the late neurula stage onward. Well before this stage, endoderm cells are specified [i.e., they form only endoderm derivatives as explants (6, 7)] and are determined [i.e., they form only endoderm derivatives if transplanted to ectopic sites (8)]. Yet nuclei transplanted from more advanced-stage endoderm cells into unfertilized eggs form functional muscle and nerve cells in ≈20% of all cases (Table 1). Even at the heartbeat stage, when the endoderm has begun regional differentiation (9, 10), 13% of nuclear transfers from the endoderm can form functional muscle and nerve cells (Table 1). Likewise, the region of the mesoderm destined to form muscle expresses the myogenic genes *Myf5* and *MyoD* by the late gastrula stage (11, 12), and cells from this region continue to express muscle markers even when these cells are transplanted singly to the endoderm (13). Yet the nuclei of myogenic cells generate a functional nervous system in >5% of nuclear transplants. These results cannot be attributed to escaped germ cells or other rare cell types residing in the endoderm or muscle, because the success rate is too high.

Using nuclei from differentiated or adult cells, the success rate of nuclear transfers is much lower than from larval or embryo cells (Table 1). In the case of adult *Xenopus* tissues, the cells that grow out from explants are of fibroblastic morphology and often do not express differentiation markers. However, for the eventual purposes of cell replacement, the accessibility of adult tissue, as in the case of skin or blood, is much more important than the definition of cell type. In *Xenopus* experiments, cells from adult skin have been obtained by outgrowth in culture and retain expression of an epidermal keratin marker. Nuclei from these cells give nuclear transfer results with the same efficiency as cells from other adult organs (14). About 1% of eggs receiving transplanted nuclei from cells of adult skin reach the muscular response stage and therefore have functional muscle and nerve cells (Table 1). Work with mammals has given comparable results (15), although relatively few experiments have been done in which nuclei of defined cell types have been transplanted to enucleated eggs.

The overall conclusion from these direct nuclear transfer experiments is that a substantial proportion of nuclei from specified or determined embryonic cells expressing differentiation markers undergo major reprogramming when transplanted to enucleated eggs.

Serial Nuclear Transfers and Grafts

The question arises as to whether the low percentages of nuclear transfer success shown in Table 1 for nonendoderm nuclei mean that only a minority of cells in a tissue have the capacity to be reprogrammed or that this capacity exists but has not been demonstrated for technical or other reasons. In Amphibia, a

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Table 1. First nuclear transfers using determined and differentiated cells

Species	Donor tissue	Donor stage	% of total nuclear transfers reaching muscular response stage	Ref.
Xenopus laevis	Endoderm	Muscular response stages 23–26	17–24	3
X. laevis	Endoderm	Heart beat stage 36	9–13	3
X. laevis	Muscle	Muscular response stage 26	2	42
X. laevis	Intestinal epithelium	Early feeding tadpole stage 47	1–3	4
X. laevis	Skin outgrowth	Adult	1.3	14
Cow	Inner cell mass cells	Embryonic	0.6	43
Primate	4–32 cell embryos	Embryonic	1.4	44
Sheep	Mammary	Adult	0.4	45
Mouse	Cumulus	Adult	2.8	46

substantial proportion of nuclear transplant embryos cleave abnormally and die as partial blastulae within 24 h of nuclear transfer. In the case of nuclei from differentiated or adult cells, partial cleavage results from one-quarter to one-third of all nuclear transfers and is far more frequent than complete cleavage (4). However, it has been found that the normal-appearing cells of partial blastulae can be used as donors for a second, serial set of nuclear transfers to more enucleated eggs, an experimental design first used by King and Briggs in 1956 (16). When this is done with partial blastulae derived from intestinal epithelium cells of *Xenopus*, it is found that many of the serial nuclear transplant embryos develop remarkably well, sometimes reaching the normal tadpole stage (4). These serially derived tadpoles reflect the developmental potential of the originally transplanted intestinal epithelium cell nucleus, even though this potential was not revealed by the first nuclear transfers.

The best explanation for this apparent improvement in nuclear transfer success is the following. It is believed that nuclei from slow-dividing somatic cells cannot complete their chromosome replication in time for the first cleavage of a recipient egg, which always takes place according to the time schedule of the egg, for example at 1.5 h for the first cleavage in *Xenopus*. Somatic cells take some 6 h to complete chromosome replication. When their transplanted nuclei are forced into early mitosis with incompletely replicated chromosomes, they are likely to suffer chromosomal damage (17, 18) and generate chromosomally defective embryos, which cannot survive. However, it sometimes happens that a transplanted nucleus fails to undergo chromosome segregation when the recipient egg divides into two cells and the whole replicating transplanted nucleus moves into one of the first two blastomeres. It then has a second chance to complete the replication of its chromosomes before undergoing mitosis when the egg goes from a two- to four-cell stage. As a result, partial blastulae are obtained because one of the first two blastomeres undergoes cleavage with the transplanted nucleus, while the other blastomere, having no nucleus, dies. These partial blastulae are more likely to contain nuclei with completely replicated chromosome sets than are those nuclear transplant embryos that undergo chromosome segregation at the first mitosis.

The result of carrying out serial nuclear transplantation shows that a substantial proportion of the partially cleaved blastulae contain nuclei with wide developmental potential. When this is taken into account, the proportion of original intestinal epithelium cells whose nuclei can promote muscle and nerve differentiation rises to 20% (Table 2 and ref. 4).

Using a similar line of thinking, it was found that the normal-appearing cells of partial blastulae can be grafted to host embryos and then reveal a wide range of developmental potential. Using GFP-marked donor nuclei, it has been calculated that at least 16% of differentiated larval intestinal epithelium cells contain nuclei capable of completely different pathways of differentiation (19). In conclusion, the use of serial nuclear transfer and grafts shows that a much higher proportion of differentiated cells contain nuclei that undergo a major reprogramming by egg cytoplasm than the 1–3% apparent when considering only first nuclear transfers.

Possible Reasons for Failures

After the transfer of nuclei from differentiated cells to enucleated eggs, whether in Amphibia or mammals, only a few of the nuclear transplants develop into adult animals. There are several possibilities that could account for this low success rate, and the primary reason for developmental failure may differ between Amphibia and mammals. In Amphibia, the majority of eggs receiving transplanted nuclei from differentiated or adult cells undergo only a few irregular cleavages or fail to divide at all. Thus, the primary loss in amphibian somatic cell nuclear transfer is during the very earliest cleavages. In mammals, the developmental loss tends to be greater after the initial cleavages. In primates, development to the early eight-cell stage has been shown to be similar in somatic and embryonic cell nuclear transfers and after intra-cytoplasmic sperm injection (ICSI) (between 80% and 90% in every case). However, the development of cloned embryos to the later blastocyst stage was markedly different; only 1% of somatic cell nuclear transfers reach the blastocyst stage, whereas 34% of embryonic nuclear transfers and 46% of ICSI controls reach this stage (20).

Table 2. The combination of first and serial nuclear transfer results

Species	Donor tissue	Donor stage	% of total nuclear transfers reaching muscular response stage	Ref.
X. laevis	Intestinal epithelium	Early feeding tadpole stage 47	20	4
X. laevis	Skin outgrowth	Adult	11–12	14
X. laevis	Kidney, lung, heart	Adult	13	47

These variations in the stage of developmental loss probably reflect different problems encountered after somatic cell nuclear transfer in Amphibia and mammals. In Amphibia the primary problem may be the previously mentioned difficulty that somatic cell nuclei have in completing chromosome replication within the very limited time (only 90 min in *Xenopus*) before eggs undergo cleavage (21). In mammals there are 20 h for the mouse, or longer for humans, between the time of fertilization and the first cleavage division. This makes it unlikely that incomplete chromosome replication is a problem in mammals. However, mammals have imprinted genes (22) that Amphibia do not (23), and it has been suggested that the developmental loss observed after mammalian somatic cell nuclear transfer could be caused by the incomplete reprogramming of various imprinted genes (as well as *Oct4*) (24). Another recent suggestion for mammalian developmental failure is that enucleation removes maternal spindle proteins required to maintain ploidy through the initial cleavages (25).

Although amphibian cloned embryos tend to suffer from chromosomal damage caused by incomplete chromosome replication and mammalian cloned embryos tend not to express imprinted genes correctly, there is also a range of other factors that may affect both amphibian and mammalian nuclear transplants. Quantitatively incomplete/incorrect reprogramming of gene expression has been found in both amphibian (19) and mammalian (26) cloned embryos, and it has been suggested that this may affect development (27). Also, it has been suggested that the stage of the donor cell cycle may be critical to avoid aneuploidy through re-replication of the donor genome (28). Another idea concerns the centriole; this is normally introduced with the sperm at fertilization and the eggs of most animals do not contain their own centriole. Perhaps most differentiated cells that are no longer required to divide do not contain a fully functional centriole. It is possible that technical factors may also be important. Failure to rupture a donor cell would certainly account for the total lack of cleavage. On the other hand, the position in an egg at which a transplanted nucleus is deposited is presumed not to matter on the grounds that sperm, entering from the surface of the egg, always find their way to the correct central position of the egg.

In conclusion, there is no definitive explanation for the high frequency with which nuclei transplanted from differentiated or adult cells fail to elicit any cleavage or development of recipient eggs. It is probably a combination of aneuploidy, genetic damage, and incomplete epigenetic reprogramming. For the purposes of cell replacement, this is a serious problem only if the supply of recipient eggs is strictly limited, as might be the case for humans.

The Cell Differentiation Potential of an Imperfect Genome

A fundamental idea behind the original vertebrate nuclear transfer experiments was that a complete genome is required for an egg to develop to a normal adult. This is very likely to be true; in fact, the completeness of a genome might be defined in this way. But this does not at all mean that each individual pathway of cell differentiation also depends on a complete genome. Nuclei that lack essential genes for one developmental pathway may nevertheless be able to proceed along other differentiation routes. Nuclear transfer experiments in both Amphibia and mammals have given support to this idea. In *Xenopus* it has been found that many of the partially cleaved nuclear transplant embryos (that are developmentally defective) have quantitatively aberrant expression of early zygotic genes (19). Despite this, healthy cells from such embryos, genetically marked by GFP, were able, after grafting to host embryos, to participate in the differentiation and growth of normal muscle, notochord, epidermal, and other cells (19). In mice, it was discovered that whereas only 2% of nuclear transplant embryos could develop into adult animals, 9% of nuclear transplant embryos could produce embryonic stem cell lines (29). This finding suggests that defective cloned mammalian embryos, which are incapable of developing into an entire mouse, can still produce useful stem cell lines. An interesting future direction of research will be to investigate the differentiation capacity of transplanted nuclei carrying known chromosomal or gene deficiencies. Perhaps genetically deficient cells may be entirely suitable for somatic cell replacement.

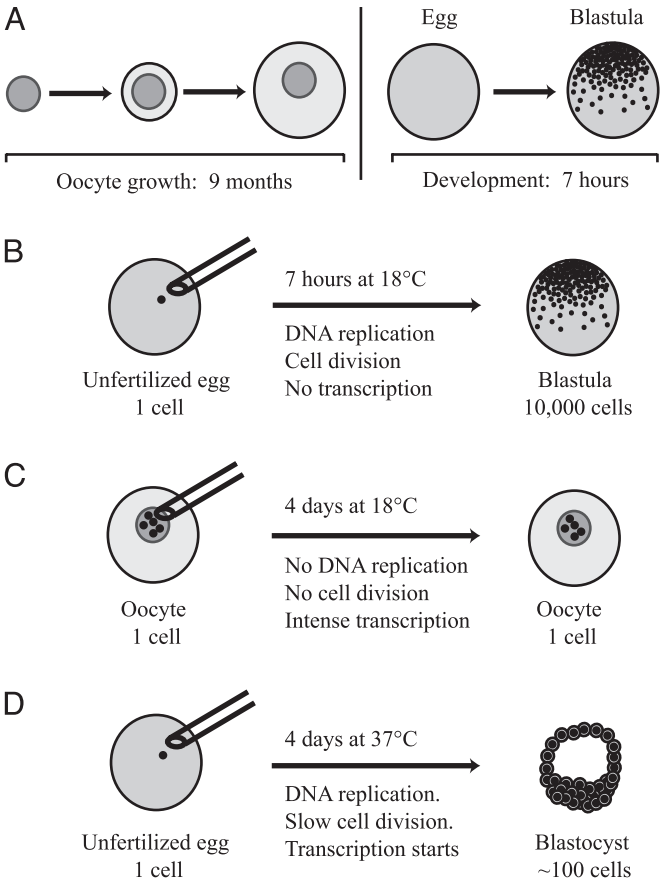

Fig. 1. Diagrams to show the time scale of nuclear reprogramming in *Xenopus* and the mouse. (*A*) Oogenesis and early development of *Xenopus*. (*B*) In *Xenopus* egg nuclear transfers, reprogrammed gene expression is seen at the late blastula stage after at least 12 cell division cycles. (*C*) In *Xenopus* oocyte nuclear transfers, reprogrammed gene expression is seen in the complete absence of DNA replication and cell division. (*D*) In mouse nuclear transfer to eggs, reprogrammed gene expression has been seen at the blastocyst stage.

Reprogramming Without Replication

The great majority of nuclear transfer experiments in both mammals and Amphibia have been carried out with eggs in second meiotic metaphase as recipient cells. In all of these cases, the first response of an enucleated egg to a transplanted nucleus is the induction of DNA synthesis and cell division (Fig. 1). In Amphibia, new transcription, and hence evidence of nuclear reprogramming, commences after 12 cell cycles at the midblastula transition, 5 h after nuclear injection. In mice, new transcription starts at the early two-cell stage, ≈24 h after nuclear injection, and new transcription starts later in other mammalian species. This situation raises the possibility that nuclear reprogramming requires DNA replication and/or cell division to reset an epigenetic program, a suggestion made by Tada and Tada (30).

To test this possibility, we have transplanted somatic cell nuclei into nondividing amphibian oocytes in the prophase of first meiosis. These cells cannot be fertilized, are inactive in DNA synthesis, but are intensely active in transcription (Fig. 1). Their active genes are maximally packed with RNA polymerase, as seen in the spectacular transcription complexes of Miller (31). We transplanted between 10 and 100 somatic cell nuclei to a single oocyte to obtain a detectable response, and the results were assessed by 2D protein analysis. The injected nuclei underwent a large increase in volume and dispersion of their chromatin over the several days for which injected oocytes can be cultured. Protein analysis showed that new proteins were synthesized by oocytes injected with mammalian nuclei from cultured HeLa cells, although the identity of the proteins was not known (32, 33). In the case of nuclei from one species of amphibian (*Pleurodeles*) transplanted to oocytes of *Xenopus*, some new proteins were synthesized with the size and charge properties of oocyte expressed genes (34). In other experiments, oocyte-specific 5S genes were activated when *Xenopus* somatic cell nuclei with inactive oocyte-type 5S genes were injected into oocytes (35) and liver-specific enzymes were inhibited in experiments with two species of Ambystoma (36).

Very recently we have extended our analysis of nuclear transfer in *Xenopus* oocytes. Quite surprisingly, we find that the nuclei of differentiated adult cells of mice (thymocytes) and humans (white blood cells) can be to some extent reprogrammed by *Xenopus* oocytes (37). In particular, the diagnostic pluripotency stem cell marker gene *oct4* is induced in these mammalian nuclei after injection into the germinal vesicle (enlarged oocyte nucleus). The *oct4* transcripts have the human or mouse sequence when oocytes are injected with human or mouse somatic cell nuclei, respectively. The ability to activate *oct4* expression in the nuclei of adult somatic cells may increase the probability of deriving embryonic stem cells from nuclear transplant embryos (38). Evidently amphibian oocytes contain molecules and conditions that can at least partially reprogram nuclei of adult mammalian cells. Because one *Xenopus* oocyte has 4,000 times the protein content of a mammalian egg, and because one female *Xenopus* contains some 25,000 oocytes, the *Xenopus* ovary constitutes favorable material for the identification of reprogramming molecules and mechanisms.

Proliferation of Reprogrammed Cells

To be clinically useful, it will be necessary to be able to extensively proliferate reprogrammed cells. The remarkable discovery, primarily of Evans (39), that mouse blastocyst cells can be made to proliferate almost indefinitely in culture, as embryonic stem cells, without losing their potential for differentiation into most, and sometimes all, cell types of an adult gives great encouragement in this requirement. We heard at this meeting about much current research directed toward understanding the mechanisms and control of embryonic stem cell proliferation and differentiation. It may eventually be necessary to build into cells destined for replacement a finite proliferative capacity to reduce the likelihood of donated cells becoming cancerous.

In conclusion, we suggest that the extraordinary reprogramming capacity of eggs and oocytes may lead to the identification of reprogramming molecules and mechanisms. These may facilitate a route toward cell replacement in humans, by a combination of nuclear transfer, stem cell creation, and embryonic stem cell proliferation, as suggested by the work of Munsie *et al*. (40) and Rideout *et al*. (41).

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