Somatic Cell Cloning: The Ultimate Form of Nuclear Reprogramming?

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Abstract. With the increasing difficulties associated with meeting the required needs for organs used in transplantation, alternative approaches need to be considered. These include the use of stem cells as potential sources of specialized cells, the ability to transdifferentiate cell types in culture, and the development of complete organs that can be used in humans. All of the above goals will require a complete understanding of the factors affecting cell differentiation and nuclear reprogramming. To make this a reality, however, techniques associated with cloning and genetic modifications in somatic cells need to be continued to be developed and optimized. This includes not only an enhancement of the rate of homologous recombination in somatic cells, but also a thorough understanding of the nuclear reprogramming process taking place during nuclear transfer. The understanding of this process is likely to have an effect beyond the area of nuclear transfer and assist with better methods for transdifferentiation of mammalian cells.

The ever-expanding gap between availability of organs and the number of patients awaiting an organ transplant is of great concern. In 2003, 14,880 transplantations were carried out between January and August, yet more than 83,000 patients were in the organ waiting list (based on data from the Organ Procurement and Transplantation Network as of November 1, 2003). This increasing divergence between availability of organ donors and the increasing number of patients in waiting lists has caused researchers to look into alternative methods to fulfill these needs. Whereas for complete organ replacement, xenotransplantation with pig organs is the main alternative, for tissue repair, other alternatives exist. The most promising to date is the use of embryo-derived and adult-derived stem cells (1). Cloning by nuclear transfer has a key role to play both by assisting in the generation of swine with complex genetic manipulations, and in the generation of human stem cells. Although ethical concerns need to be considered for the application of these technologies, such discussion is beyond the scope of this review. This review will focus on the development of the cloning technology, the basic concept in nuclear reprogramming, and recent advances in both understanding the epigenetic process of nuclear reprogramming and in enhancing the ability to undertake complex genetic manipulation in this species.

The goals of generating a complete organism by nuclear transfer of a nuclei into an oocyte, or reproductive cloning, has been pursued for many years. By use of frog (*Rana pipiens*) eggs, Briggs and King (2) described the first nuclear transfer experiment. Briefly, ovulated MII eggs were enucleated with a handmade needle, and cells from a developing frog were lysed and their nuclei injected into the egg. Over the next 4 to 11 d, the majority of the cleaved blastulae developed into normal postneurula and tadpole stages, demonstrating the ability of the injected nuclei to at least partially complete normal development. These experiments were the first to demonstrate that the genes, which served as the templates for RNA production and which were responsible for the production of proteins for early embryonic development, were not lost or irreversibly modified during differentiation, allowing for reprogramming of their fate if placed in the proper environment. The development of techniques for microsurgery, microscopy, activation, enucleation, and embryo culture and the process of nuclear transfer were modified for mammalian oocytes and blastomeres. Initial experiments attempted to introduce a somatic nucleus by Sendai virus–mediated cell fusion or a cell nucleus by direct injection into rabbit eggs; however, at best, these eggs only underwent a few divisions (3,4). Development of the technology continued with partial successes along the way (5,6) until Tsunoda *et al.* (7), produced live mice from the transfer of nuclei from the one-, two-, four- and eight-cell stage. Willadsen (8) was the first to report successful nuclear transfer in nonmurine species and developed the framework for the methods in use today; his protocol was very similar to current cloning techniques in that cytochalasin B is used for enucleation, the first polar body and the adjacent potion of the cytoplasm is removed, and the cell is fused to the cytoplasm. His report of cloned lambs was quickly followed by the pro-

duction of the first cloned cow (9) and a cloned piglet (10). However, in all cases, embryonic cells were used as nuclear donors. The conceptual breakthrough came with the generation of Dolly, the cloned sheep (11). In this instance, the nuclear donor was not an early embryonic cell but a fully differentiated mammary gland cell. Dolly was the living proof that the nuclei of a fully differentiated cell still contains all of the information required for he development of a full organism if placed in the proper environment or "reprogrammed" properly. After Dolly, sheep, cattle, goats, pigs, zebra fish, rabbits, cats, rats, and a mule have been added to the list of animals generated through somatic cell nuclear transfer (12–17). However, generation of all species through cloning is hindered by low viable birth rates and high incidences of abnormalities. In a recent article, a comprehensive analysis of the health profiles of cloned animals produced to date indicates that 64% of cattle, 40% of sheep, and 93% of mice exhibit some form of abnormality (18). A large percentage of these animals die during gestation or shortly after birth (19–22). Various abnormalities are manifested in animals derived from nuclear transfer. In cattle they include large offspring syndrome, diabetes, pulmonary hypertension, dilated cardiomyopathy, internal hemorrhaging umbilical artery, viral infection, dystocia, kidney problems, leg malformations, pneumonia, heart defects, liver fibrosis, osteoporosis, joint defects, anemia, and placental abnormalities. In sheep, abnormalities include large offspring syndrome, arthritis, and kidney, liver, and brain defects. Mice exhibit obesity, large offspring syndrome, enlarged placentas, umbilical hernias, respiratory failure, and failure to foster pups. Goats and pigs exhibit relatively few abnormalities, but they include bacterial infections of the lungs (goats) and abnormal teat numbers, cleft lips, and malformed limbs (pigs) (18,23). These abnormalities are likely associated with inaccurate nuclear reprogramming of the injected nuclei. But what exactly is nuclear reprogramming, and what does it entail?

Conceptually nuclear reprogramming is simple to understand. It consists of a series of epigenetic DNA modifications including cytosine methylation, and histone protein modifications such as acetylation, methylation, and phosphorylation. Essentially, it entails modifying the expression program of a nuclei from one state to another. Thus, the generation of Dolly required the reprogramming of a mammary cell nuclei into a nuclei of an early embryo. That means gene expression patterns unique to the mammary cell line had to be shut down and a completely new set of genes activated. This entailed both the movement of transcription factors to and from the donor nuclei and the remodeling of chromatin. Another factor in the nuclear reprogramming equation is played by methylation of DNA: methylation patterns of sperm and oocyte during normal fertilization are not analogous to those of somatic cell nuclei. Thus, nuclear reprogramming requires drastic changes in gene expression profiles, reorganization of chromatin, and recapitulation of normal methylation patterns.

Chromatin remodeling is needed as terminally differentiated cells undergo a chromatin reorganization that includes regional heterochromatization (24). This regional heterochromatin packing makes it more difficult for the nuclear reprogramming event to occur by limiting accessibility of the required nuclear factors. Embryonic cells and stem cells, in contrast, must be able to respond quickly to developmental signals and activate and deactivate specific regions of the genome. As a result, the chromatin structure is in a more open configuration and thus more readily available for reprogramming. In mice, extensive experimentation has demonstrated that embryonic stem cells are more easily reprogrammed than other cell types (25). Similarly, fully differentiated cells such as mature B lymphocytes could not be fully reprogrammed by nuclear transfer alone (26).

Additionally, multiple investigators have reported significant clonal differences derived from the same cell line. These differences have been attributed to DNA changes occurring during culture (27). We feel this interpretation does not fully account for clonal differences because the cells remain karyotypically normal. An alternative explanation is partial differentiation of specific subclones, accompanied by chromatin compaction, and the subsequent problem in reprogramming. Although attempts at modifying chromatin organization before cloning are ongoing, results to date are inconclusive. However, because of the availability of chemicals such as 5-azacytidine and trichostatin A, both capable of remodeling chromatin (28,29), it is likely that eventually a protocol will be developed that will permit unpacking of the heterochromatic regions in terminal differentiated cells before cloning.

A more troubling drawback in nuclear reprogramming is the differences associated with methylation patterns of donor cell nuclei compared with sperm and oocyte DNA. Before that can be addressed, however, some understanding of DNA methylation and its complexity is required. Methylation at cytosine-guanine nucleotides (CpG dinucleotides) exists in virtually all vertebrates, many invertebrates, and most plants (30). CpG dinucleotides are typically clustered in GC-rich regions, termed CpG islands, but methylation is not constrained to these regions (31). Four DNA methyltransferases and derivatives have been identified in mice and humans: Dnmt1, Dnmt2, Dnmt 3a, and Dnmt3b (31). These methyltransferases can be further classified into two categories: maintenance DNA methyltransferases (Dnmt1 and Dnmt2), and *de novo* DNA methyltransferases (Dnmt3a and Dnmt3b).

De novo methylation occurs during gametogenesis in both male and female germ cells, where it is believed to play an important role in the establishment of genomic imprinting in the gametes. Additionally, *de novo* methyltransferases are responsible for remethylation of the genome after the asymmetric demethylating events that occur in the preimplantation embryo. Why are all these waves of demethylation and remethylation during development so critical? Essentially, methylation of CpG islands can have drastic effects on gene expression. More importantly, the genome contains a set of "imprinted" genes where the methylation pattern is dependent on the parental origin. Thus, the same gene can be differentially methylated when one compares the copy that came from the mother from the copy that came from the father. This phenomenon results in alleles of particular genes being repressed or expressed on the basis of parental inheritance. Although it has been known for

some time that DNA methylation is involved in the mechanisms of imprinting, it is still unclear how exactly these modifications interact with genes to regulate expression (32). Regions residing in all imprinted genes identified to date, termed differentially methylated regions (DMR), are believed to regulate the expression of alleles on the basis of the presence or absence of CpG methylation that resides as islands within these DMR (33). Thus, differential methylation of DMR is coincident with the differential expression of alleles.

Of more relevance to nuclear reprogramming is the observation that after imprinting in the germ line, the parental genomes exhibit differential modes of demethylation in the newly formed zygote. Upon entry of the sperm into the oocyte, maternal cytoplasmic factors obtain access to paternal chromosomes for approximately 5 h, at which time decondensation of the tightly packaged sperm DNA occurs resulting from the protamine to histone exchange required of the paternal chromosomes. During this time, the paternal genome undergoes dramatic demethylation, and the maternal genome, which contains most of the marks associated with imprints, undergoes further *de novo* methylation up to the blastocyst stage and is most likely regulated by passive demethylation via lack of maintenance methyltransferases (34,35). Afterward, the paternal pronucleus forms, thereby regulating access of cytoplasmic factors to the genome. Asymmetric demethylation of the parental genomes has also been observed in cattle, pigs, and humans (36). So in a normal event, both incoming DNA—sperm and oocyte—are not treated equally. Remethylation of the paternal and maternal genome resumes after implantation and formation of the epiblast and trophectoderm (37).

This situation differs drastically from that which occurs in somatic cell nuclear transfer because there are no differences in chromatin composition and packing and thus accessibility to modifying enzymes between the paternal and maternal chromosomes. Experiments by us and others have indicated that this leads to methylation defects that are particularly evident in the trophectoderm derive cells lines. Kang *et al.* (38) examined the methylation patterns of repeat elements in cloned bovine embryos, donor cells, and control embryos, produced *in vitro* and *in vivo*. This experiment determined that the methylation patterns observed in cloned embryos were similar to that present in the donor cells, and this pattern was dramatically different than that exhibited in both *in vitro* and *in vivo* derived control embryos. Our results indicate that the methylation patterns in cloned cattle at midgestation differ drastically in the chorion (trophectoderm-derived tissues) but not in the fetus proper when compared with noncloned controls (personal communication). We have interpreted this as being a result of a rapid differentiation of the trophectoderm cell line, with a concomitant reduction in the activity of the demethylases and methylases, when compared with the cells of the inner cell mass (ICM). As a result, the reprogramming of tissues derived from the trophectoderm is incomplete. In contrast, the cells of the ICM continue to express high levels of the required methylation-related enzymes, allowing the nuclei more time to reestablish a normal methylation pattern. Examination of phenotype of cloned animals supports our molecular observations.

From a basic science standpoint, cloning by nuclear transfer is helping us elucidate the complex interaction between chromatin remodeling, DNA methylation, and gene expression during development. All these advances will eventually lead to a greater understanding of reprogramming cells by artificial means, and this could have drastic implications in tissue and organ transplantation. The ability to reprogram the phenotype of one cell for another would essentially lead to our ability to take easily accessible material such as skin fibroblasts and reprogram it to become tissues such as specific neuronal types, cardiac muscle, and hepatocytes.

Additionally, somatic cell cloning has many advantages when generating transgenic swine. By using cloning as the basis for generation of transgenic animals, somatic cells are genetically modified and selected *in vitro*, and only those containing the transgene are used for nuclear transfer procedure. Thus, all animals born from the procedure are transgenic (*i.e.,* 100% efficiency). It also allows, for the first time, the application of gene targeting by homologous recombination in species other than mice. Unfortunately, because of its high degree of specificity, the frequency of homologous recombination is very low and varies from 10^{-7} to 10^{-8} in primary somatic cells (39–41). Although it has not been possible to target some loci because of this inefficiency (42), there have been reports of successful targeting in somatic cells and production of genetically modified animals by using the same strategy as used in mouse embryonic stem cells (43–45). Recently chimeric nucleases have been used to stimulate gene targeting in human somatic cells by creating double stranded-breaks in the genomic target (46). Increased gene targeting efficiency has also been demonstrated by using adenoassociated virus and DNA double strand breaks (47). We developed a very efficient method of gene targeting for primary somatic cells by using bovine hypoxanthine phosphoribosyl transferase as a test locus (48). This approach uses the incorporation of nuclear localization signal in the targeting construct and cell synchrony to boost the gene targeting efficiency. These methods should have wide applicability and will facilitate the process of targeted modification in somatic cells for the generation of genetically modified swine as well as for somatic cell therapies.

In addition to reproductive cloning, therapeutic cloning, or the generation of human stem cells via cloning, also has tremendous potential applicability in tissue transplantation. Therapeutic cloning entails the reprogramming of a somatic cell donor nuclei such as a skin cell into a blastocyst stage embryo. The embryo is then cultured *in vitro* under conditions that generate stem cells capable of differentiating into multiple types (49). This in essence creates a potential source of multiple tissue types with perfect histocompatibility to the patient, thus bypassing issues associated with rejection (50). Although progress in this area has been hampered by regulatory constrains, its potential should not be overlooked. Neither should its difficulties, as control of embryonic stem cell differentiation is far from fully developed, and the abnormalities in nuclear reprogramming that are presently being "filtered" by fetal death in reproductive cloning would not exist by using his

approach. Thus, it is critical that methods to evaluate the proper reprogramming of stem cells generated through therapeutic cloning be developed.

In summary, somatic cell cloning will continue to play a major role in the development of transplantation technology, both through a better understanding of the nuclear reprogramming process and by facilitating the generation of transgenic animals with complex genetic manipulations.

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