

Prospects for the use of nuclear transfer in human transplantation

Robert P. Lanza*, Jose B. Cibelli, and Michael D. West

*Advanced Cell Technology, Worcester, MA 01605. *Corresponding author (rlanza@advancedcell.com).*

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The successful application of nuclear transfer techniques to a range of mammalian species has brought the possibility of human therapeutic cloning significantly closer. The objective of therapeutic cloning is to produce pluripotent stem cells that carry the nuclear genome of the patient and then induce them to differentiate into replacement cells, such as cardiomyocytes to replace damaged heart tissue or insulin-producing β cells for patients with diabetes. Although cloning would eliminate the critical problem of immune incompatibility, there is also the task of reconstituting the cells into more complex tissues and organs in vitro. In the review, we discuss recent progress that has been made in this field as well as the inherent dangers and scientific challenges that remain before these techniques can be used to harness genetically matched cells and tissues for human transplantation.

Keywords: therapeutic cloning, nuclear transfer, transplantation, tissue engineering, stem cell

The modern era of clinical organ transplantation was ushered in nearly 40 years ago, when Joseph Murray and his colleagues performed the first successful long-term renal transplant between identical twins (monozygotic clones) at the Peter Bent Brigham Hospital in Boston, MA. This technology was subsequently extended to transplantation between more distantly related and unrelated donors through the use of immunosuppressive agents, such as imuran, glucocorticoids, and more recently cyclosporine. Medical applications have since expanded to include heart, liver, lung, pancreas, and cells and tissues, such as bone marrow and pancreatic islets¹. However, these successes have created a pressing need for more donor organs and tissues.

According to the United Network for Organ Sharing, over 63,000 patients are currently awaiting an organ of one type or another in the United States. This number is steadily rising, and a new name is added to the waiting list every 18 min. In addition to patients with heart, liver, kidney, and lung disease, over 16 million patients worldwide suffer from neurodegenerative disorders such as Parkinson's and Alzheimer's disease, over 120 million patients suffer from diabetes, and millions more from arthritis, AIDS, strokes, and other diseases that may one day be treatable with cell transplants.

Advances in cloning have resulted in therapies with the potential to eliminate immune responses associated with the transplantation of these various tissues, and thus the requirement for immunosuppressive drugs that carry the risk of a wide variety of serious complications, including cancer, infection, renal failure, and osteoporosis. Nuclear transfer (NT) techniques pioneered in amphibians and mice²⁻⁴ are now routinely used in our own and other laboratories to clone ungulates, such as cattle and sheep⁵⁻⁸.

The concept, termed "therapeutic cloning," is to transfer the nucleus from a patient's cell (e.g., a skin cell) into an enucleated donor oocyte (see Fig. 1). After reprogramming, the donated somatic nucleus regains its totipotency and is able to initiate a round of embryonic development. Pluripotent stem cells derived from the resulting embryo carry the nuclear genome of the patient, and are then induced to differentiate into replacement cells, such as cardiomyocytes to replace damaged heart tissue, insulin-producing β -cells for patients with diabetes, chondrocytes for osteoarthritis, or dopaminergic neurons to treat Parkinson's disease, among others.

Toward human therapeutic cloning

To accomplish the goal of cloned human tissues, it will first be necessary to modify NT technology to produce human preimplantation embryos. Recent breakthroughs in animal cloning have greatly increased the feasibility of achieving this aim. A range of large animal species have been cloned using fetal and adult somatic cells⁵⁻¹¹. Wilmut et al.⁵ were the first to use the NT technique to clone a wholly viable mammal from quiescent (G0) adult somatic cells. Soon after, Cibelli et al.⁶ used differentiated cells that were nonquiescent to produce the first cloned transgenic calves. The latter methodology has also been applied recently to the propagation of transgenic goats¹¹. It is noteworthy that, in the latter two programs, transgenic somatic cell lines functioned as karyoplast donors for the NT procedure. The ability to carry out genetic manipulations before embryo reconstitution permits the introduction of transgenes (e.g., antiapoptotic or protective genes) that may facilitate the success of the technique in other mammalian species, such as humans and pigs.

Meng et al.¹² have provided tantalizing evidence that nuclear transplantation may indeed be possible in humans. They have successfully produced two healthy rhesus monkeys, one male and one female, by transfer of cleavage-stage blastomere nuclei into enucleated oocytes. Genotypic analysis of both infants using short tandem repeat (STR) markers confirmed that the animals were derived from the nuclear donor cells. The demonstration that NT is a viable technology for the cloning of nonhuman primates is of particular significance because of their genetic and physiological similarity to humans. However, donor nuclei from embryonic blastomeres are thought to be relatively undifferentiated, readily reprogrammed, and likely to support full-term fetal development¹³. The cloning of primates, including humans, using differentiated (fetal or adult) donor cells clearly poses a greater challenge, and has yet to be reported.

Efficient human embryo reconstitution by NT will require the optimization of several parameters. Our understanding of oocyte maturation is still incomplete, and protocols for in vitro maturation of human oocytes are unreliable and need to be optimized. The parameters for cell fusion and activation must also be improved. These events are poorly understood, and the optimal combinations for induction of embryonic development have yet to be determined.

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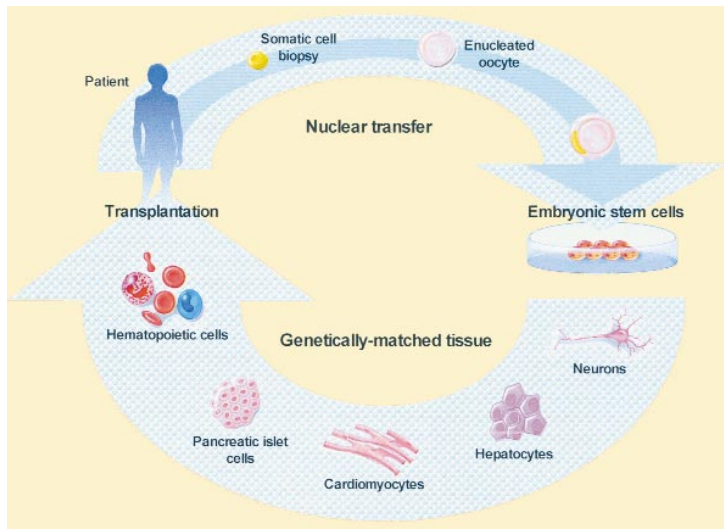


Figure 1. Procedure for human therapeutic cloning. A cell from the patient is fused with an enucleated donor oocyte using the NT techniques pioneered in murine and livestock species. Stem cells are isolated from the resulting clone and then differentiated in vitro into genetically matched cells and tissues for transplantation. For example, cardiomyocytes could be used to treat patients with heart disease, insulin-producing cells could be used for patients with diabetes, dopaminergic neurons for Parkinson's disease, or hepatocytes used in a tissue-engineered liver.

However, unlike “reproductive cloning” (which aims to produce wholly viable organisms), human therapeutic cloning does not seek to take embryo development beyond the earliest (preimplantation) stages. Rather, the goal is to derive pluripotent stem cells, such as embryonic stem (ES) cells from the inner cell masses of blastocysts (those cells that form the somatic lineages) as a source of replacement cells for tissue engineering and transplantation therapies.

Rejuvenating cells

A recent study in *Nature*¹⁴, involving Dolly the cloned sheep, has raised questions as to whether cells obtained from embryos created by NT will undergo premature senescence, potentially limiting the efficacy of therapeutic cloning. The authors noted that telomeres were smaller in the NT animals studied than in age-matched controls, and that restoration of telomere length did not occur because these animals were produced without germline involvement. “The smaller [telomere length] in [Dolly] is consistent with the age of her progenitor mammary tissue (six years old) and with the time that [sheep mammary] cells derived from that tissue spent in culture before NT¹⁴. Unfortunately, these measurements of telomere length are neither precise nor well controlled, and more data will be required before any definitive conclusion can be made about Dolly's cellular age. Indeed, Dolly appears to be healthy and typical for a sheep of her age. She has undergone normal pregnancies and has successfully delivered healthy lambs¹⁴.

In contrast to the report by Shields et al.¹⁴, our group has shown that the somatic cell NT procedure can be used to restore the life span of primary cultured cells⁶ (see Fig. 2). When fibroblasts from six-week-old fetuses were cultured until senescence, they underwent approximately 30 population doublings, with an average cell cycle length of 28–30 h. To test whether these cells could be rescued from senescence by NT, a 40-day-old fetus was generated using cells within 0.8 population doublings from senescence. Fibroblasts derived from this fetus underwent 31 population doublings, as compared to 33 doublings for fibroblasts from a same-age fetus conceived normally. These data suggest that NT is capable of rejuvenating senescent cells. More detailed studies of telomere dynamics are currently underway.

Genetic and developmental problems

In addition to the issue of premature aging, questions have also been raised as to the safety of the NT procedure itself^{16–19}. In reproductive cloning studies in livestock and laboratory animals, a large number of abnormalities and deaths have been observed during fetal and neonatal development. Animals are often born oversized and unhealthy, sometimes with placental abnormalities, genetic defects, and/or immunological deficiencies. For instance, in our first experiment with cloned transgenic calves, we transferred 28 embryos to 11 recipient cows⁶. Of the six cows that became pregnant, one cow aborted before day 60 of gestation, whereas a second animal aborted at day 249 of gestation. The placenta of the latter animal manifested abnormalities such as hydroallantois and enlarged placentomes. On necropsy, the fetus and umbilical vessels were observed to be oversized, the lung lobes edematous, and the right heart ventricle enlarged.

Overall, 14% of the embryos reconstructed by NT using fetal somatic cells produced live offspring⁶, as compared to 8% (ref. 5) and 3% (ref. 11) reported in sheep and goats, respectively. Although four calves in the above study continued development to term, one of the animals died five days after birth as a result of pulmonary hypertension. The animal also exhibited a dilated ventricle, a patent ductus arteriosus, and umbilical vessels three times normal size.

Similar developmental problems have been observed in other cloning studies^{17–20}. Accumulating evidence suggests that some of this pathology could be attributed to disruption of the imprinting system—a genetic mechanism whereby maternal and paternal genes balance one another at the molecular level. For instance, the paternal imprinting pattern may turn on genes that foster growth of a large placenta, whereas the maternal genes may be imprinted to suppress placental growth. Abnormal placenta growth due to imprinting imbalances could in turn cause fetal hypertension, with subsequent enlargement of the heart and other abnormalities seen in cloned animals. Of course, other mechanisms may also be causative in the genetic and developmental defects seen in clones. Animals produced by standard in vitro fertilization techniques, for example, exhibit similar problems (albeit at a lower frequency), as do cattle that have been manipulated as embryos in the laboratory²¹.

It is unclear whether the increased incidence of problems in clones is due to genetic damage caused by the NT process itself (such as the electrical pulse used to fuse the oocytes with the donor cells), to other in vitro manipulations, or to the accumulation of somatic mutations in the donor nucleus used for transfer. Whatever the cause, the embryonic and fetal mortality observed in clones should be looked upon as part of the normal process whereby maternal surveillance filters out genetically defective embryos. Unfortunately, this mechanism has its limits, and many defects in the genome may not be fully expressed until after birth. This clearly poses problems for reproductive cloning, where the procedure would almost certainly lead to increased risks of infant death and malformation.

As many of these abnormalities relate to the placenta or to other complex events that occur during animal development, they would not be expected to affect the use of cloning for therapeutic purposes. Unlike reproductive cloning, the preimplantation embryo, or blastocyst, is not implanted in the uterus. Rather, pluripotent stem cells are derived from the inner cell mass to produce replacement cells and tissues for transplantation. Still, therapeutic cloning has certain risks, such as the introduction of genetic mutations in vitro during cell expansion and differentiation. Also, major chromosomal abnormalities, and other somatic mutations that occur throughout a patient's lifetime, would not be eliminated through spontaneous abortion and other selective pressures on the conceptus during normal development. Such defects could remain concealed within the genome until after the time of transplantation, possibly leading to

abnormal cell growth and differentiation, and to the development of cancers and other diseases.

Intra- and interspecies nuclear transfer

Cibelli et al.²² were the first to demonstrate that stem cells could be produced using somatic cell nuclear transplantation in a large mammalian species. In their approach, transgenic bovine fibroblasts were fused with enucleated oocytes to clone blastocyst-stage NT embryos. These embryos were used to generate cell colonies with morphology and cytoplasmic markers that characterize ES cells. After undifferentiated proliferation *in vitro* for several months, these ES-like cells were reintroduced into preimplantation embryos where they formed derivatives of all three embryonic germ layers, including ectoderm, mesoderm, and endoderm. This study demonstrates that somatic cells can be dedifferentiated by NT to produce pluripotent stem cells that could, theoretically, be used to grow any type of cell or tissue needed for transplantation. Indeed, 15 of 41 (37%) bovine embryos reconstructed in our laboratory using transgenic adult somatic cells were able to develop into ES cell-like colonies.

Although more controversial, NT methods have also been developed to generate primordial cells by transferring nuclei from adult somatic cells into surrogate oocyte cytoplasm. In an experiment performed in 1996, human lymphocytes and oral mucosal epithelial cells were fused with enucleated bovine oocytes to form a preimplantation embryo that could have, in theory, been used to create replacement cells for transplantation²³. Of the 56 NT units produced, six grew to the 4- to 16-cell stage, whereas only one reached the 16- to 400-cell stage. The latter clone was plated onto a fibroblast feeder layer, and began to propagate as a colony with an ES cell-like morphology.

More recent studies have confirmed the ability of bovine oocyte cytoplasm to support mitotic cell cycles under the direction of differentiated somatic cell nuclei of several mammalian species²⁴. Nuclear transfer units between sheep, pigs, monkeys, and rats and enucleated bovine oocytes, all underwent transition to interphase accompanied by nuclear swelling and further progression through the cell cycle. As in our own studies, some embryos progressed further and developed to advanced stages, as demonstrated by successive cell division and formation of a blastocoele cavity at the time appropriate for the species of the donor nuclei.

Certainly, the use of interspecies NT to generate stem cells raises many scientific and ethical questions. Do the resultant embryos have the potential to develop into viable offspring? Could such an organ-

Table 1. Some potential applications of therapeutic cloning

Disorder	Cells transplanted
Alzheimer's disease	Nerve cells
Atherosclerosis	Endothelial cells
Burns	Skin cells
Chronic pain	Chromaffin cells
Diabetes	Islet cells
Epilepsy	Nerve cells
Heart disease	Cardiomyocytes
Huntington's disease	Nerve cells
Hypocalcemia	Parathyroid cells
Hypocholesterolemia	Hepatocytes
Kidney disease	Kidney cells
Leukemia	Hematopoietic cells
Liver disease	Hepatocytes
Macular degeneration	Retinal cells
Multiple sclerosis	Glial cells
Muscular dystrophy	Skeletal muscle cells
Osteoarthritis	Chondrocytes
Parkinson's disease	Dopaminergic neurons
Rheumatoid arthritis	Chondrocytes
Spinal cord injuries	Nerve cells
Strokes	Nerve cells

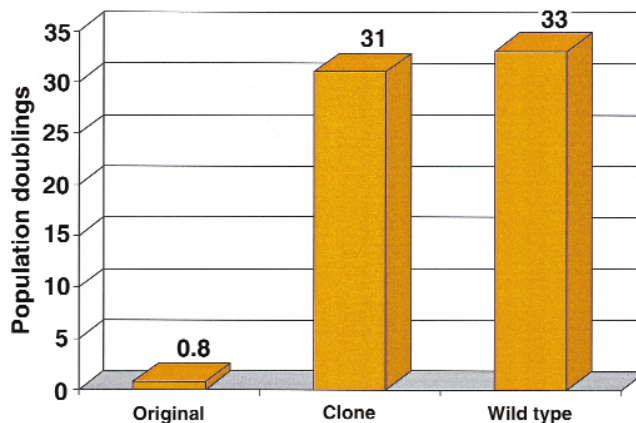


Figure 2. The ability of NT to reset the life span of senescent somatic cells⁶. A bovine fetus was cloned using fibroblasts at 0.8 population doubling from senescence (original). Fibroblasts derived from it underwent 31 population doublings (clone), as compared to 33 doublings for fibroblasts from a same-age (wild type) fetus conceived normally.

ism be considered a chimera—part human and part cow—even though all nuclear DNA is eliminated from the bovine oocytes?

On the scientific side, questions have been raised as to the ability of surrogate mitochondria to support human embryonic development. It is clear that mitochondrial DNA alone does not contain enough genetic information to code for all mitochondrial components, and that the nuclear and mitochondrial genetic systems must interact in the formation of the protein systems in the mitochondria^{25,26}. The mitochondrial genome of vertebrates is extremely specialized, and incompatibilities are likely between distantly related species²⁷. This may account for why high proportions of interspecies units failed to progress beyond the eight-cell (transcription-requiring) stage, and why, ultimately, it may be desirable to replace the recipient mitochondria with mitochondria isolated from the patient's own (biopsy-grown) cells. On the other hand, the transfer of human mitochondria with the donor cell may be sufficient for the viability of the resulting cells.

Reprogramming and programming

Intra- and interspecies nuclear transfer techniques share the common ability to reprogram the nucleus of a differentiated somatic cell using the maternal components found in oocyte cytoplasm. By understanding how these factors direct development, we may eventually be able to reprogram the cells of a patient, and convert them into stem cells, without the need for an enucleated egg cell. As well as being rich in maternal mRNAs and proteins, the cytoplasm of the unfertilized oocyte contains all the components required to direct the earliest phases of embryonic development up to the activation of the zygotic genome. In most large animal species, activation of the embryonic genome occurs between the four- and eight-cell stages of preimplantation development²⁸⁻³¹. After successive cleavage divisions, a small fluid-filled cavity appears inside this ball of dividing cells. The cavity becomes progressively larger, until the embryo resembles a hollow sphere, the blastocyst, with a single peripheral layer of epithelial-like cells, and an inner cell mass displaced to one pole of the sphere. This knob of undifferentiated cells (from which embryonic stem cells are derived) will eventually give rise to the adult organism.

Ultimately, the success of human therapeutic cloning will depend on coaxing these primordial stem cells to differentiate *in vitro* into the appropriate replacement cell types³². Our knowledge of the mechanisms underlying the differentiation of human tissues is still limited, and it may take many years before we understand the morphogenic signals and factors that control phenotypic programming

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well enough to produce specific functionally specialized cell populations. As ES cells have the potential to be tumorigenic, growing into teratomas and teratocarcinomas when injected into mice³³, it will also be important to separate the uncommitted ES cells from the desired, differentiated progeny. Fortunately, it is possible to insert transgenes into the donor genome before the NT procedure⁶. In addition to facilitating the design of a fail-safe "suicide" mechanism, which would signal the transplanted cells to self-destruct if they become tumorigenic, this approach could also be used to genetically select specific differentiated cell lineages in culture (automatically killing off any remaining ES cells). In fact, Klug et al.³⁴ successfully used this strategy to generate essentially pure (>99%) cultures of cardiomyocytes from differentiating murine ES cells. Cardiomyocytes expressing an MHC-*neo*^r transgene survived G418 selection and formed stable intracardiac grafts when injected into the hearts of adult dystrophic mice.

De novo cloned tissues and organs

The usefulness of cardiomyocytes and other differentiated cell types in medicine is obvious (see Table 1). However, to realize the full potential of therapeutic cloning, it will be important to understand how to reconstitute more complex tissues and organs in vitro. Although cloning would eliminate the most critical problem—immune incompatibility—there is still the task of putting the cells together to create or recreate functional structures.

For example, myocardial infarction is one of the commonest diagnoses occurring in hospitalized patients in western countries³⁵. Whereas injections of individual or small groups of cardiomyocytes could aid in the treatment of some localized infarcts, this approach is unlikely to be of value in patients with more extended ischemic injury, where the risk of scar formation, cardiac rupture, and other complications is much greater. However, tissue engineering offers the possibility of organizing the cells into three-dimensional myocardial "patches" that could be used to repair the damaged portion(s) of the heart³⁶. For myocardium and other relatively simple tissues, such as skin and blood vessel substitutes, this may involve seeding cells onto masses or sheets of polymeric scaffold. Creating more complex, vital organs, such as the kidney, the liver, or even an entire heart, on the other hand, will be a much greater challenge, requiring the assembly of different cell types and materials in great combinatorial complexity.

Future prospects

As this new field progresses, we will move from marveling at the enormity of the task that lies ahead, to marveling at the human capacity to imitate nature. The ability to design tissuelike constructs from cloned cells could soon give doctors exactly the same advantage Joseph Murray had when he carried out the first successful transplant between identical twins: the potential to replace a failing body part with genetically matched tissue. Rapid progress is being made in the laboratory to engineer tissues and neo-organs, and already tissue-engineered skin, cartilage, bone, and blood vessels are being tested in patients³⁶. However, our knowledge of the molecular pathways of tissue growth and differentiation are limited, and we still have a long way to go before we can understand and control the process well enough to channel the in vitro differentiation of human ES cells into all of the various cell replacement types.

But the future of therapeutic cloning will depend on more than just unraveling these scientific processes. We must also take a hard look at the ethical and moral issues that surround the use of ES cells derived from cloned blastocysts^{37,38}. Is the preembryo, for instance, a person, even though developmental singleness (individuality) has not been established? Does a blastocyst have the same rights as an

adult, or even a fetus or an early gastrula? Do the potential therapeutic benefits of the procedure outweigh the harm? Our ability to address these questions satisfactorily will be of profound importance in determining the development and adoption of NT approaches for use in human transplantation.

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