Minireview

Can mammalian cloning combined with embryonic stem cell technologies be used to treat human diseases?

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Abstract

Cloning is commonly perceived as a means of generating genetically identical individuals, but it can also be used to obtain genetically matched embryo-derived stem cells, which could potentially be used in the treatment of patients. A recent report offers the first 'proof of principle' of such cloning for therapeutic purposes, referred to as nuclear transplantation to produce stem cells for autologous transplantation.

Cloning is a mode of asexual reproduction in which all offspring have an identical nuclear genome to that of the parent. In recent years, mammalian cloning has been achieved by the introduction of somatic cell nuclei into fertilized eggs from which the zygotic nucleus has been removed. In our anthropocentric society, the recent success in animal cloning and its implications for humanity have captured the public's attention and imagination. But even though cloning has now been accomplished in several mammalian species, there are often severe complications associated with the procedure, and cloned animals are never quite the same as their parent. For instance, cloned embryos often exhibit developmental abnormalities, usually including excessive growth [1], referred to as large-offspring syndrome (LOS); in some cases, epigenetic aberrations have been reported, such as inappropriate X chromosome inactivation in cloned bovine fetuses and placentae [2]. Thus, only a very small proportion (less than 1%) of cloned mammals make it to birth. Many of the offspring that are born suffer from various defects, including obesity [3] and liver and immunological defects [4]; their chromosomes often have telomeres with variable lengths, possibly correlating with the donor cell type used for generating clones [5-7]. Either individually or in combination, these symptoms may drastically shorten the lifespan of clones. It is issues such as these that have raised considerable concern about the cloning procedure and highlighted our lack of understanding of the basic biology of cloning.

Mammalian cloning has far-reaching consequences, beyond the generation of cloned adults. One potentially powerful application of cloning technology is for the generation of cloned embryos that will never be implanted in a uterus - embryos that will not develop beyond a hundred or so cells but will be used to generate genetically matched, immunologically compatible (autologous) stem cells that can potentially differentiate in such a way as to replace damaged or diseased tissues or organs in an adult (see Box 1 for definitions of terms used). In light of the uncertainties and debate on the subject, a distinction must be made between cloning with the intent of generating live animals (reproductive cloning) and cloning in order to facilitate the derivation of stem cells from early embryos, which can ultimately be incorporated into therapeutic regimes. In this article, we will refer to the latter procedure as nuclear transplantation to produce stem cells, as suggested in the guidelines put forward by the US National Academies [8] after a workshop on human reproductive cloning held in August 2001. (It is also sometimes called therapeutic cloning or non-reproductive cloning.) Although there is a long way to go before nuclear transplantation to produce autologous stem cells becomes a

Box 1

Definition of terms used

Cloning	A procedure that generates individuals with identical nuclear genomes
Nuclear transfer (NT)	The transplantation of a nucleus derived from one cell into a second enucleated cell, usually an oocyte
Nuclear reprogramming	The epigenetic re-instruction of the nucleus to undertake an alternative genetic program, usually a program of embryogenesis
Stem cells	Cells that have the dual capacity to self-replicate and to differentiate into specialized derivatives
Nuclear transplantation to produce stem cells	Derivation of stem cells from the product of the nuclear transfer procedure (usually the blastocyst). Also referred to as therapeutic cloning, research cloning or non-reproductive cloning
Autologous	Derived from the same individual (usually referring to stem cells cloned from an individual to be used as donor cells for transplantation to that same individual)
Totipotent	Able to give rise to all cells derived from the zygote (namely all fetal and extraembryonic lineages)
Pluripotent	Able to give rise to a wide variety of lineages but not all (usually all fetal and a subset of extraembryonic lineages)
Chimera	An animal generated from, and comprising, several genetically distinct populations of cells derived from more than one individual

reality for humans, several recent reports demonstrate that we are heading in this direction.

Nuclear transfer and stem cells

The technique of vertebrate somatic-cell nuclear transfer (also referred to as nuclear transplantation) was first developed half a century ago in amphibians [9], and the first cloned adult amphibians were described a decade later [10]. Only in the last five years has the technique been used successfully for the production of viable cloned mammals [11]. There are currently two elegantly simple protocols for the cloning of mammals by nuclear transfer. The first relies on the fusion of a somatic cell and an enucleated egg and has been used to clone sheep, mice, goats, cows and pigs [11-17], whereas the second (schematized in Figure 1) is based on nuclear microinjection and has been extensively used to generate cloned mice [18] and also cloned pigs and goats [19,20]. Both protocols involve the removal of the nucleus from an unfertilized egg (an oocyte) and its replacement with a nucleus from an adult cell or a cultured cell line; both rely on the premise that the microenvironment of the host oocyte - presumably its cytoplasm - can re-instruct the donor nucleus to adopt the behavior of the removed oocyte nucleus. Thus, the donor nucleus is reprogrammed so that it becomes developmentally versatile (totipotent) and able to direct and execute the embryonic developmental program.

Cloning entire individuals by nuclear transfer is not the aim of most studies at present, however. Of far more interest is the potential to produce stem cells and, by combining the production of stem cells with nuclear transfer, to produce autologous stem cells that match the donor of the adult nucleus. Stem cells are cells that have the unique dual capacity for self-renewal and differentiation; in other words, they can not only divide to give identical stem-cell progeny, they can also differentiate into a wide variety of other cell types. There are several categories of stem cell, including embryo-derived and lineage-specific stem cells: the former usually have a broader repertoire for differentiation than the latter. Stem cells isolated from the inner cell mass of the blastocyst-stage preimplantation mammalian embryo, known as embryonic stem or ES cells, can contribute to most but not all lineages (reviewed in [21]); this pluripotency mirrors that of the inner cell mass. If included in embryos derived from more than one fertilized egg (chimeras), ES cells can contribute to the fetus itself (including the germ line) and extraembryonic mesoderm. ES cells can also be maintained as permanent, undifferentiated cell lines *in vitro* while still

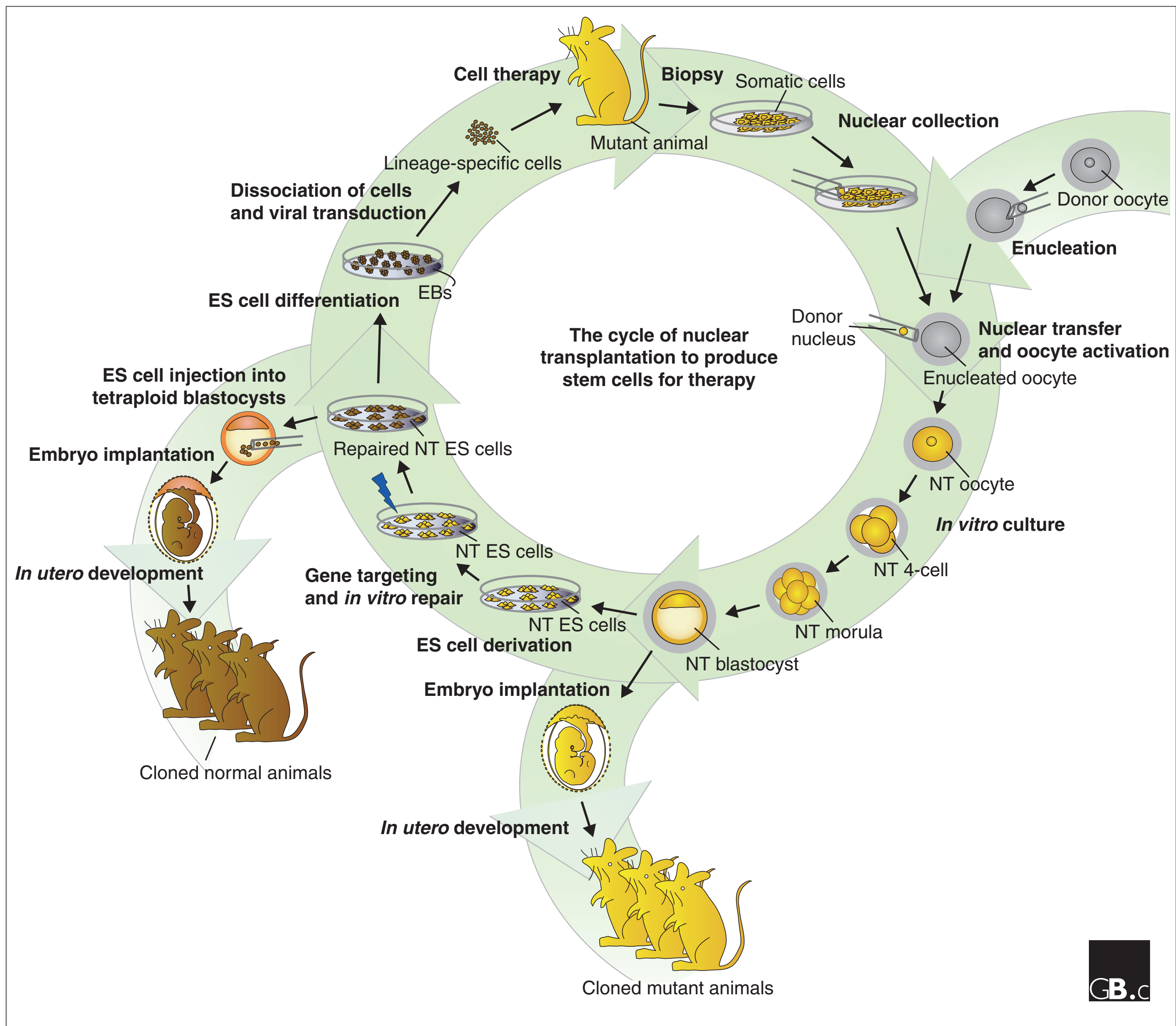

Figure 1

A representation of the procedures used to produce autologous stem cells with a corrected genetic defect for the purpose of cell-based gene therapy in adult animals, based on the experiments reported by Rideout *et al.* [29]. First (top), biopsy samples are obtained from a mutant animal. These are used to establish primary cultures of somatic cells, which will provide donor nuclei. Donor oocytes, arrested at the metaphase II stage of meiosis, are enucleated, and a somatic-cell-derived donor nucleus is transferred into an enucleated oocyte in a procedure known as nuclear transfer. The resulting nuclear-transfer (NT) oocytes are activated and embryogenesis initiates. NT embryos are allowed to develop *in vitro* up to the blastocyst stage, the stage at which mammalian embryos normally implant into the uterus. For embryonic development to continue, the blastocysts must be reintroduced into the uterus of a (surrogate) female, where they will undergo embryogenesis and ultimately produce cloned mutant offspring. Alternatively, pluripotent NT ES cell lines can be derived from the NT blastocysts. NT ES cells bear all the hallmarks of standard ES cell lines, in that they exhibit broad (pluripotent) developmental potential, their genome can be manipulated *in vitro* by routine gene targeting and other transgenic approaches, and they can be differentiated *in vitro* when grown under appropriate conditions. Gene targeting can be used to repair specific genetic defects in the mutant NT ES cells. The corrected NT ES cells can subsequently be introduced into tetraploid blastocysts to generate chimeras that, if implanted into the uterus of a surrogate female and allowed to undergo embryogenesis, can develop into cloned normal offspring. Alternatively, corrected NT ES cells can be differentiated *in vitro* to obtain lineage-specific stem cells, in this case by growth of embryoid bodies (EBs) followed by differentiation to yield hematopoietic stem cells. Finally, the genetically corrected, autologous cells can be used for cell therapy of the mutant animals.

preserving their developmental potential. It has been two decades since ES cells were first isolated, and they remain the mainstay of mouse genome engineering because their genes can easily be manipulated *in vitro* - even down to individual base pairs - by standard gene-targeting and transgenesis techniques, while their developmental potential is

retained. Genome modifications introduced into ES cells *in vitro* can be re-introduced into mice via inclusion in chimeric embryos. ES cells can also be induced to differentiate into defined-lineage cell types under appropriate conditions *in vitro*. Given that ES cells are unequivocally pluripotent stem cells and can be genetically modified, differentiated *in vitro,* and reintroduced into animals, they may be suitable reagents for use in cloning and cell-therapy regimes that aim to repair defects such as the loss of dopaminergic neurons in Parkinson's disease or islet cells in diabetes mellitus.

Embryonic stem cells are not the only type of stem cells - nor are they necessarily the most appropriate type for therapeutic purposes. Lineage-specific stem cells are the progenitors of specific differentiated cell lineages and are present in later-stage embryos and adults in organs such as skin, intestine, brain, and bone marrow. One issue that arises from the presence of such cells is that in animal cloning studies, donor nuclei have been taken from ostensibly differentiated somatic cells, but most donor cell populations are probably heterogenous, and it is not clear whether it is differentiated cells or rare lineage-specific stem cells in the population that give rise to clones. If the latter is the case, it may be that the rarity of stem cells leads to the low efficiencies of cloning - lower than when ES cells are used as nuclear donors [22,23]. Protocols that can distinguish stem cells from differentiated cells would then need to be developed in order to increase overall efficiencies. A key question that has therefore persisted, and remained unanswered until recently, is whether highly specialized lineage-specific cells can be reprogrammed such that they can adopt a totipotent state, with the potential to differentiate into all possible cell types, and thus direct the developmental program used to generate a complete individual.

Reprogramming differentiated cells

Rudolph Jaenisch and colleagues, who have been at the forefront of nuclear-transfer work in mice, designed an experiment to address the issue of reprogramming differentiated lineage-specific cells [24]. They chose lymphocytes as nuclear donors, as these are one of the few cell types of adult mammals whose genome is irreversibly changed as they mature, thereby making them genetically distinct and recognizable [24]. B and T cells are the two classes of mature lymphocytes, expressing immunoglobulins (antibodies) and T-cell receptors, respectively. The type of antibody or T-cell receptor expressed is dictated by the rearrangement of each cell's genomic DNA; mature lymphocytes express only one specific antibody or receptor. Thus, in clones generated from B or T cells, the signature genomic rearrangement present in each donor cell nucleus would be preserved in all the cells of the cloned progeny.

Jaenisch and colleagues' study [25] investigating the developmental potential and reprogramming of lymphocyte nuclei combined the technologies of mammalian cloning and ES cells in a two-step procedure that improved the efficiency of generating clones. First, they generated nuclear-transfer (NT) embryos by transfer of lymphocyte nuclei, but instead of re-implanting the embryos directly into the uteri of foster mothers, they used NT blastocysts to derive NT ES cells (see Figure 1). They then took advantage of the 'tetraploid complementation' technique [26] and injected their NT ES cells into tetraploid host blastocysts [27]. Tetraploid cells preferentially form the extraembryonic tissues trophoblast and extraembryonic endoderm and are excluded from fetal tissues and extraembryonic mesoderm, whereas ES cells exclusively form the latter two tissues [28]. Thus, the chimeric mice generated by Hochedlinger and Jaenisch [24] consisted of a fetus and extraembryonic mesoderm derived from NT ES cells with trophoblast and extraembryonic endoderm derived from tetraploid cells.

These experiments [25] resulted in the production of cloned mice from adult lymphocyte nuclei, as could be recognized by the signature genomic rearrangements of lymphocytes. Jaenisch and colleagues [24] have thus answered the previously unresolved question of whether terminally differentiated cells can provide nuclei for the production of clones, by demonstrating that at least some specialized nuclei can be reprogrammed. Perhaps the tetraploid extraembryonic component used in this procedure may be pivotal in helping overcome some of the defects that have otherwise consistently been observed in cloned embryos (such as enlargement of the placenta). Additionally, the nature of this experimental setup is less demanding of the NT cells, as they do not need to be truly totipotent because all the trophoblast and extraembryonic endoderm derivatives are derived from the tetraploid cells. The NT cells therefore need only to achieve pluripotency to generate the fetus. Also, the extended period of time in culture inherent in the ES-cell-derivation procedure may allow further or more complete reprogramming of the differentiated donor nuclei, ultimately leading to increased developmental potential.

Given that some differentiated cells can indeed donate nuclei that can drive the development of a fetus, how close is the goal of autologous stem-cell therapy? Jaenisch and colleagues have now reported the first successful application of nuclear transplantation to produce stem cells that could be used for cell-based treatment in a model organism [29]. They used *Rag2* mutant mice, which have a defined genetic disorder in immunoglobulin gene rearrangement that renders them immunodeficient, as nuclear donors. *Rag2* mutant NT embryos were generated, from which *Rag2* mutant NT ES cells were derived, and gene targeting was then carried out on the NT ES cells to repair one of the *Rag2* mutant alleles. The potential of the repaired NT ES cells was then tested in two ways. First, repaired NT ES cells were injected into tetraploid blastocysts for the generation of offspring. Normal embryos developed to birth from the

tetraploid chimeras, indicating that the NT ES cells with the repaired *Rag2* gene retained their pluripotency. Furthermore, the presence of normal T and B cells in these mice proved that the repaired *Rag2* allele was functional. Second, repaired NT ES cells were differentiated in culture into hematopoietic stem cells (which form blood and immune cells), and the latter cells were transplanted into adult *Rag2* mutant mice. (Incorporation of the cells into the immune system was not entirely successful because of an immune barrier peculiar to the Rag2-deficient recipients, but this barrier was partially overcome by further manipulation of the immune system of the recipients.) Thus, the procedure was successful in restoring a modest degree of immune function in the mutant mice, but the difficulties encountered suggest that even genetically matched cells derived by nuclear transplantation may still face barriers to effective transplantation in some situations.

Studies carried out in mice routinely pave the way for work in other mammals, including humans, and nuclear transplantation to produce stem cells seems likely to be no exception. For example, a recent paper reports some success in the production and development *in vitro* of a cloned human embryo using cumulus cells (the cells that surround the oocyte) as nuclear donors [30,31], presumably with the goal of using such NT embryos for the generation of human NT ES cells. Also, the issue of histocompatibility has recently been tested in tissues generated from bovine NT embryos cloned from adult bovine fibroblasts [32]. In order to make cloning a feasible approach for generating reagents for the treatment of human diseases, however, the overall efficiency of the procedure needs to be drastically improved. Human oocytes are hard to come by, and nuclear reprogramming is still pitifully inefficient. Headway must therefore be made toward understanding the biology of nuclear reprogramming, and this knowledge must be applied to increasing the efficiency of cloning procedures and stem-cell derivation. Improved understanding should also help avoid the phenotypic aberrations observed in cloned animals. In order to increase efficiencies, surrogate oocytes from other mammals might be suitable as donors in which nuclear reprogramming could take place, or conditions that direct nuclear reprogramming in a non-oocyte environment (ooplasmic transfer) could be developed [8]. Alternatively, protocols for the transfer of oocyte cytoplasm into specialized cells, in the converse of nuclear transfer, may yield further insights. Only when a fundamental understanding of the molecular nature of the biological events underlying animal cloning is gained, however, will cloning represent a truly viable option for cellular therapies aimed at treating disease.

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