Therapeutic cloning in the mouse

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Nuclear transfer technology can be applied to produce autologous differentiated cells for therapeutic purposes, a concept termed therapeutic cloning. Countless articles have been published on the ethics and politics of human therapeutic cloning, reflecting the high expectations from this new opportunity for rejuvenation of the aging or diseased body. Yet the research literature on therapeutic cloning, strictly speaking, is comprised of only four articles, all in the mouse. The efficiency of derivation of embryonic stem cell lines via nuclear transfer is remarkably consistent among these reports. However, the efficiency is so low that, in its present form, the concept is unlikely to become widespread in clinical practice.

Cloning of mammals by nuclear transfer was introduced in 1986 (1) and gained attention by the report of the birth of Dolly the sheep in 1997 (2). To date, seven mammalian species have been cloned from adult cells (3), with the notable exception of primates (4). Mouse cloning has been possible since 1998 (5). The versatility of this small mammal has propelled the mouse as the experimental model of choice for developing new cloning strategies and applications.

Therapeutic cloning, in its current embodiment, entails the derivation of embryonic stem (ES) cell lines from an already born organism (6). This individual may suffer from a disease that is potentially responsive to autologous cell replacement. ES cell precursors appear transiently at the blastocyst stage in early embryonic development, and their cultured ES cell derivatives have the unrivaled ability to differentiate, *in vitro* and *in vivo*, into any specialized cell type. How can cells with ES properties then be derived from a fully grown organism that no longer contains ES cells? Nuclei isolated from a tissue biopsy of an adult organism can be transferred into oocytes. Some of these reconstructed embryos develop into blastocysts, from which ES cell lines can be derived. Provisionally, these cell lines are considered to harbor the same special and highly desirable properties as "conventional" ES cell lines derived from normal embryos produced by fertilization; but to distinguish them from such lines, they are referred to as nuclear transfer ES (ntES) cell lines. The ntES cell lines are expected to be genetically identical to the nucleus donor (the fully grown organism) except for the mitochondrial genome, which is derived from the oocyte. This near-genetic identity holds the promise of circumventing immune rejection of cells originating by differentiation from ES cells, derived from a random embryo that will differ genetically from the diseased individual. Although rejection of foreign cells can be prevented or contained, it remains the bane of transplantation medicine.

An attractive scenario has thus emerged in which each of us could have a few ntES cell lines derived while we are young and healthy, perhaps even shortly after birth. When a disease arises that can be treated by cell replacement, or when our aging body is wearing out due to cell loss, our personal ntES cells would be thawed out, expanded, and differentiated into the desired cell type. Cells would be purified and transplanted to restore the compromised cell function. Various differentiated cell types could be combined to form tissue, organ parts or entire organs, replacing, for instance, a defective heart valve.

Mouse ntES Cell Lines

The first report of a mouse ntES cell line appeared in 2000. Munsie *et al.* (7) used cumulus cells, somatic cells that surround the oocyte when it ovulates in the female, to generate 10 blastocysts, from which a single ntES cell line was developed. This cell line was able to differentiate *in vitro* and *in vivo*, and generated chimeric mice when injected into blastocysts. Shortly thereafter, a total of five ntES-cell like lines were reported, derived from 101 blastocysts produced from nuclei of neural cells. Because the donor cells were of fetal origin, this paper does not fall within the concept of therapeutic cloning, in its strict sense (8). A third study appeared in 2001 and reported the generation of 35 ntES cell lines from 398 blastocysts, generated both from cumulus cells and tail tip cells (9). The distinct advantages of using tail tip cells are that males also have tails, and that a tail biopsy is relatively noninvasive compared with the isolation of cumulus cells; a tail tip biopsy can be regarded as the mouse equivalent of a human skin biopsy. The mouse ntES cells could be induced to differentiate massively into dopaminergic neurons, the cell type lost in Parkinson's disease in human.

The final two articles appeared in 2002 (10, 11). Two ntES cell lines were produced from 41 blastocysts generated by nuclear transfer from B and T lymphocytes of peripheral lymph nodes, demonstrating that the genome of these terminally differentiated cells can be reprogrammed (10). The culmination of the history of therapeutic cloning is a report of the first "therapeutic" effect in the final study (11): one ntES cell line was generated from 27 blastocysts generated by nuclear transfer from tail tip cells of a profoundly immunodeficient mouse, homozygous for a knockout mutation in the recombination activating gene 2. The knockout mutation was "cured" by gene targeting, and the manipulated ntES cells were differentiated *in vitro* into hematopoietic cells, which partially repopulated the crippled immune system of other knockout mice with the same mutation. This is a proof of principle of "therapy," although the experimental design was somewhat artificial.

A high priority is now to demonstrate significant therapeutic benefit of ntES cell derivatives in a model of a natural disease such as type 1 diabetes. Moreover, differentiated cells derived from a ntES cell line established from a sick mouse must be transplanted into the same individual, not in another mouse of the same inbred strain.

Efficiency

These reports make use of the same nuclear transfer technique: piezo-actuated microinjection of somatic cell nuclei into enucleated oocytes. The efficiency, or perhaps better, the lack thereof, is remarkably consistent (Table 1). Not all data are

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Abbreviations: ES, embryonic stem; ntES, nuclear transfer ES.

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Table 1. Efficiency of n+ES cell derivation

Ref.	Reconstructed oocytes	Blastocysts	ntES cell lines	Efficiency, % Per oocyte	Efficiency, % Per blastocyst
7	362	10	1	0.3	10
9	1,016	398	35	3.4	8.8
10	980	41	2	0.2	5
11	202	27	1	0.5	4
Total	2,560	476	39	0.15	8.2

available for each experiment, and some groups report the number of successfully reconstructed oocytes but not the total number of oocytes used for nuclear transfer. With these limitations in mind, 4–16% of cloned blastocysts generated an ntES cell line. Overall the efficiency is 8.2%: 1 ntES cell line can be derived from 12 cloned blastocysts. Unfortunately, none of these studies contains control data for ES cell lines derived in parallel from blastocysts produced from fertilized oocytes, but this value is expected to be >8.2%. The number of oocytes required to generate one ntES cell line in these reports, a number that is critical for human applications, as argued below, varies from as high as ≈1,000 to as low as ≈15 (for cumulus cells from B6D2F1 females; ref. 9). In the most comprehensive study, performed by a single experimentator over a short period, ≈30 reconstructed oocytes were needed to produce one ntES cell line (9).

The testing of the ntES cell lines is incomplete in these reports, and germ-line transmission, the gold standard for pluripotency of an ES cell line, has been demonstrated only for a few lines and not in all reports. A cautious objective for clinical applications is to produce two to three ntES cell lines per individual, realizing that some of these may be aneuploid or not sufficiently pluripotent. This requires, in the best case and in the best hands, 60–90 successfully reconstructed oocytes, say, 100 oocytes available for micromanipulation.

Extrapolation to Humans

This analysis of the limited body of literature raises concerns about the feasibility and relevance of therapeutic cloning, in its current embodiment, for human clinical practice. Nuclear transfer is unlikely to be much more efficient in human than in mouse. Optimistically, ≈100 human oocytes would be required to generate customized ntES cell lines for a single individual. A crucial difference is that, although 100 mouse oocytes can be obtained from a few superovulated females at a cost of ≈$20, human oocytes must be harvested from superovulated volunteers, who are reimbursed for their participation. Add to this the complexity of the clinical procedure, and the cost of a human oocyte is ≈$1,000–2,000 in the U.S. Thus, to generate a set of customized ntES cell lines for an individual, the budget for the human oocyte material alone would be ≈$100,000–200,000. This is a prohibitively high sum that will impede the widespread application of this technology in its present form.

Despite major efforts, the efficiency of nuclear transfer has not increased over the years in any of the mammalian species cloned. Little hope should be placed in a dramatic (say, 10-fold) increase in efficiency in the near future. It becomes imperative to develop alternative strategies for therapeutic cloning, if this approach is ever to make a significant impact on medicine.

Alternative strategies can be divided into oocyte-dependent and oocyte-independent approaches. First, oocytes could be differentiated from existing ES cell lines, so that they can be produced in essentially unlimited numbers. This would eliminate completely the need for human oocyte donors. This exciting new approach has become realistic with a recent report of oocytes derived from mouse ES cells (12). For therapeutic cloning purposes, the oocyte is essentially a processor for reprogramming the inserted nucleus, and its nuclear genome is not carried over in the ntES cells. Another strategy would be to use oocytes from another species, ideally a nonprimate species such as rabbit. However, the idea of generating embryos with mixed human/animal properties, even transiently, is offensive to many people.

In the long run, efforts should be concentrated toward developing oocyte-independent systems, for instance by fusing somatic cells with enucleated ES cells, or by injecting ES cell- or oocyte-derived reprogramming factors into somatic cells. A major benefit of the complete elimination of oocytes and embryos from the concept of therapeutic cloning is that the ethical debate would vaporize instantaneously. In this way, scientific progress may provide a solution to ethical concerns.

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