Inter-species therapeutic cloning: the looming problem of mitochondrial DNA and two possible solutions

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The concept of therapeutic cloning, proposed by Gurdon and Colman,\(^1\) entails the synthesis of tissues or organs for transplantation into a patient starting from embryonic stem (ES) cells that are genetically identical to that patient, due to having been derived from an oocyte whose nucleus was replaced by one taken from the patient (a process termed somatic cell nuclear transfer, SCNT). It remains the leading proposal for exploiting stem cell technology in the clinic in a manner that avoids the severe drawback of immune rejection. However, it presently faces three formidable hurdles. One, our ability to manipulate the differentiation of ES cells along a desired lineage, is progressively being overcome. The others, unfortunately, are more sociological than technical and are thus, perhaps, even greater. These are (a) the profound ethical concerns voiced by highly influential policy-makers and (b) the inadequate supply of human oocytes to permit synthesis of autologous tissues and organs at a rate comparable with demand. Two recent papers\(^2\,^3\) would seem to cast doubt on the feasibility of the only currently available proposal for escaping both these latter problems. In this perspective I will explain that proposal, the problem with it that is highlighted by these papers, and two possible ways to avoid that problem.

First it is appropriate to mention two other proposals, each of which avoids one of the sociological issues mentioned above but not the other. The supply problem might in principle be met by isolating large numbers of oocytes from females who have died in infancy, since the infant ovary contains many times more oocytes than in adulthood. However, this seems likely to encounter even more ardent ethical opposition than the use of oocytes donated by consenting adults. Conversely, the ethical problem may in principle be met by our recently developed ability to differentiate ES cells into oocytes,\(^4\) but the limited availability and variety of the cell lines presently authorised for federally-funded work is potentially a barrier to high-volume production of such oocytes.

The proposal that meets both objections is to use oocytes from non-human species. The idea here is simple. A non-human oocyte is made of non-human proteins, but when its nucleus is replaced by a human one and development is begun, those non-human proteins are progressively diluted and degraded, so that the ES cells that are the source of the eventual tissue or organ are entirely human in composition.

Well, almost entirely—but not, in this protocol, quite. The problem is that not all our proteins are encoded in the nucleus: a paltry 13 of them are encoded in the mitochondrial DNA. Paltry in number but not in function: these proteins are essential subunits of the respiratory chain, without which the cell cannot use oxygen to derive energy from nutrients. The ES cells derived from oocytes with a nucleus originating elsewhere are thus genetic hybrids.

This fact has been discussed extensively in the context of standard intra-species SCNT, because despite being normally encased in the mitochondrion the mtDNA-encoded proteins do somehow find their way to the immunoproteasome and thence to the major histocompatibility complex, which presents them as antigens on the cell surface. The inter-individual differences in amino acid sequence of these proteins could thus, in theory, lead to an immune response even in the absence of any incompatibility of nuclear-coded proteins. However, these sequence differences are so few that this prospect has been deemed highly unlikely.
In the case of interspecies SCNT, however, the sequence disparity is considerable and the immune rejection risk thus very real. This has actually not been much discussed as a drawback of such an approach, possibly because another drawback is much more palpable. The 13 mtDNA-encoded proteins are all subunits of enzyme complexes that also have nuclear-coded subunits. The quaternary structure of enzyme complexes changes during evolution while preserving function, and this occurs by co-evolution of the tertiary structure of the individual subunits. Thus, a hybrid cell derived by inter-species cell fusion, such that the mtDNA is all from a different species than the nuclear genome, can be predicted to exhibit respiratory dysfunction due to inaccurate interlocking of mtDNA-encoded and nuclear-coded subunits. This has indeed been observed.\(^5\)

There might seem to be a ray of hope for SCNT in this regard, however. The micromanipulation of a nucleus from one cell into another necessarily entails the concomitant transfer of a small quantity of cytoplasm from the donor cell, and that cytoplasm contains intact mitochondria. The result is that the mitochondrial DNA of typical oocytes after SCNT is only about 98% oocyte-derived; the remaining 2% is from the donor. Since the propagation of mitochondria and their DNA is thereafter mediated by proteins all encoded in the nucleus (the 13 mtDNA-encoded proteins all being respiratory chain subunits not involved in mitochondrial biogenesis), one might hope that the oocyte-derived mitochondria would be progressively lost in ES cell culture by virtue of inferior propagation. This could happen on two levels: first, mitochondria with oocyte-derived mtDNA would have impaired respiration compared to those with donor-derived mtDNA, since the latter would possess entirely donor-derived (and hence functional) enzyme complexes whereas the former would have hybrid ones; and second, the (donor-derived) machinery for mtDNA replication is somewhat sequence-specific in its binding sites and will thus be less efficient at replicating oocyte-derived mtDNA. Such inefficiency has been shown in cytoplasmic hybrid cells using even very closely-related species.\(^6\)

Unfortunately, two recent papers\(^2,3\) appear to dash these hopes. Careful monitoring of the relative and absolute abundance of donor- and oocyte-derived mtDNA in the early SCNT-derived embryo as it develops to the blastocyst stage shows that, when the donor is a primate and the oocyte is from either cow or rabbit, the donor mtDNA is not merely out-replicated but is actually lost in the few cell divisions following the initiation of mitochondrial biogenesis.

Is this surprising? The most obvious point not yet mentioned might be thought to make it even more surprising than it may seem thus far: that donor-derived mitochondria consist initially of donor-derived proteins as well as DNA, so might be expected to exhibit perfectly healthy mtDNA replication by the donor-derived enzymes that they harboured at the time of SCNT. Oocyte-derived mitochondria might also replicate their DNA efficiently at first, using oocyte-derived proteins already present at the time of SCNT, but these would be progressively diluted by newly-synthesised, hence donor-derived, replication machinery that would work less well on the oocyte-derived DNA than on donor-derived DNA. Thus the embryo would surely gravitate to the desired wholly donor-derived state.

A phenomenon well known to mitochondrial biologists, but perhaps not well enough to biologists in general, provides a simple explanation: mitochondrial fusion. Mitochondrial biogenesis does not turn on in the early embryos analysed in these studies\(^2,3\) until at least four cell divisions have occurred. (This is no surprise, as the oocyte is a large cell with over ten times as many mitochondria as a typical cell.) It is very likely that all mitochondria will have undergone extensive fusion and fission in the period between the SCNT procedure and the onset of mitochondrial biogenesis. This is especially true given the much higher oxygen concentration in the laboratory than the fallopian tube, since oxidative stress is known to induce fusion.\(^7\) Fusion is presumed to result in the mixing of all components of mitochondria. Thus, when mtDNA replication begins, most of the donor-derived mtDNA will be replicated by oocyte-derived proteins from the more numerous oocyte-derived mitochondria. This replication will be inefficient, as noted above. It may be worse than that: replication may actually misbehave, with proteins detaching from DNA or otherwise generating illegitimate replication intermediates, which may be
targeted for degradation, thus explaining the reduction in absolute as well as relative abundance of mtDNA molecules in the embryo over the few cell divisions following the start of mitochondrial biogenesis.

The practical impact of this sequence of events for inter-species therapeutic cloning seems to have been neglected hitherto. Perhaps this is because it has proven possible to generate ES cells from such embryos.\(^8\) That is not enough, however, because ES cells are probably no different from other cells in culture in being highly glycolytic and making only modest use of their respiratory chain. That would not be true of the differentiated cells that would compose a tissue or organ grown from ES cells for transplant into a patient. Thus, such tissues are likely to prove impossible to generate, on account of the impaired respiratory capacity of the hybrid mitochondrial enzyme complexes.

All is not lost, however: two solutions to this conundrum seem evident (Figure 1). The first exploits the fact that the period during which the donor mtDNA is at a replicative disadvantage should be quite short, lasting only from the time that mitochondrial biogenesis begins to that by which most nuclear-coded mitochondrial proteins (and, in particular, mitochondrial biogenesis machinery) is donor-derived. If even a few percent of the mtDNA is donor-derived at the point when it starts to be selected for, all will shortly be well. Thus, donor mtDNA could simply be introduced in greater quantity than occurs by SCNT per se, by a separate process of fusion with an enucleated donor-derived cell. This fusion could most straightforwardly be performed after the hybrid embryo has given rise to an ES cell line (strategy 1A). If it transpires that such embryos are often too respiration-impaired to allow the creation of such a line, an alternative would be to fuse ES cells that are non-human in both genomes with enucleated human cells, then immediately differentiate those cells into oocytes before their human mtDNA has been lost, and then perform SCNT on those oocytes (strategy 1B). The latter procedure would at no point involve cells containing mostly mitochondria from a different species than the nucleus, so bioenergetic competence should not be an issue.

The second possible solution is technically ambitious but has two advantages over the first: it entirely avoids issues of bioenergetic competence, and it solves another aspect of aging (in the eventual transplanted tissue) at the same time. This is to incorporate suitably modified copies of the 13 protein-coding genes of the mtDNA into the nuclear DNA of a donor cell before SCNT (strategy 2). The modifications necessary to make such genes work are now virtually proven to be restricted to lessening the encoded proteins’ hydrophobicity enough to allow import through the mitochondrial membrane without impairing their tertiary structure or stability within the inner membrane; progress in doing just this has been rapid in recent years.\(^9\)\(^-\)\(^12\) A donor nucleus containing all such genes would be entirely autonomous, capable of maintaining complete mitochondrial function even if the mitochondrial DNA is incompatible or is lost: indeed, such loss will probably be rapid and would be positively beneficial, as it would remove competition with the non-human, mtDNA-encoded proteins in complex assembly.

The ethical and logistical challenges to therapeutic cloning are presently considerable. Inter-species SCNT offers a uniquely clear escape from these challenges, if it can be made to work. It is thus essential to explore candidate solutions to the mtDNA problem without delay.

References


Figure 1. Three possible ways to create ES cells containing only human genes from a prospective patient, and hence capable of differentiation into autologous respiration-competent tissues, without destroying a human oocyte. In strategy 1A, SCNT creates an oocyte with human nuclear but non-human mitochondrial DNA, which develops far enough to isolate ES cells that are then fused with enucleated cells from the patient, leading to loss of the non-human mtDNA. In strategy 1B, the order of introduction of the patient’s nuclear and mitochondrial DNA is reversed: a non-human ES cell line is fused with enucleated cells from the patient, giving it a large proportion of human mitochondrial DNA, and is then differentiated into oocytes, which after SCNT are stimulated to develop through early embryogenesis, by which time the selective disadvantage temporarily suffered by the human mtDNA has been reversed and ES cells can be isolated. In strategy 2, patient cells are transfected in vitro with engineered constructs causing them to express all 13 normally mtDNA-encoded proteins from the nucleus, and a nucleus from such a cell is used for the SCNT procedure; the eventual ES cells and tissues have no mtDNA at all. Flasks indicate ES cells, thick borders indicate oocytes. Red indicates non-human; black, human patient; dark red cytoplasm, mtDNA hybrid; blue nucleus is human and expresses human mtDNA-encoded proteins from transgenes.