Regenerative medicine and embryonic stem cells: are there alternatives?

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Use of human embryonic stem (ES) cells in regenerative medicine is associated with ethical problems because these cells can only be obtained from human embryos generated outside the human body. A recent discovery suggests that human ES-like cells can be obtained without generating human embryos thus providing a problem free solution for regenerative medicine.

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Introduction

Regenerative medicine can be broadly defined as development of innovative therapeutic approaches that will enable the human body to repair and regenerate damaged cells, tissues or organs. Current scientific evidence favours the use of one specific cell type for therapy, namely human embryonic stem (ES) cells. These cells can only be obtained from human embryos generated outside the human body. Historical evidence suggests that experimentation on parts of human body will inevitably create ethical problems. As a result, the proposed use of ES cells in therapy is currently one of the most hotly contested areas of biomedical research. Some ethical issues associated with the use of ES cells in regenerative medicine will be highlighted here and recent scientific developments that can potentially eliminate ethical problems will be presented.

Stem cells are usually defined as cells capable of both self renewing and producing different cell types. On the basis of their potential to generate different cell types, stem cells are classified into several categories (Table 1). In mammalian organisms only zygote and early blastomere are totipotent. Totipotent cells can generate all cell lineages of an organism, including extra-embryonic tissue. Embryonic stem (ES) cells, on the other hand, are pluripotent and they can generate all cell types of the body in vivo and in vitro

Table 1 Classification of stem cells according to their developmental capacity

Potency	Developmental capacity	Cell type
Totipotent	All cell lineages including extra-embryonic tissue	Zygote and first cleavage blastomere
Pluripotent	All cell lineages but no extra-embryonic tissue	Embryonic stem cells
Multipotent	One cell lineage	Adult stem cells (e.g. hematopoietic cells which will produce all blood cells)
Unipotent	One cell type	Cells that produce terminally differentiated cells (e.g. spermatogonial stem cells which will produce sperm)

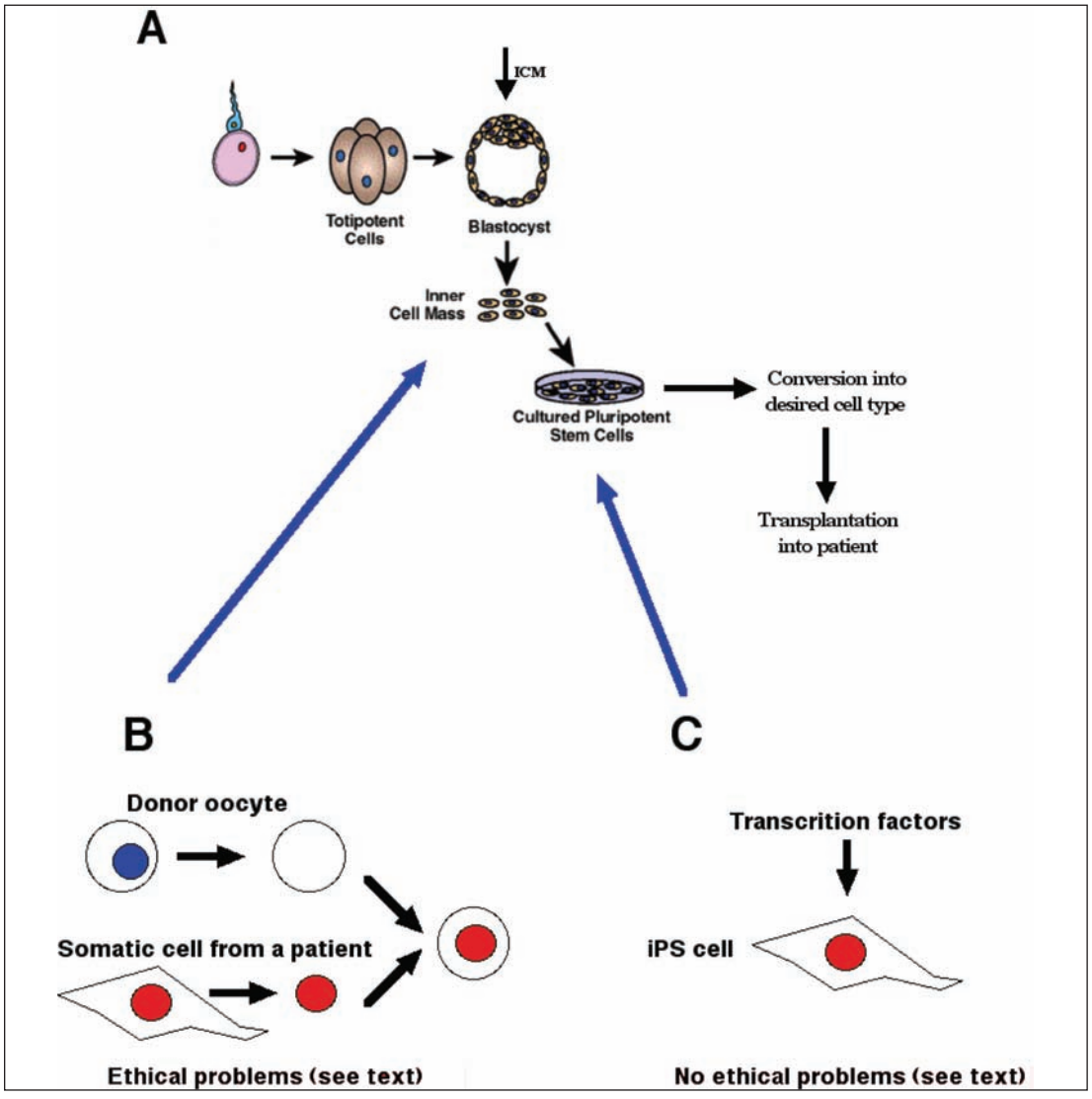

Figure 1 **A**. Isolation of human ES cells from human embryos. ES cells are depicted as Cultured Pluripotent Stem Cells. These cells should be converted into a desired cell type before transplantation into patients. ICM (Inner Cell Mass). **B**. Procedure for Somatic Cell Nuclear Transfer or therapeutic cloning. Nucleus removal from a donor oocyte and a patient somatic cell are depicted, as well as the subsequent insertion of the patient cell nucleus (red) into the enucleated donor oocyte. The resulting hybrid cell now has the same fate (blue arrow) as the naturally fertilized egg in panel A. ES cells obtained in this way are an exact genetic match for the patient. **C**. An iPS cell generated by cell reprogramming (addition of transcription factors into a somatic cell). The blue arrow indicates that iPS cells are equivalent to pluripotent stem cells and can be used in therapy.

but cannot generate extra embryonic tissue. Each tissue has a pool of adult stem cells which are multipotent and can generate all cell types of one lineage, for example hematopoietic cells. The ability of ES cells to generate all cell types of the body makes them a far more attractive target for regenerative medicine than adult stem cells. For example, ES cells can be experimentally induced to differentiate into any desired cell types (e.g. heart muscle cells) and then transplanted into damaged tissues/organs (e.g. heart) to fully regenerate them. Adult stem cells, on the other hand, are restricted to only one tissue type.

The proof of principle for the above ES cells-based theoretical framework which underpins regenerative medicine (Figure 1 A) already exists in the case of laboratory animals such as mice. For example, mice used as a model of Parkinson's disease (1) show significant improvement after transplantation of dopamine neuron cells, damaged in Parkinson disease, into the midbrain of Parkinsonian mice. Therapeutic dopamine neuron cells have been obtained from genetically matched mouse ES cell lines by somatic cell nuclear transfer (SCNT) followed by neural induction and differentiation into midbrain dopamine neurons (for details of SCNT see below). This promising animal study raises an important question: would the same approach work in humans? At present, there is no scientific reason to believe that it would not. However, as discussed above, ethical issues make the above approach questionable in humans.

Ethical issues

One of the major ethical and practical issues that seriously undermine the use of ES cells in regenerative medicine is the fact that human ES cells can only be obtained from human embryos. Human embryos can be generated outside the human body in *in vitro* fertilization (IVF) clinics during the course of infertility treatment. Unused fertilized eggs and/or unused donor oocytes that remain after IVF are the exclusive resources for generating human ES cell lines. A five day old human embryo, known as a blastocyst, has roughly 100 cells. Approximately 30% of blastocyst cells form the so called inner cell mass (ICM). ICM contains pluripotent ES cells (Figure 1 A). The standard procedure for generating ES cell lines includes removal of ICM from blastocysts and expansion of ES cells *in vitro* (Figure 1 A). The resulting ES cell lines can be stored indefinitely as therapeutic material. This procedure was successfully used for the first time in 1998 (2). In most countries it is permitted to generate ES cell lines for research. One of the rare exceptions is the US. The federal US government prohibits funding of scientific work that aims to create human ES cell lines. However, funding from non-governmental resources is allowed for this type of work.

A prerequisite for any successful regenerative therapy is that ES cells must be genetically matched to the patient in order to avoid tissue rejection. Exact genetic matching can only be achieved by using the patient's own cells. A laboratory technique known as SCNT, which was instrumental in generating the first cloned mammal, Dolly the sheep, is at present the only way to obtain genetically matched therapeutic ES cells (Figure 1 B). Scientists working in the field of regenerative medicine frequently refer to SCNT as therapeutic cloning. In this procedure a somatic cell (e.g. skin cell) is taken from a patient, the nucleus of this cell removed and inserted into a donor oocyte whose nucleus has also been removed (Figure 1 B). The resulting hybrid cell is allowed to divide to form a blastocyst from which ES cell lines will be obtained (Figure 1B). These ES cell lines are the exact genetic match for the patient's tissues, thus eliminating prob-

lems associated with tissue rejection. The ES cell lines can be used to obtain any single human cell type that may be required for therapy.

However, therapeutic cloning could inadvertently pave the way for reproductive cloning. Reproductive cloning can occur if the cloned blastocyst is allowed to be implanted into a uterus. The General Assembly of United Nations has officially adopted a document (The 2005 UN Declaration on Human Cloning) which calls upon all member countries to prohibit reproductive cloning. Some countries including the UK have allowed scientists to proceed with therapeutic cloning by issuing appropriate licences. All human embryos generated during the course of this procedure must be destroyed before they become 14 days old. However, in many countries therapeutic cloning has not been formally allowed yet.

In summary, the current theoretical framework behind regenerative medicine is almost entirely based on (a) human ES cell lines as the key therapeutic material and (b) therapeutic cloning as a way of preventing tissue rejection upon transplantation (Figure 1 A and B). This theoretical framework has both practical and ethical problems. Practical problems are highlighted by the fact that there is no unlimited supply of human oocytes or fertilized eggs required for therapeutic cloning. The key ethical problem is the danger that therapeutic cloning could be misused and attempts made for reproductive cloning in people.

Negative developments

The single event that added the greatest element of controversy to the already controversial field of human therapeutic cloning was the case of scientific fraud by the Seoul University research group led by Dr. Hwang Woo-Suk. In a scientific paper published in the prestigious journal Science Dr. Woo-Suk and his colleagues claimed that the above theoretical framework behind regenerative medicine (Figure 1) works in practice. In other words, they claimed success in transferring the human somatic cell nucleus into the human oocyte, propagating the resulting hybrid cell in vitro until the stage at which they were able to isolate human ES cells (blastocyst) and subsequently establishing human ES cell lines, perfect genetic matches for 11 different patients. However, detailed scrutiny of the published material by the scientific community raised some doubts about the authenticity of the published photographs of cultured ES cells. This led to a full investigation by the Seoul University which concluded that the entire study was fabricated. The published paper was eventually retracted by the journal Science and Dr. Woo-Sook was suspended. This case of scientific fraud seriously damaged the credibility of therapeutic cloning. Also, some scientists questioned whether therapeutic cloning will ever be practical, given problems such as its low success rate (see below).

At present, there is evidence that some aspects of human therapeutic cloning work in practice, namely production of cloned blastocysts (3). However, no human ES cell lines have been obtained yet from cloned blastocysts and future studies will show if this is possible. Recent developments in primates provide strong support for the notion that human therapeutic cloning will work in practice. For example, the journal Nature published a study last year in which scientists were able to verify therapeutic cloning for the first time in a species close to humans – rhesus monkeys (*Macaca mulatta*) (4). The research group led by Shoukhrat Mitalipov from Oregon National Primate Research Centre used several hundred monkey oocytes to obtain 35 cloned blastocysts. In order to generate ES cell lines the 20 best cloned blastocysts were selected resulting in 2 monkey ES cell lines. It is not difficult to

spot the major problem here: the low success rate of therapeutic cloning in primates. Although Mitalipov's study used an improved procedure for therapeutic cloning the success rate of this technique (oocyte to ES cell line ratio) is still extremely low. In order to obtain 2 ES cell lines Mitalipov and colleagues used a total of 304 oocytes (0.7% success rate). This is a serious practical problem which becomes ethically questionable in the context of human therapeutic cloning. For example, if the success rate of therapeutic cloning in humans is similar to that of monkeys, and without significant improvements, many oocyte donors may be required to produce a single genetically matched ES cell line for a single patient.

Are there alternatives to human therapeutic cloning?

Given the success with therapeutic cloning in Parkinsonian mice and their significant improvement after transplantation of ES cell-derived dopamine neurons into their midbrains (see above) and clear indications that human cloned blastocysts can be generated (3) some scientists argue that it is still worth pursuing human therapeutic cloning. However, it is also reasonable to search for alternative approaches that may eliminate the ethical problems discussed.

Modifications of therapeutic cloning have recently been developed in order to address some of the ethical problems. For example, Meissner and Jaensich from MIT developed a technique in mice which prevents implantation of a cloned blastocyst into the uterus (5). If this technique is replicated in humans it would essentially prevent the use of cloned blastocysts for reproductive cloning. However, it is likely that the technique will be difficult to implement in human oocytes because they are sensitive to experimental treatments. More recently, scientists in the UK have applied for a licence to create animal-human hybrid embryos by using animal instead of human oocytes. In September 2007 the UK Human Fertilization and Embryology Authority approved these applications. By using animal oocytes the problem of large numbers of human oocyte donors required for therapeutic cloning can certainly be eliminated. However, creation of animal-human hybrid embryos raises further ethical concerns and causes new problems e.g. whether animal viruses can spread into the human genome.

These proposals are only minor amendments of the present technology for therapeutic cloning and none of them really provide problem-free solutions to serious ethical issues raised by therapeutic cloning. Ethical problems can be fully eliminated only by providing source(s) of therapeutic cells which do not originate from human embryos. A reasonable alternative is the use of human adult stem cells which are present in small numbers in each adult tissue. However, these cells have limited developmental potential (see Table 1). In addition, raising a sufficient number of adult stem cells for therapy may not be practical. An alternative scenario would be reprogramming somatic human cells in order to convert them into cells which will have the properties of human ES cells. Is this possible?

Many scientists have been trying to develop protocols that could convert somatic cells into ES-like cells but without much success. However, after painstaking work, the Japanese scientist Shinya Yamanka and his PhD student Kazuthosi Takahashi from Kyoto University have recently been able to reprogram mouse somatic cells so that they essentially become cells with all the major characteristics of mouse ES cells (6). They managed this by simply introducing four transcription factors into mouse somatic cells: Oct3/4, Sox2, c-myc and Klf4. Apparently, these factors alter the genetic programme of mouse somatic cells and lead

them to dedifferentiate and become ES-like. To make them distinct from ES cells, reprogrammed cells are now called iPS (induced pluripotent stem) cells. This important discovery led Yamanaka to try the same procedure with human cells. Not surprisingly, Yamanaka and colleagues have been able to generate human iPS cells from human skin fibroblasts, using the same combination of transcription factors as in the case of mouse cell reprogramming (7). In addition, a research group led by James Thompson from Wisconsin University reproduced Yamanaka's results and obtained human iPS cells using a slightly different combination of transcription factors (8). Tests have shown that human iPS cells have all the major characteristics of human ES cells including self-renewal and pluripotency, suggesting that iPS cells may be suitable for therapy.

Taken together, studies by Yamanaka, Thompson and their colleagues unequivocally show that human somatic cells can be converted into ES like cells. Many scientists now believe that iPS cells constitute a superior option for regenerative medicine in comparison with therapeutic cloning-generated ES cells because the iPS cell technology is free from major ethical problems (Figure 1 C). For example, Dr Ian Wilmut, credited as the scientist behind creation of Dolly the sheep and the pioneer of therapeutic cloning, has recently abandoned therapeutic cloning in favour of iPS cells. In addition, many US and European laboratories are switching their work to iPS cells. However, there are still some unanswered questions that must be addressed before iPS cells can be used in therapy. For example, iPS cells contain viruses which are used for introduction of transcription factors. Some of these viruses may cause tumorigenicity of iPS cells after transplantation into patient bodies. In addition, one of the transcription factors originally used to reprogram human skin cells by Yamanka's team is c-myc, an oncogene. Oncogenes are genes that promote tumour growth suggesting that the presence of this transcription factors may increase the risk of tumour formation by therapeutic cells. However, Yamanaka's team have recently managed to reprogram human skin cells without the c-myc transcription factor (9). It is also possible to select viruses for introduction of transcription factors that will be harmless to humans, thus eliminating problems of tumorigenicity.

Do the above developments mean the end of therapeutic cloning? Not necessarily. Proponents of therapeutic cloning believe that it still represents a viable option for therapy. Their main argument is that iPS cells are not 100% equivalent to ES cells and until it is unequivocally proven that iPS cells are safe for therapy in humans the work on human therapeutic cloning should continue. This is understandable since human ES cells represent the golden standard against which all potentially therapeutic cells should be measured. However, one thing is clear. There is now a hope that in the near future the technology for production of therapeutic cells in regenerative medicine will be free from the ethical problems that undermine therapeutic cloning.

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