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## Derivation of embryonic stem cells for cellular therapy: Challenges and new strategies

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### Summary

Cellular therapy is the replacement of unhealthy or damaged cells or tissues by new ones. Embryonic stem (ES) cells are undifferentiated cells that can generate all the cell types of the body, and therefore hold the potential to cure a broad range of diseases and injuries, ranging from diabetes, liver and heart diseases, to neurological diseases, such as Alzheimer's and Parkinson's diseases. The derivation of human ES (hES) cells has been a major step toward bringing ES cell research to therapy. However, there are several challenges to the advent of ES cell research to therapy. Among them, the derivation of hES cell lines devoid of animal contaminants, the maintenance of their normal karyotypes, their potentials to form tumors upon grafting, and the derivation of isogenic hES cell lines. Stringent ethical and political guidelines are also limiting the use of human embryos for research, thereby limiting progress in ES cell research. Recently, several investigators have devised protocols to derive hES cells free of feeder layer and animal serum, reported that some established cell lines remain stable overtime, pre-differentiated ES cells *in vitro* to circumvent the risk of tumor formation, and derived ES cell lines without destroying embryos. In this manuscript, we will review and discuss these developments that may unlock ES cell research and therapy.

**key words:**

nuclear transfer • cloning • tumor • isogenic • development • embryo

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## BACKGROUND

ES cells have the ability to remain undifferentiated and proliferate indefinitely *in vitro* while maintaining the potential to differentiate into derivatives of all three embryonic germ layers [1]. They are derived from the inner cell mass (ICM) of blastocysts at a stage (4 to 5 day of a human embryo) before it would implant in the uterine wall. ES cells have been isolated from primate human, – but also non-human (rhesus monkey) – blastocysts, cultured and maintained *in vitro*; thereby providing an unlimited source of ES cells for cellular therapy [2–4]. The derivation and maintenance of ES cells *in vitro* require stringent culture conditions to remain undifferentiated (Figure 1). Human ES (hES) cells are grown on mouse embryonic fibroblast feeder layers, and in the presence of various reagents of animal origin, e.g., coating substrates, serum. Such culture conditions are not without limitations. Recently, it was reported that mouse feeder layers and/or media used for culturing hES cells could lead them to incorporate N-glycolyl-neuraminic acid residues present in these animal sources [5]. N-glycolyl-neuraminic acid is a sugar present on the surface of most mammal and rodent cells, but is lacking in humans [6], and against which most humans have circulating antibodies [7]. The incorporation of N-glycolyl-neuraminic acid residues on hES cell lines would result in the rejection of the graft, thereby, limiting the use of existing hES cell lines for cellular therapy, and mandating for the generation of new cell lines devoid of animal contaminants [5]. To circumvent such limitation, protocols have been devised for culturing hES cells on autogeneic feeder layer [8,9], free of feeder layer [10]. Recently, new hES cell lines have been derived free of feeder layer and animal serum [11]. New hES cell lines free of animal contaminants may offer a source of tissue for cellular therapy.

## FROM ES CELLS TO THERAPY: THE CHALLENGES AHEAD

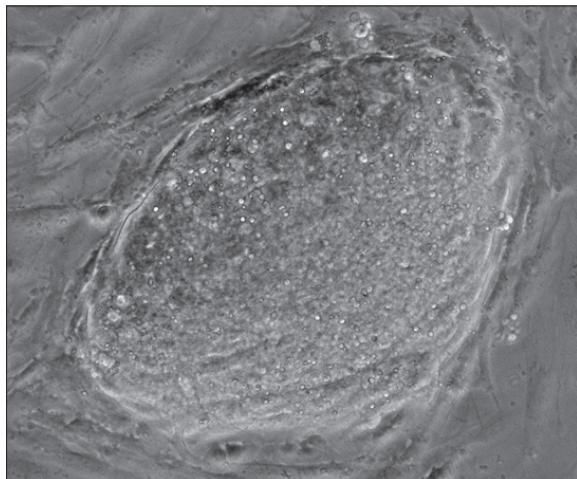
There are several challenges to the advent of ES cell research to therapy. Some investigators have reported that hES cells do not maintain their normal karyotypes [12–14], while others have confirmed that some established cell lines remain stable overtime [13,14]. Though the incidence of such instability on the behavior of the cells and their ability to differentiate is not well understood, established cell lines must be maintained under stringent culture standard, and be checked overtime for normal chromosomal content. ES cells have also the potential to form tumor upon grafting [1]. The formation of teratoma would be associated with the undifferentiated state of the ES cells. To circumvent the risk of tumor formation, it is proposed to pre-differentiate the ES cells *in vitro* to the desired lineage, and to remove the cells that have not differentiated from the cellular graft prior to grafting. Protocol leading to a 100% differentiation, or purification by positive selection –by isolating the differentiated cells from the bulk culture– would provide alternative strategies to this aim. Cell surface markers and fluorescent activated cell sorting are strategies that are considered for eliminating undifferentiated cells, and have been successfully tested in experimental set-up [15], as well as protocols leading to high yield of differentiated ES cells, such as differentiated oligodendrocytes [16]. A third challenge is the potential immunogenicity of the hES cell lines. hES cell lines are allogenic cell lines, de-

rived from blastocysts. To limit the risk of rejection by the patient, upon transplantation, would require matching the donor and recipient genetic make-up. Patients may also follow immunosuppressive treatments, such as treatment with cyclosporine. An alternative would be to generate isogenic hES cell lines from the patients by somatic cell nuclear transfer (SCNT) [17]. SCNT consists in isolating nucleus of a somatic cell type (fibroblast for example) harvested from the future recipient into an enucleated oocyte. By mechanisms still unknown, the cytoplasm of the oocyte reprograms the chromosomes of the somatic cell's nucleus. The cloned cell develops into a blastocyst from which ES cells can be derived, that carries a set of chromosomes identical to that of the donor, and therefore is unlikely to be rejected by that donor/future recipient [17,18]. Recently, 11 hES cell lines were established by SCNT of skin cell nuclei from patient with various diseases into donated oocytes, setting the stage for future studies assessing the potential of isogenic hES cells and SCNT, as a strategy to derive ES cell lines, for therapy [19]. However, though SCNT has such potential, unknown regarding the behavior of the generated cell lines and tissues, in term of viability, development are major hurdles to overcome for therapeutic cloning, the cloning of individuals to get matching cells, tissues, or organs from the resulting embryos [17].

Asides from these challenges, major constraints faced by ES cell research, and SCNT, are the ethical and political concerns over the use of embryos for research [20]. Recently, several investigators have proposed several alternatives to the derivation of ES cells without destroying embryos or cloned embryos.

## OVERCOMING THE ETHICAL AND POLITICAL CHALLENGES: ALTERNATIVE PROTOCOLS TO DERIVE ES CELLS

Two recent manuscripts have reported the derivation of ES cells without destroying embryos. In a first manuscript, Chung et al. (2005) reported a protocol to derive ES cells by applying a technique of single-cell embryo biopsy [21], similar to pre-implantation genetic diagnosis (PGD) of genetic defects used in fertility clinics. This genetic diagnosis consists in extracting a cell –to be used for genetic testing– from an 8 cells stage embryo (blastomere), a procedure that does not interfere with the developmental potential of embryos. In the report, the investigators extracted single cells from eight-cell mouse blastomeres (2 days old); the researchers cultured the isolated cells and found they behaved like ES cells. Meanwhile, the embryos went on to produce mice. The result suggests that when clinics perform PGDs, the isolated cells could be grown. Resulting cultures would then be used for genetic testing, and to establish stem cell lines. In addition to deriving ES cells from the “donors” –that would be stored–, the different cell lines established could make up a stem cell bank. A second report by Meissner and Jaenisch (2005) used, a variation of SCNT, called altered nuclear transfer (ANT), to generate ES cell lines [22]. ANT has been proposed as a variation of nuclear transfer because it would create abnormal nuclear transfer blastocysts that are inherently unable to implant into the uterus but would be capable of generating customized ES cells. ANT concept is based on the premise that the inactivation of a gene crucial for trophoblast development will eliminate the potential to form the fetal-



**Figure 1.** Embryonic stem cells. Mouse ES cell colony on mouse fibroblasts. ES cells remain in an undifferentiated stage upon rigorous culture conditions. ES cells are undifferentiated cells that can generate all the cell types of the body. They have the ability to remain undifferentiated and proliferate indefinitely *in vitro*, thereby providing an unlimited source of cells for cellular therapy. They are derived from the inner cell mass (ICM) of blastocysts.

maternal interface, but will spare the ICM (inner cell mass) lineage [23]. The investigators chose *CDX2* as a candidate gene; this gene encodes the earliest-known trophoblast-specific transcription factor that is activated in the 8-cell embryo and is essential for establishment and function of the trophoblast lineage, so that the eggs could not produce an embryo that can implant in a uterus. The cloned blastocysts were morphologically abnormal, lacked functional trophoblast and failed to implant into the uterus. Yet, the eggs divided and grew enough to yield stem cells [22]. Since the cell lines are derived from a variant of SCNT, if applicable, such technique would allow the generation of ES cell lines that would match the patient genetic make-up, thus allowing autologous transplantation. However, one of the limitations in the reported procedure is the use of virus (lentil virus) to inactivate the gene *CDX2*; such genetic manipulation may affect adversely the ES cells, and may present some risks for the recipient.

These reports proposed alternative protocols to derive ES cells. The acceptance of these strategies by the scientific, ethical and political communities may impact the future of ES cells for cellular therapy. These studies also demonstrate new properties of ES cells, and will contribute to our understanding of ES cells and in turn to bring ES cell research closer to therapy.

## CONCLUSIONS

Various cell types have been considered for cellular therapy. Among them, ES cells represent a major area of research, as the potential of ES cells to generate all the cell types of the body make them a valuable source of cells for therapy. Data discussed here show that though ES cells have such potential, there are major challenges to overcome to bring ES cells to therapy, particularly ethical and political concerns [24]. Other ethical issues, such as the possible shortage of oocytes

and their possible non-ethical origin remain the source of concerns, particularly for SCNT. The recent revelations regarding the procedures for the recruitment of oocyte donors [25], force us to ensure strict compliance with ethical standard and particularly with consent donors. Further, in the case of ANT, it has been argued that finding acceptable to destroy a *CDX2* mutant embryo but not a normal embryo is “a flawed proposal”, as there is no basis for concluding that the action of *CDX2*, or any other gene, represents a transition point at which a human embryo acquires moral status [26]. Thus, whether ANT solves the ethical dilemma of whether the mutant embryo is equivalent to normal embryo remain the source of debates and controversies [27]. Stem cells can also be isolated from adult tissues. Adult-derived stem cells may thus offer alternative strategies for cellular therapy, particularly for the central nervous system [28–31].

## Note in addendum

Since the submission of this manuscript, the Journal *Science* issued the following statement on 04 January 2006, regarding the manuscript entitled “Patient-specific embryonic stem cells derived from human SCNT Blastocysts” [19]: “All authors have indicated a willingness to retract the 2005 paper (“Patient-specific embryonic stem cells derived from human SCNT Blastocysts” May 19 2005, *Science Express*; June, 2005, *Science*)”.

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