DO WE NEED MORE HUMAN EMBRYONIC STEM CELL LINES?

B. Arabadjiev1,2, R. Petkova2, S. Chakarov1,2, A. Momchilova1 and R. Pankov1
1Sofia University “St. Kliment Ohridsky”, Faculty of Biology, Department of Cytology, Histology and Embryology, Sofia, Bulgaria
2Scientific Technological Service Ltd., Sofia, Bulgaria
3Institute of Biophysics, Bulgarian Academy of Sciences, Sofia, Bulgaria
Correspondence to: Stoyan Chakarov
E-mail: stoian_chakarov@abv.bg

ABSTRACT
The enormous potential of human embryonic stem cells is fueling continuous research aimed at establishment of new lines of these cells. Currently, research groups from 24 countries have reported derivation of over 1000 human embryonic stem cell lines. Because of the controversy surrounding the derivation of these cells from human embryos it is important to clarify whether the existing hESC lines are sufficient for basic research and future therapeutic applications. Here we briefly review some of the most important arguments justifying the need for continuing derivation of human embryonic stem cell lines.


Keywords: embryonic stem cells, induced pluripotent stem cells

Abbreviations: hESC- human embryonic stem cells; ICM- inner cell mass; IVF- in vitro fertilization; HPC- hematopoietic stem cells; PDG- preimplantation genetic diagnosis; iPSCs- induced pluripotent stem cells; GMP- good manufacturing practice

Introduction
The establishment of the first five human embryonic stem cell (hESC) lines by Thompson and colleagues in 1998 (39) generated great excitement over the opportunities for new breakthroughs in human biology and development of new and fundamentally different approaches for improvement of human health. This finding may also provide new tools for drug discovery and toxicity testing as well as for studying human development, human pathology and regulation of gene expression. The unique ability of hESCs both to proliferate indefinitely in their undifferentiated state and to differentiate supposedly to all of the 220 different cell types that are present in the adult organism holds extraordinary promise for development of cellular therapies to treat debilitating or fatal conditions. The latter ability of hESCs, which is known as pluripotency (4, 30) is normally restricted to cells of the inner cell mass (ICM) that only exist for a few days in the early embryo. However, if isolated in vitro, the ICMs can give rise to human embryonic stem cell lines (Fig. 1) that can be propagated or kept frozen for long periods of time, thus serving as an unlimited source of cells for scientific studies, pharmacology testing and cell therapy.

Fig. 1. A typical colony of human embryonic stem cells, isolated from ICM of 5 day old IVF embryo, grown on feeder of inactivated mouse STO cells

Human embryonic stem cell lines inventory
Since 1998 a number of laboratories all over the world reported derivation of new human embryonic stem cell lines. Up to date, more than 1000 hESC lines have been announced, originating from 24 countries, although only 223 are available for further research (Table 1).

This seemingly large number poses the question whether we have reached a sufficient amount of hESC lines necessary for the scientific studies and therapeutic purposes. The issue becomes even more important taking into consideration the ethical concerns surrounding the derivation of these cells. Despite the established procedure for hESC line derivation without embryo destruction (7), the traditional and most used method involves isolation of the ICM from whole surplus IVF embryos, which inexorably eliminates their potential
for further development. The latter has motivated the pro-life movement, whose members are concerned with the rights and status of the embryo as a person, therefore having the right to live, and resulted in a variety of national legislations regulating human ESC research. Although hESC lines are derived under strict regulation and from “discarded embryos,” that is, from embryos that were left over from fertility treatments and therefore at least some of them were not supposed to live anyway, this ethical debate strengthens the need to carefully evaluate the necessity for establishment of new human embryonic stem cell lines.

Such evaluation is a difficult task considering the fact that only a limited number of the existing hESC lines have been studied extensively. According to a recent study by Loser et al. (25), only three hESC lines- H9, H1 and H7, derived by Thomson and colleagues and provided by WiCell Research Institute, have been used in more than 50% of the scientific papers published between 1998 and 2008. Another eight cell lines (BG01, BG02, HES-2, HES-3, HES-6, HUES7, HUES9 and i6) account for more than 40% of the research articles that appeared during the same period. Despite this low number of thoroughly evaluated and tested hESC lines, recent data suggest that the existing cell lines may have serious shortcomings (21, 27, 37) that may restrict their use for some scientific and most of the therapeutic purposes.

### TABLE 1

Overview of the established hESC lines worldwide

<table>
<thead>
<tr>
<th>Region</th>
<th>Country</th>
<th>Total number of established hESC lines</th>
<th>Number of hESC lines, currently available for research</th>
</tr>
</thead>
<tbody>
<tr>
<td>EU countries</td>
<td>Denmark</td>
<td>27(31)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Netherlands</td>
<td>4(4)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>United Kingdom</td>
<td>38(42)</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>13(18)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>France</td>
<td>20(20)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Belgium</td>
<td>24(29)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Czech Republic</td>
<td>7(7)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Sweden</td>
<td>72(90)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Finland</td>
<td>10(10)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>215(251)</strong></td>
<td><strong>161</strong></td>
</tr>
<tr>
<td>Non-EU countries</td>
<td>Canada</td>
<td>2(4)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>USA</td>
<td>219(434)</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Brazil</td>
<td>(1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Switzerland</td>
<td>1(1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Israel</td>
<td>15(14)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
<td>18(18)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Russia</td>
<td>3(16)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Iran</td>
<td>6(6)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>India</td>
<td>9(15)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Japan</td>
<td>3(3)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>South Korea</td>
<td>32(37)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>China (including Taiwan)</td>
<td>42(236)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Singapore</td>
<td>14(15)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Australia</td>
<td>22(19)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Thailand</td>
<td>(1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>386(820)</strong></td>
<td><strong>62</strong></td>
</tr>
</tbody>
</table>

Data from: European Human Embryonic Stem Cell Registry (until August 2009) and Loser et al. 2010 (the numbers in brackets) who determined the number of hESC lines reported up to November 24, 2009.
The role of histocompatibility antigens in assessing the minimal size of hESC lines inventory

The number of the embryonic stem cell lines has grown at a fairly rapid rate during the last couple of years. Whether these cell lines could be actually used for research and potential clinical applications, however, depends on a number of factors. The first and foremost of these is the genetic variability found among humans which explicitly requires a tissue antigen match between the donor and the recipient. This variability ought to be mirrored by the hESC lines in order to gain maximal benefits from stem cell research and their medical applications. New studies, however, demonstrate that most of the lines used by stem cell researchers are obtained from donors of Northern and Western European ancestry. Several of the lines are from donors of Middle Eastern or Southern European ancestry and none of the studied 47 lines are derived from individuals of recent African ancestry, Eastern Europeans, Pacific Islanders, or from populations native to America (33). Another study by Laurent et al. (21) confirms the restricted ethnic diversity of the existing hESC lines by indicating that the majority of the studied lines (43 out of 47) are of European and East Asian ethnicity. Thus, limited ethnic representation of the available cell lines significantly depreciates the potential benefits for underrepresented patient populations.

Optimally, transplantation of embryonic stem-derived cells would require availability of histocompatible hESC lines for every patient. Since significant differences in histocompatibility types exist among various ethnic populations (14, 20), establishment of new lines representing human genetic diversity will be vital for successful therapeutic purposes (1, 38). The ways to achieve this could be establishment of national banks of stem cell lines derived from embryos with genotypes which are representative for the specific population, or, as it has already been suggested, establishment of a readily accessible global human embryonic stem cell bank (26). In either case it is obvious that more efforts need to be invested in creation of new human embryonic cell lines for more accurate representation of human genetic diversity.

As of the present moment, should the embryonic stem cells become a legitimate therapeutic tool, a significant proportion of Earth’s population, namely, about 25% (Africans, Native Americans, Pacific Islanders, Eastern Europeans, etc.), would be severely underrepresented in the embryonic stem cell banks. As for the other 75%, the chance of obtaining a compatible embryonic stem cell sample would heavily depend on the number of samples in a stem cell bank. Generally, a 5-out-of-6 histocompatibility alleles (5/6) match is considered to be compatible enough for solid organ and routine HPC transplantations. For cord blood it has been established that somewhat lower degrees of compatibility do not affect significantly the survival rate (provided that the cell dose is high enough) as HPC from cord blood are known to engraft well and to cause manageable post-transplantation complications with only a 4/6 match between the donor and the recipient (42). Currently, there is not enough information yet about histocompatibility antigen mismatch-patient survival relationship for human embryonic stem cells but one may speculate that in order to be successful, ESC transplantation may require even lower degrees of histocompatibility.

However, even in the best case scenario, where it is required at least 4/6 matched embryonic stem cell transplant from an unrelated donor with ethnicity that is represented in the available human embryonic stem cell banks, according to data from European and Asian research groups, the chances of finding a suitable sample are not very high unless the searched inventory is larger than what the international research community has to offer as of now, according to Table 1. Specifically, the group of Taylor (38) reports that the chance for a full match in the UK population is estimated at about 20% with inventory size of 150 cell lines, increasing to 40% if one or two mismatches are allowed (assuming that all of these stem cell lines fit the criteria for use for transplantation in humans and are readily available). The authors speculate that should the number of embryonic stem cell lines is reduced but the selection of the histocompatibility types is made more stringent and more tailored to the structure of the studied population (more specifically, a selection for stem cell lines who are homozygous for the HLA types which are commonly seen in the population in question), about 10 embryonic stem cell lines may suffice to ensure about the same degree of compatibility. Similar results are reported by Lee et al. (23) for the Korean population as they conclude that depending on the resolution of HLA typing, a moderately-sized bank of embryonic stem cells containing about 30 human embryonic stem cell lines will serve the needs of about 15-25% of the recipient population (provided that no more than two HLA mismatches are allowed). Therefore, to ensure a compatible transplant even for a patient that matches the ethnic profile for most commonly donated embryos, the number of stem cell lines that might constitute a usable inventory is estimated to be between roughly 10 and 200; and that is valid only for a population-sized study. On global scale, the number of the needed hESC cell lines could be expected to be higher.

On the other hand, it must be kept in mind that not each and every one of the derived cell lines is suitable for transplantation. There are some unavoidable losses of cell lines during maintenance and many may be established but subsequently disqualified for storage and use (see below). These cell lines must be replaced, therefore, in order to maintain the required inventory size, an embryonic stem cell bank must create and maintain many “additional” cell lines.

Discrepancy between differentiation properties of hESC lines- is what you see what you really get?

The genetic variability issue also sets the important scientific question about the relationship between hereditary background and cellular characteristics. Several reports indicate that,
depending on their genetic makeup, hESC lines may differ substantially in their differentiation properties (32, 37), although they may appear similar in the undifferentiated state (18). In 2006, it was shown that a common origin of cell lines does not guarantee their identity in regard to differentiation characteristics and expression profile, as it was demonstrated that three clones originating from the same parent cell line and cultured under the same conditions may have nonidentical differentiation and expression profiles (35). This may also bring forth other unforeseen issues, as, for example, only a couple of months ago it was discovered that the first human embryonic stem cell line ever made in Switzerland (CH-ES1), (10) exhibited properties of a malignant cell line (17). The existing data which at present are limited by the low number of hESC available for research are insufficient to clarify whether genetic variations have statistically significant effect on experimental results (12). This issue will remain open until more studies on new and genetically diverse hESC lines provide adequate data to assess the genetic impact on important biological properties like self-renewal and pluripotency.

Of mice and men, or why do we need more disease-specific hESC lines

Another argument in favor of development of new hESC lines is based on the possibility of creation of disease-specific embryonic stem cell lines. The importance of this branch of stem cell research is often undervalued in the light of recent achievements with animal model systems - usually, mouse models. These have provided exclusive insight into the intimate mechanisms of the pathogenesis of human diseases but they may (and do) fail to represent truthfully some aspects of human pathology. The situation is about the same in the field of drug development as many apparently working therapeutic strategies tailored out in animal models do poorly in human patients. Therefore, it has become increasingly important to develop additional physiologically relevant human disease models for the purposes of mechanistic studies and drug development. Disease-specific embryonic stem cell lines are usually derived from embryos at high risk of specific genetic disorders that have been identified using preimplantation genetic diagnosis (PGD) after in vitro fertilization (13, 31, 34, 40). Such hESC lines carry naturally inherited mutations and can be used as model systems for understanding human genetic diseases for which there are no adequate research models. They also have great value for the exploration of new therapeutic strategies and can provide proper testing systems for disease-oriented drug discovery. To date, 116 hESC lines with genetic disorders have been developed, representing 33 heritable human diseases (25). These lines represent only a small fraction of the large number of hereditary disorders. For example, only single-gene disorders are estimated to be the cause of more than 4000 human diseases. Thus, successful modeling and development of treatments for genetic disorders would require derivation of more hESC lines representative for specific human genetic diseases.

Induced pluripotent cells vs. embryonic stem cells - not quite the panacea

A recent breakthrough in stem cell biology allowed scientists to re-program successfully human somatic cells into pluripotent cells by forcing them to express once-silent developmental genes (36, 44). These reprogrammed cells, termed “induced pluripotent” stem cells (iPSCs), are believed to have similar developmental potential as authentic hESCs. The iPSCs are of particular value since they can provide autologous, immune-matched cells for transplantation. As these cells do not pose the ethical concerns, associated with derivation of embryonic stem cells, they are likely to be accepted as a general research tool and source for therapeutic purposes.

The discovery of iPSCs raises the reasonable question of whether they can substitute completely for the hESCs, thus eliminating the need to derive more hESC lines. This issue can be clarified only if extensive scientific proof of interchangeability between the two cell types is provided. However, several lines of evidence demonstrate that iPSCs differ from embryonic stem cells. Studies by Chin (6) reveal that the two types of cells differ in DNA methylation and microRNA expression profiles. A recently published, long-term evaluation and comparison of 25 hESC and 8 iPSC lines show that the iPSCs have notably decreased growth and differentiation efficiency and higher tendency to senescence and programmed cell death (11). It is evident that further methodological and functional studies are necessary to improve reprogramming technique in order to produce better quality iPSCs (3). Earlier this year, Ghosh et al. demonstrated that reprogramming of iPSC as it is now could be best described as incomplete as the pattern of gene expression characteristic of the donor cellular type persists in the iPSCs (15). Also, recently it has been shown that the expression profile of iPSC cells may exhibit cancer hallmarks (16, 28). More specifically, abnormally high level of cancer-related microRNAs was found in re-programmed induced pluripotent cells- about 10 times higher than in hESCs. This could potentially be a great impediment to further development of iPS-based treatments, or, at least, until the related mechanisms are studied thoroughly so as to invent a strategy to counter such effects. Therefore, induced pluripotent cells are not the perfect substitute for hESCs (at least, the way they are made at the moment) and the data accumulated so far indicate that it would be premature to abandon research using hESC. The latter still remain the “gold standard” for defining and monitoring pluripotency.

To improve is to change - but is it?

Among the arguments pertaining to the necessity of creating more human embryonic stem cell lines it is important to note that many of the existing hESC lines may have been altered by the culture conditions used to propagate them. Although all of the lines derived worldwide share the expression of specific pluripotency markers, many differences are emerging between lines (2) that may not be associated with the inherent genetic variation of the embryos from which these cells were derived.
Some cell lines show differences in their population doubling rates which have been reported to range from 28 to 48 h, as well as in their ability to form specific lineages in vitro (22, 37). Direct evidence for the effect of culture conditions have been provided by demonstration that adaptation to trypsin passing is associated with abnormalities of chromosomes 12 and 17 (5, 9). Besides such gross genomic alterations, several groups have reported many small genomic changes, using more sensitive karyotyping methods, showing that hESCs are prone to acquire significant genomic abnormalities in culture (24, 43). These alterations were found not to be random, suggesting that it is essential to set up standards shared by multiple laboratories for routine analysis and especially for propagation of existing and newly derived hESC in culture.

GMP compliance and quality control in derivation of hESC lines- does that new broom really sweep clean?
The growing possibility of developing cellular therapies that are based on human embryonic stem cell demands cells with defined quality characteristics and that are safe for the patient. Such cell lines can be produced by employing good manufacturing practice (GMP) that eliminates all sources of contamination- animal-derived or human-derived. Using embryos cultured in a GMP-standardized laboratory, inner cell mass isolated by mechanical means rather than by immunosurgery, GMP grade human feeder cells and xeno-free media, GMP-quality xeno-free hESC lines could be derived. Since most of the existing lines of hESC do not meet these criteria it has been suggested that it would be safer to start over again and establish new hESC lines that have never been exposed to any animal products (29). In 2007, the first six cell lines, produced in compliance with international regulatory requirements and suitable for therapeutic use were reported by Crook et al. (8). The same year another two hESC lines were derived in xeno-free conditions though one of them has been shown to have genetic abnormalities (41). Actually, the unfortunate first Swiss hESC line that exhibited teratocarcinoma-like traits was derived in xeno-free conditions (10) so GMP compliance is not a guarantee that the cell line would be usable for therapeutic purposes. Nevertheless, it is obvious that currently available GMP grade xeno-free hESC lines remain grossly insufficient for putative therapeutic use even if the most optimistic criteria are used

Conclusions
The accumulation of new data about the specific characteristics of the established human embryonic stem cell lines convincingly demonstrates that the simplistic approach “one size fits all” cannot be applied for the expected diverse utilization of hESC. Despite sharing a number of basic characteristics, defining their stemness and pluripotency, each hESC line has its particular qualities that characterize and in most cases restrict its use. For instance, cell lines that resemble as closely as possible the embryo in vivo would be ideal for studying early human development, but most probably they would not be suitable for modelling certain diseases or for therapeutic purposes. The existence of such specificity indicates that full exploitation of the enormous potential of human embryonic stem cells requires continued establishment of new and more potent hESC lines. In our opinion, regardless of seemingly large number of the existing hESC lines, the derivation of new ones will remain one of the “hot” topics of hESC research.

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REFERENCES


