

PART I

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DERIVATION METHODS FOR HUMAN EMBRYONIC STEM CELLS: PAST, PRESENT, AND FUTURE

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HUMAN EMBRYO CULTURE FOR HUMAN EMBRYONIC STEM (hES) CELL PRODUCTION

The first hES cell lines were derived from blastocyst-stage embryos nearly 10 years ago (1, 2). So far, more than 500 successful hES cell line isolation from human fresh or frozen morula and/or blastocyst-stage human embryos have been reported by numerous groups worldwide (3, 4). However, in nearly all of these reports, the possible correlations between embryo development parameters and successful derivation were poorly defined, making a true efficiency for hES cell derivation impossible to ascertain from embryology aspects (5).

Irrespective of the isolation methodology used to derive hES cell lines, embryos that are used for derivation should, in principle, be produced by assisted reproductive technologies (ARTs). Therefore, the material to be used is a product of a complex process involving controlled ovarian hyperstimulation, *in vitro* fertilization (IVF) procedures, and intense ethical clearance protocols. These techniques have been in clinical use for nearly three decades, and

are still continuously being improved to increase pregnancy rates and healthy delivery rates worldwide.

Despite acceptable take-home baby rates that have been obtained, results of human ART have shown that during *in vitro* culture, only 30%–40% of the fertilized human oocytes can in fact have the capacity to develop successfully to blastocyst stage. That is, approximately 70%–75% of these zygotes/embryos exhibit varying degrees of developmental abnormalities, including unequal cleavage and excessive fragmentation leading to developmental arrest. Even for those that can become expanded blastocysts, some eventually lose viability before or shortly after implantation, possibly due to induced apoptosis in both inner cell mass (ICM) and trophectoderm lineages.

In most cases, the etiology behind this extensive embryonic loss is unknown, and several studies have so far pointed to a variety of possible causes, including inadequate oocyte maturation, suboptimal culture conditions, and chromosomal/nuclear abnormalities during early cleavage stages, pointing out the need for improved techniques/protocols that can minimize these negative factors *in vitro* (6–13). At different stages of development during which embryonic stem cells have been derived—that is, morula, late blastocyst, and epiblast—at every stage, there is possible embryonic loss and hence there is scope to refine the methodology.

Current research that is aimed at optimizing human embryo development and minimizing this embryonic loss thereby increasing the success rate in ART is in turn expected also to improve the hES cell derivation process. Compared to the late 1990s, today's human embryo culture systems provide more standardized and optimal ingredients and protocols that are more beneficial for human embryo as well as hES cell survival. Recent experience has shown that improvements in human embryo culture conditions help hES culture derivation in two major ways: improvements in embryo quality that may lead to better ICM development and improvements in culture conditions that can provide culture environment that can be closer to *in vivo* (14). It recently has been reported that low O₂ concentration increases the viability of preimplantation embryos, assists their normal development, and forms healthy blastocysts with well-formed ICMs with greater cell number (15). Although current studies on human embryos have not shown relatively increased pregnancy rates with low oxygen culture, numerous authors have indicated a beneficial role of low O₂ on embryo quality, even in early cleavage stages (16).

Although *in vitro* culture systems are continuously being perfected to improve the quality of the materials cultured, intrinsic (paternal) factors that are inherited from infertile couples can also affect the human embryo quality and outcome of hES cell derivation. That is, in many cases, the nature of infertility resides on the quality and quantity of oocytes/spermatozoa to be retrieved, and embryos that are produced from inferior quality oocytes and/or spermatozoa can carry numerous metabolic and/or genetic problems that may affect their development and implantation. Excess embryos from these couples, if used in hES cell derivation, may reach blastocyst stage but still carry abnormal

developmental patterns that can affect the derivation as well as differentiation profile.

Increased rate of imprinting and other epigenetic abnormalities in human *in vitro*-produced preimplantation embryos (17) is a clear example of such consequence. Furthermore, the significantly higher incidence of Beckwith–Wiedemann Syndrome in IVF babies, an imprinting disorder caused by loss of imprinting (LOI) of insulin-like growth factor (IGF2) and other imprinted genes, raises the prospect that the brief *in vitro* culture of human embryos as part of the infertility treatment may cause epigenetic abnormalities (18). This issue may indicate that initial hES cell culture may create relatively diverse epigenetic profiles that can lead to different developmental and differentiation profiles in extended *in vitro* culture. Whether this finding can explain the differences in cultural behaviors of hES cell line in similar *in vitro* settings remains to be seen in the near future.

Although direct association of these problems with a specific ART technique or culture characteristics has not yet been specifically defined, a possible use of human embryos obtained from fertile couples can in theory be a superior source for hES cell derivation.

Nowadays, the allocation of human embryos for hES cell isolation from fertile couples can be realized in two different ways: First, in order to obtain a healthy and human leukocyte antigen (HLA)-compatible child with his/her sibling, fertile couples carrying a specific single gene disorder can undergo IVF and preimplantation genetic diagnosis (PGD) combined with (HLA)-typing procedures. Second, fertile couples can undergo IVF and PGD in order to obtain a child with a desired gender. Although the latter approach is strictly banned in many countries worldwide, in some it can be practiced for family balancing purposes. Therefore, healthy but HLA-incompatible embryos or embryos carrying undesired sex chromosome constitution can be donated for hES cell derivation purposes, thereby allowing the production of hES cells that can be obtained from fertile couples (19).

HUMAN EMBRYOS AS hES CELL SOURCES

So far, nearly all hES cell derivation studies have used either inferior-quality or excess fresh or frozen/thawed human embryos that are donated for research after ART treatments, or donated human ova and sperm cells to produce embryos to be used for this purpose. Several recent studies have also utilized embryos that have shown developmental arrests and discarded from routine IVF treatments, embryos that are produced and donated after PGD, as well as embryos that are parthenogenetically created from donated human oocytes. Very recently, successful generation of hES cell lines from biopsied human blastomeres was also reported. These alternative sources of hES cell lines are depicted in Figure 1.1.

In the majority of hES cell isolation studies, fresh or frozen/thawed spare human embryos were used as a source material. Very few of the fresh spare

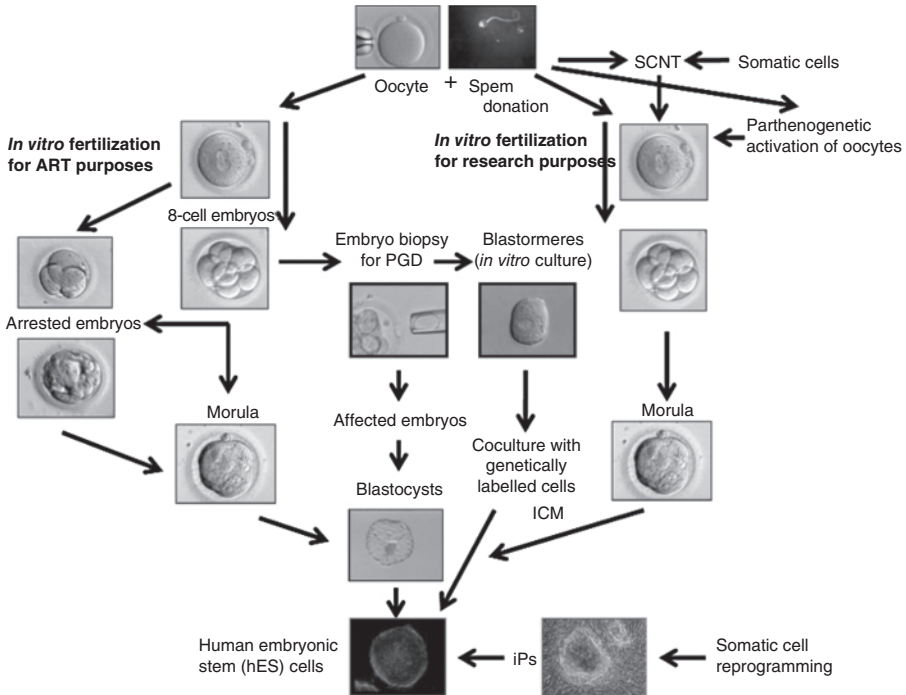


Figure 1.1. A schematic representation of possible hES cell derivation strategies that have so far been practiced worldwide. (See color insert.)

embryos can survive and form blastocysts in extended culture, which results in lower rate of embryonic stem cell derivation compared to frozen/thawed counterparts (20).

hES cell isolation from embryos that were specifically created for this purpose from donated human sperm and oocytes was reported by Lanzendorf et al. (21). In their study, insemination of 142 donated oocytes gave 68% fertilization and 50% blastocyst development rates, respectively. Forty blastocysts were used for immunosurgery, resulting in 18 distinct ICM structures and eventually 3 hES cell lines. Since embryos were produced from gametes of healthy donors, it remains to be argued whether better embryo development and hES cell derivation rates can be obtained with current technology.

Utilization of arrested human embryos for hES cell isolation has recently been reported by Zhang and his colleagues (22). This approach corroborates with several proposals and alternative ways to derive new hES cells without destruction of viable human embryos (23). Especially in countries with a non-flexible policy, arrested embryos can provide a more ethical source for hES cell derivation and hence can resolve some of the political issues surrounding research using human embryos.

PGD technique, in its simplest terms, involves the screening of preimplantation embryos for chromosomal abnormalities or for single gene defects. In this context, it becomes an alternative option to traditional prenatal diagnosis. Besides its diagnostic value and expanding indications such as cancer predisposition, dynamic mutations, and late onset disorders, a new feature, namely PGD combined with HLA typing, also demonstrates its novel therapeutic role in contemporary medicine (24, 25).

The application of PGD has not only helped couples carrying specific genetic problems to have disease-free children, but has also created a novel source for hES cell research. After biopsy and genetic analysis, embryos that are diagnosed as chromosomally abnormal or carrying a specific monogenic disease can be donated for research, thereby resulting in hES cell lines that can be the earliest *in vitro* models for that particular genetic anomaly. On the other hand, a recent report by Munne and his colleagues has further proposed that embryos that were diagnosed as abnormal after PGD could in part have a self-correction ability, that is, revert to a normal karyotype after prolonged culture, resulting in hES cell lines with normal and stable karyotypes (26). However, this finding still remains to be confirmed by other independent studies.

To date, PGD is the only technique that allows the removal of a cell from a developing embryo, without impairing its potential to create pregnancy (27). If in theory, removed cells could be cultured and expanded for several days, they would be an ideal and ethically acceptable hES cell sources for future research and therapy. Retrieval of individual human blastomeres through PGD and expanding them directly in culture mainly for detection of chromosomal aneuploidy have been documented by some groups, and proof-of-concept study has been performed on mice (28–31). Several studies initially aimed to culture individual human blastomeres *in vitro*. However, the results were hampered by the fact that culture conditions and techniques were inefficient to successfully culture and expand diploid human blastomeres *in vitro*.

The fact that cleavage during preimplantation stage as well as in hES cell cultures largely depends on the presence of adjacent neighboring cells as well as several unknown factors produced by them requires further attention, and technical improvements and alternatives should be sought to clarify the feasibility of this approach. In mice, whether a blastomere is to become either a trophoblast or an ICM cell appears to be specified by its position during first cleavage (32). It was also demonstrated that asymmetrical distribution of Cdx2 gene product in mouse oocytes and embryos defines the lineage of trophectoderm (33). In humans, this process happens to occur during compaction stage. This observation is in correlation with recent studies in that a signal for ICM or trophectoderm lineage can be present in some blastomeres far more earlier than the phenotypical characteristics would have emerged (34, 35).

Despite all the limitations and initial reports indicating unsuccessful attempts to derive stem cells from biopsied human blastomeres, Klimanskaya

et al. were first to report the derivation of new hES cell lines from isolated human blastomeres (36, 37). Although their technique was later extensively argued that the approach was not the same as human embryo biopsy for PGD purposes, the same group has very recently announced the extension of their initial reports on embryos obtained from routine PGD cases (38). These results can indicate that if one or two biopsied blastomeres would be enough to expand the line, the same embryo would also be implanted in the uterus, creating a viable offspring and comparable pregnancy rates as well as an unlimited stem cell source for that sibling, hence opening a new era in both clinical applications and ethically permissive hES cell isolation and derivation studies.

Unless derived from the same embryos as mentioned above, creating clinical grade hES cells was considered to be of limited use unless there existed a similar HLA match between the cells and the potential recipient. For this reason, recently, somatic-cell nuclear transfer (SCNT) technology has been applied in the creation of patient-specific human and primate embryonic stem cell lines by several groups (39, 40).

Compared to SCNT, parthenogenetic activation of oocytes is a relatively simple method to create histocompatible stem cells since the technique does not require complex instruments and micromanipulation experience. In mice and primates, parthenogenetic embryonic stem cell lines have been derived and their pluripotency has been demonstrated by different groups (41, 42). Patient-specific stem cell lines from human parthenogenetic blastocysts have recently been reported by different groups (43, 44).

As a valid alternative to patient-specific hES cell line derivation by SCNT, it was proposed that a minimum of 40–50 homozygous hES cell lines would be necessary to cover 50% of the HLA isotypes in the American population and 150 cell lines to cover the UK population, thus minimizing the immune rejection of hES cell-derived transplants (45, 46). However, although these numbers may be underestimated due to the ethnically diverse nature of the populations in question, the creation of master hES cell banks for future therapeutic as well as research applications seems to be a realistic approach which will be helpful to the standardization of hES cell cultures and the reduction of the cost and unnecessary derivation of new hES cell lines in the near future (47–49).

Apart from human gametes and embryos as hES cell sources, several groups have recently announced that they have produced induced pluripotent (iP) cells from human adult somatic cells (50, 51). Although they have used slightly different protocols, their approaches commonly involved overexpression of a group of four genes (Oct-3/4, Sox2, Klf4, and c-Myc) that are known to be actively expressed in hES cells. The results have shown that these induced cells show common pluripotency and differentiation characteristics of hES cells, although they are not identical as shown by DNA microarray analyses. However, if this “reprogramming of differentiated cells” is possible, there would be no need for blastocysts to be disintegrated to extract ICMs, hence

the technology would be ethically permissive and there becomes no need for therapeutic cloning.

Besides many important issues such as epigenetic status, safety issues related to gene modification and tumorigenic potential of these cells remain to be explored; the approach could at least in theory be an alternative way to produce hES cells from human embryos and hence could be more acceptable from an ethical point of view.

ISOLATION METHODS

So far, the derivation methodology of the reported hES cell lines included isolation of ICM cells from trophectoderm (TE) cells by immunosurgery, mechanical, chemical, or laser-assisted removal of ICM, and direct plating of intact blastocysts on feeder cells/dishes coated with extracellular matrix proteins without prior ICM dissection and proper staging of blastocysts for hES cell derivation (1, 2, 52–55).

Immunosurgery method utilized the exposure of embryos to pronase enzyme, animal-derived complement system reagents, and antibodies raised against human cells (Figure 1.2). Recent experiences, however, have shown that this technique is not the optimal derivation method when poor-quality, spare embryos with small or nearly visible ICMs are used. Moreover, hES cells that have been isolated with this method will eventually not be suitable for therapeutic use due to their risk of carrying animal pathogens (56).

Compared to immunosurgery, the application of “whole culture method” or “direct culture method” was also reported to be equally effective in the derivation process in several recent reports (20, 57–59). This method has been mentioned to be superior to immunosurgery since it not only shows better success rates, but it also clearly avoids the use of animal-derived antibodies, hence making the culture system—one step—more suitable for therapeutic use. Several recent publications also reported novel isolation strategies, which indicate that isolation technique can be optimized according to the quality of the embryo used in the derivation process; therefore, a combined strategy that utilizes varying culture and isolation methods could be the method of choice (60).

Chemically removing zona pellucida by means of acid Tyrode’s approach is generally thought to be superior to expose the embryo to pronase, an enzyme that is extracted from bacteria. However, exposure of embryos to the acidic solution (pH of 2.5–3) can be hazardous for ICM cells unless the incubation time is carefully optimized and the procedure itself is done by an experienced staff. Laser is widely used in contemporary ARTs for artificial opening of zona pellucida before intracytoplasmic sperm injection (61), embryo transfer (assisted hatching) (62), or embryo biopsy during PGD applications (63). The use of laser technology in ICM isolation for hES cell derivation has recently been reported by Turetsky and his colleagues (55). Their study indicates that

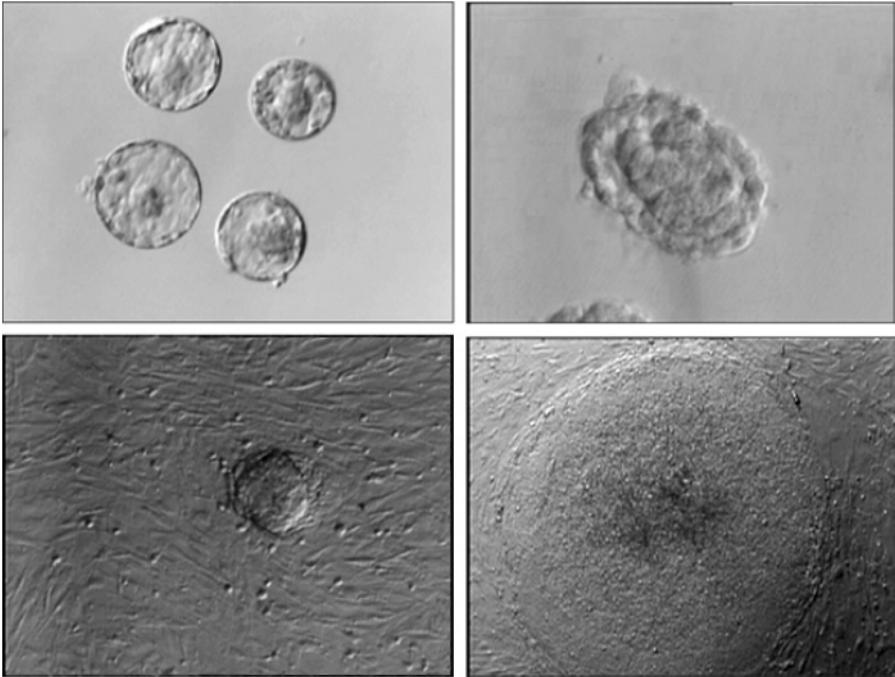


Figure 1.2. Establishment of an hES cell line by immunosurgery. (a) Good quality human blastocysts that are suitable for immunosurgery; (b) A zona-free human blastocyst in culture media containing complement proteins. Note the degenerating trophoblast cells due to complement action; (c) A picture of growing ICM cells attached on feeder surface after immunosurgery; (d) A good quality sixth-day human embryonic stem cell colony.

this approach can potentially be useful in xeno-free hES cell derivation since it avoids the use of animal-derived enzymes or immunological substances that have traditionally been used for this purpose. As another potentially advantageous isolation method, Strom et al. developed a mechanical isolation protocol that utilized two metal needles with sharpened tips that can cut both zona pellucida and ICM, therefore avoiding exposure of embryos to either acidic environments or animal-derived substances (54). Patented technologies for laser ablation and removal of trophoblast by mechanical means or transferring the ICM to a second plate are developed recently.

CULTURE, MAINTENANCE, AND UPSCALING OF hES CELLS FOR THERAPEUTIC USE

The proper maintenance and expansion of hES cells is one of the most important issues in hES cells biology. Although it has been nearly 10 years since the

first successful report on hES cell isolation and expansion, *in vitro* culture of these cells still need direct exposure to one or several undefined culture ingredients of nonhuman origin. The use of blood-borne complement system to isolate ICM from an expanded human blastocyst has already been discussed above. However, current hES cell culture still needs other materials such as serum, mitotically inactivated mouse embryonic fibroblasts (MEF), or MEF-derived extracts.

The feeder layer provides certain currently unknown factors, which support undifferentiated growth of hES cells. Although hES cell lines were initially cultured on mouse-derived fetal fibroblasts, recently, these feeders have been replaced with human counterparts that are isolated from various tissues (64). Another recent approach involved the culture of whole blastocysts on defined or purified cell extracts or matrix proteins such as collagen VI, fibronectin, laminin, and vitronectin of human origin (65).

Previous studies that were performed to develop feeder-free culture environments to support established hES cell lines have indicated that three factors, namely transforming growth factor (TGF β 1), leukemia inhibitory factor, and bone morphogenic protein antagonist Noggin act together to suppress hES cell differentiation and promote self-renewal (66, 67). In order to develop chemically defined media that sustain hES cell self-renewal, it is very important that signals and mechanisms controlling hES cell fate choices (such as choosing to differentiate into a particular lineage or continue to proliferate as undifferentiated progeny) should be understood in detail. Several recent studies have found out that in hES cell self-renewal, a major role is played by members of the Wnt and TGF β superfamily of signaling molecules (68, 69). TGF β family members seem to stimulate hES cell self-renewal by inducing phosphorylation of the intracellular mediators Smad2 and/or Smad3. On the other hand, bone morphogenic proteins induce hES cell differentiation to extraembryonic lineages or to germ cells by phosphorylation of Smad1/5/8 (70–72). β Fibroblast growth factor (β BFGF) and IGF-II also play important roles in hES cell self-renewal by inducing expression of TGF β family molecules such as TGF β /Activin/Nodal (73).

In a very recent study, albumin-associated lipids, which are essential ingredients in knockout serum replacement, have also been found to have strong positive effects on the self-renewal of hES cells, indicating that deeper understanding of the mechanisms will eventually lead us to produce xeno-free chemically defined culture media for hES cell self-renewal and differentiation (74). Clearly, lack of xeno-free reagents during the process of derivation of hES cell lines is a huge gap that needs to be addressed and availability of reagents such as knockout serum replacement that is made completely with xeno-free materials or with human materials would enable researchers to derive next generation hES cell lines with greater potential for human clinical cell therapy relevance.

Upscaling process involves the disaggregation of undifferentiated hES cell colony pieces from their original culture and transferring them into new

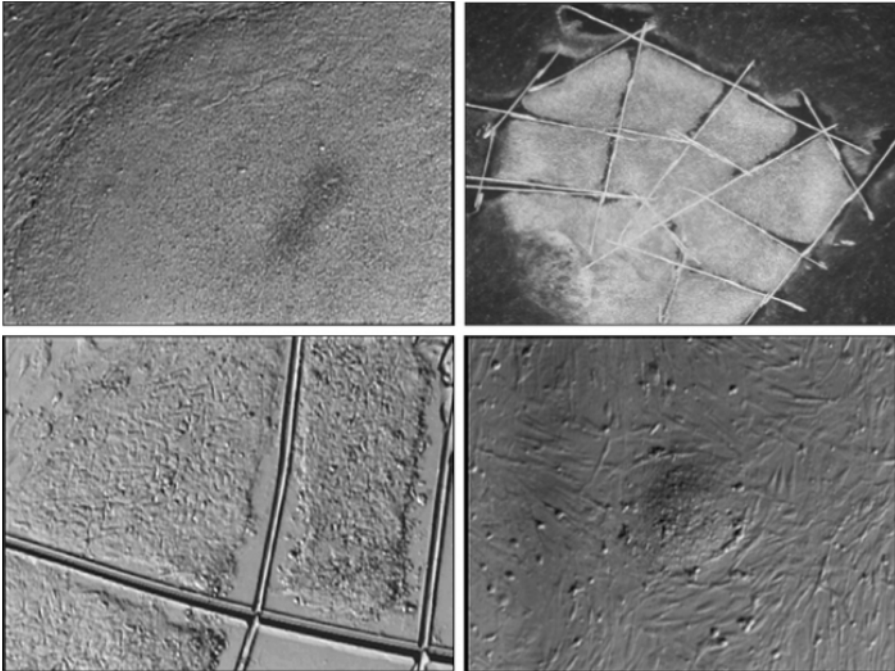


Figure 1.3. Mechanical passaging of an hES cell colony. (a) A well-expanded and undifferentiated hES cell colony that is ready for passaging; (b) Uniform cutting of a suitable colony by finely drawn glass Pasteur pipette; (c) A closer look at the picture in B: At this stage, colony pieces start rounding from the edges and pieces are dissociated from the culture plate by scratching them with a polished glass pipette; (d) A growing hES cell colony 1 d after mechanical passage.

culture environment, which again supports undifferentiated growth of hES cells. The most widely used method to maintain undifferentiated “high-quality” hES cells in culture is mechanical passaging (Figure 1.3). In this method, micropipettes or finely drawn Pasteur pipettes are used to cut the proper colonies in pieces and optimum numbers of pieces are transferred into new culture dishes every 4–7d. Although this method seems to be advantageous over enzymatic dissociation in that no animal-derived dissociation enzyme is used, it certainly becomes a limited technique when large-scale hES cell production is required. Passaging and upscaling of hES cells by enzymatic techniques has, on the other hand, recently been questioned by several reports. Compared to the ones that were mechanically passaged, cells that were treated with dissociating enzymes have shown accumulated chromosomal abnormalities, indicating a potential technique-induced genetic instability of the hES cell lines studied (75, 76).

In summary, therapeutic-grade hES cell culture requires three major technological advances:

- 1) Completely good manufacturing practice (GMP)-grade production of human embryos followed by animal- and pathogen-free ICM isolation.
- 2) Completely feeder-free (animal or human origin) hES cell culture systems, which only involve purified and screened human extracellular matrix proteins as well as growth factors, cocktails, etc.
- 3) A newer cGMP products of xeno-free nature that are clear alternatives to extracellular attachment factors such as matrigel, and completely humanized or xeno-free reagents such as knockout serum replacement.
- 4) Suitable large-scale hES cell culture expansion systems that allow mass production and upscaling of hES cells without any negative effect on cellular proliferation, differentiation, and genetic instability.
- 5) Well-defined differentiation protocols/systems that allow mass production of certain precursors/ultimate somatic cell types.

CONCLUSIONS

Since 1998, accumulated data on hES cell derivation and culture indicate that hES cell research is exponentially expanding and moving forward to keep its promise in many scientific and medical disciplines, including developmental biology, human embryology, toxicology, pharmacology, genetics, as well as regenerative and reproductive medicine.

Although so far, all reported hES cell lines have been derived by using animal-derived material either during derivation or cultivation and hence are not suitable for clinical use, recent advances in understanding embryonic stem cell self-renewal and expansion mechanisms will no doubt bring the contemporary stem cell research one step closer to xeno-free hES cell lines that can be utilized in medicine in the near future.

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