A Survey of Parameters Involved in the Establishment of New Lines of Human Embryonic Stem Cells

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Abstract Since the derivation of the first human embryonic stem cell (hESC) lines by Thomson and coworkers in 1998, more than 1,200 different hESC lines have been established worldwide. Nevertheless, there is still a recognized interest in the establishment of new lines of hESC, particularly from HLA types and ethnic groups currently underrepresented among the available lines. The methodology of hESC derivation has evolved significantly since 1998, when human LIF (hLIF) was used for maintenance of pluripotency. However, there are a number of different strategies for the several steps involved in establishing a new line of hESC. Here we make a survey of the most relevant parameters used between 1998 and 2010 for the derivation of the 375 hESC lines deposited in two international stem cell registries, and able to form teratomas in immunocompromised mice. Although we identify some trends in the methodology for establishing hESC lines, our data reveal a much greater heterogeneity of strategies than what is used for derivation of murine ESC lines, indicating that optimum conditions have not been consolidated yet, and thus, hESC establishment is still an evolving field of research.

Introduction

Pluripotent embryonic stem cells (ESC) were first derived from the inner cell mass (ICM) of mouse blastocysts in 1981 [1]. They are defined by unlimited self-renewal and the ability to differentiate—both in vitro and in vivo—into cell types of endodermal, mesodermal and ectodermal origins, rendering them a promising applicability in cell replacement therapies [2]. These characteristics also make ESCs a powerful tool for studying the molecular mechanisms underlying cellular differentiation, as well as for accessing the biological effects of pharmaceutical compounds on the normal embryo development (reviewed by [3]).

Despite continuous efforts from many laboratories, it was only in 1998 that the first lines of humans ESC (hESC) were described [4]. After that, many other groups reported on the derivation of hESCs, and the number of different lines registered on the available databases rapidly increased.

However, there is a growing evidence of the heterogeneous behavior of the distinct lines of hESC regarding their differentiation potential [5] and their epigenetic stability [6]. Moreover, if they are intended to be used as sources of tissues for transplants, there may be a need for HLA matching between the hESCs and patients, and thus the establishment of a large number of HLA-diverse hESC lines is required [7, 8]. Still regarding their clinical use,

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there is also a need to optimize xeno-free and fully defined systems in order to avoid potential contaminants. Therefore, there is a continuous interest in establishing new lines of hESC to address these issues.

The conditions described for the establishment of the first human ESC lines [4, 9] were essentially the same as those used for the derivation of mouse ESCs [1]. Since then, novel signaling pathways controlling pluripotency of hESCs have been identified and, together with the development of new reagents, they have led to the improvement of strategies for deriving new hESC lines [10]. Here we make a survey of the different protocols used in the last 12 years for the establishment of hESC lines from two international stem cell registries, looking for trends in the several steps involved in the process. Our results show that, although there were significant advances in methodology and reagents, there is still a great heterogeneity in the strategies employed for hESC derivation, indicating that optimal conditions have not been consolidated yet, and thus, this is still an intensively evolving field of research.

Gathering Information From Human Embryonic Stem Cell Registries

Data were obtained from the European Human Embryonic Stem Cell Registry (www.hescreg.eu) and the University of Massachusetts' International Stem Cell Registry (www.umassmed.edu/iscr) as of December 7th, 2010. Only those lines of hESC with pluripotency demonstrated by the capacity to form teratomas when injected into immunodeficient mice were included in the analysis.

The following parameters were analyzed: embryo—source (generated for reproduction or research), fresh or frozen, stage of development, quality, method of ICM isolation; culture conditions—basic medium, protein source, factors for maintenance of pluripotency, type of support, efficiency of derivation. Specific details regarding different aspects of the derivation process were obtained also from the corresponding publication, when available. Whenever data from the registry differed from those in the referenced publication, we considered the data described in the latter, with the exception of derivation date, which was taken from the registries. In addition, whenever derivation data differed among different publications from the same group, we considered the information that was first published.

Derivation of hESCs Around the World—When, Where and How

As of December 7th, 2010, 1,291 different hESC lines were registered in the two stem cell registries (hESCReg and

UMass ISCR). Of those, 375 lines were reported to be able to form teratomas when injected into immunodeficient mice. We listed the different parameters involved in the derivation of these 375 lines, extracting the exact data from the registries or from the corresponding publication, when available (Supplementary Table 1). Twenty six hESC lines lacked information regarding the year of derivation and were therefore excluded from the analysis of the evolution of the methodologies throughout time.

Figure 1 shows the number of new hESC lines established per year. It is interesting to note that it took 2 to 4 years since the first derivations by Thomson et al. (1998) and by Reubinoff et al. (2000) for other groups to establish new lines of hESC. The composition of the derivation medium described in those two first articles contained hLIF and MEFs as factors for maintenance of pluripotency. It was only in 2000 that the use of bFGF was described as a requirement for the prolonged culture of hESC in serum-free medium [11]. From then on, bFGF has been consolidated as the major growth factor for maintenance of hESCs' pluripotency, and this may have had a role in the raise of the number of derivations observed thereafter.

The number of new hESC lines greatly increased from 2004 to the end of 2010, as well as the number of research groups and laboratories engaged in their establishment. In fact, there was a first peak of generation of new hESC lines in 2004, contributed mostly from one group in the USA [12], one in Korea [13] and two in Sweden [14, 15], which accounted for 16, 9 and 10 of the total of 38 new cell lines derived that year, respectively (Fig. 1).

The derivations of the majority (88.8%) of those hESC lines were described in peer-reviewed journals, even in the last 2 years (Fig. 1). However, most of the latest articles tend to report only optimizations of the derivation protocols (e.g. [16, 17]) or hESC lines established from alternative
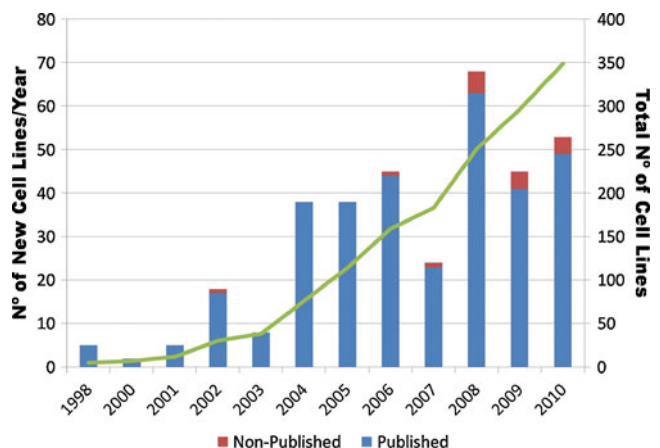

Fig. 1 Distribution of new hESC lines derived per year (*blue/red bars*—left vertical axis), and total number of hESC lines in the period (*green line*—right vertical axis)

sources of embryo, for example from poor quality embryos or morulae [18–20]. In addition, in the most recent papers, the report on the derivation of the new hESC lines usually accounted for a small part of these publications, where the cell line served as the basis for the development of the main scientific study, indicating less availability for publishing new derivations without any novel scientific aspect. One recent exception was a special issue of In Vitro Cellular & Developmental Biology—Animal, dedicated to report new derivations of hESC lines [21]. In this single issue, 25 articles described the establishment of 45 new lines. Nevertheless, given the interest of increasing the diversity of available hESC lines, it is important to have a venue for publicizing the new lines and their specific characteristics, a role well performed by human stem cell registries [22].

Source of the Embryo

Overall, the vast majority (98.4% of the informative lines) of embryos used for the derivation of hESC lines were surplus from reproductive cycles, whereas only 1.6% of the lines were derived from embryos generated specifically for research (Fig. 2a). Out of the six lines derived specifically for research, four were derived in China and two in Belgium (Supplementary Table 1), countries with permissive

legislations concerning stem cell research and somatic cell nuclear transfer for therapeutic purposes [23]. In addition, in the last 3 years none of the hESC lines analyzed were derived from embryos generated for research. Although other countries, like USA and England, also permit the generation of human embryos for research, no hESC lines registered have been derived from this type of embryo in these countries. Therefore, these data show that creating embryos exclusively to generate hESC lines is not a common practice, probably due, at least in part, to the difficulties in obtaining donors of human oocytes.

One implication of the use of surplus embryos for the derivation of new hESC lines is the possible limited genetic diversity of these embryos, which will come mostly from private clinics of assisted reproduction that may not attend to the whole ethnic admixture of a given population [24]. In fact, recent articles have described a restricted genetic background of the hESC lines established worldwide, which are mainly of European and East-Asian ethnicity [25–27]. Thus, it will be important to develop strategies to obtain ethnically diverse embryos for the derivation of new hESC lines with distinct genetic backgrounds and different HLA types.

Frozen (144 lines) and fresh (111 lines) embryos have been equally used for derivations (Fig. 2b). The use of
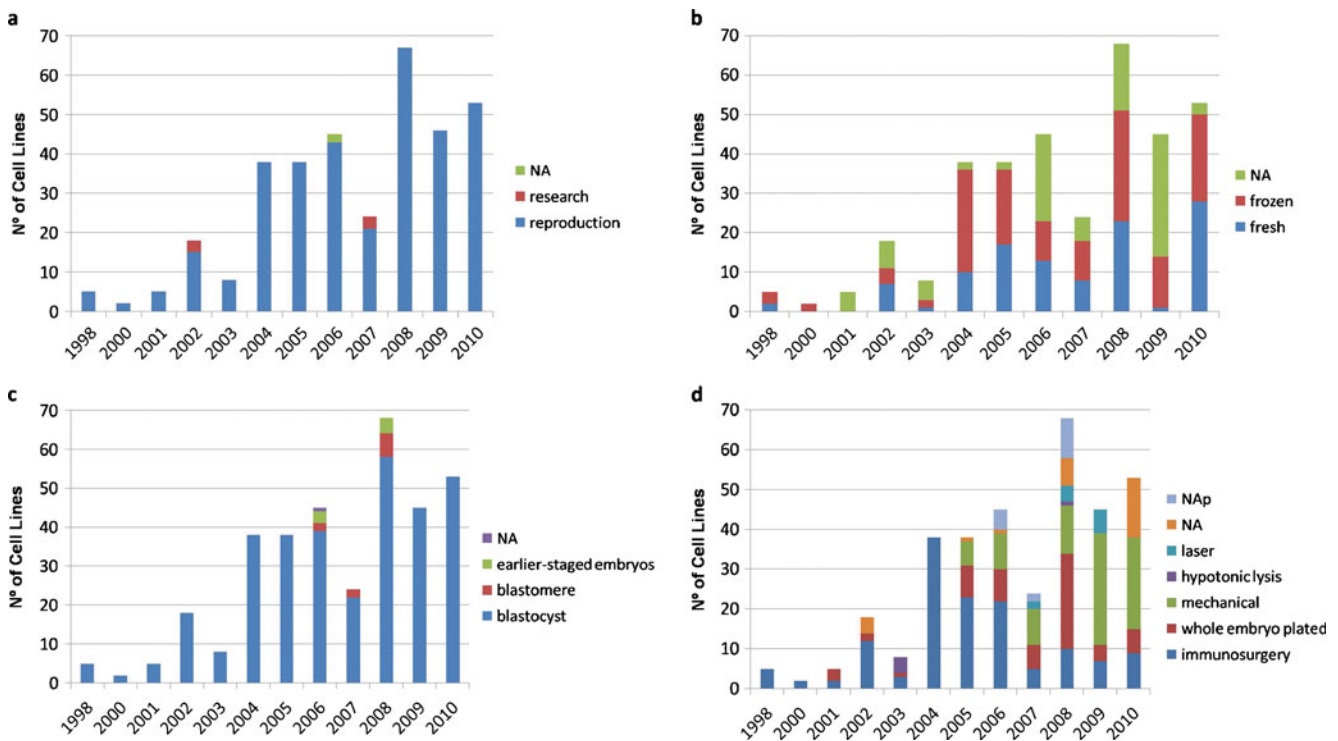

Fig. 2 Embryos used for establishment of hESC lines. Number of cell lines per year derived from **a** embryos generated for reproductive and research purposes; **b** fresh and frozen embryos; **c** stage of development of the embryos used for derivation of the hESC lines; **d** method employed for isolation of pluripotent cells. *NA* information not available. *NAp* do not apply (lines derived from blastomeres or early-staged embryos (6-cell up to morula staged embryos))

frozen embryos allows working with larger batches of embryos at once, whereas fresh embryos, if supernumerary from reproduction cycles, tend to be available in smaller numbers and at shorter notice for each experiment. Nevertheless, some researchers reported better efficiencies of hESC line derivation with fresh rather than frozen embryos (reviewed by [28]). In our survey, we could not identify this difference from the available data.

Quality of the Embryo

From the 375 cell lines listed in this review, 216 (57.6%) were informative regarding the quality of the embryo from which they were derived. However, since different methodologies for embryo scoring were employed by the different laboratories, we used in our analysis the classification provided either on the European hESC database or in the published articles, where the quality of the embryos was defined simply as "good", "intermediate" or "poor". For some of the cell lines, we adjusted the information provided by the authors for the classification format mentioned above (see Supplementary Table 1). From the 142 cell lines that met this classification, 69 (48.6%) were originated from embryos that had been classified as "good", 21 (14.8%) as "intermediate", and 52 (36.6%) as "poor". Thus, although the majority of the lines were derived from good quality embryos, a high proportion of lines were established from poor embryos, not usually suitable for reproduction. Nevertheless, the availability of more information on the quality of the embryo used to derive each cell line, as well as the use of a single common method for grading embryo quality, would be fundamental in accessing the role of this parameter in the feasibility of generating a hESC line.

Developmental Stage of the Embryo

The great majority (95.1% of the informative lines) of the hESC lines with potential to form teratoma were originated from isolated ICMs or from whole plated embryos at the blastocyst stage, whereas only 2.2% were established from earlier-staged embryos (6-cell up to morula staged embryos), and 2.7% from single blastomeres (Fig. 2c and Supplementary Table 1). In fact, four independent groups (in the UK, USA, Spain and Australia) have reported on the derivation of hESC lines from earlier-staged embryos, and four groups have described the generation of hESC lines from isolated single blastomeres (in the USA, Switzerland, Belgium, Spain—[29, 30]), demonstrating the feasibility of these strategies, which would increase the number of available embryos adequate for hESC derivation. However, it is worth noting that the main goal for the derivation of hESC lines from single blastomeres is generally to avoid

the controversies involved in the destruction of human embryos, rather than to obtain a better source of cells for hESC derivation.

Strategies of ICM Isolation

Differently from the method generally used for the establishment of murine ES cells—where the whole embryo is plated and cells from the inner cell mass are mechanically isolated from the surrounding trophectoderm within few days -, the procedure adopted until 2004 for the derivation of hESC lines included the isolation of the ICM from the embryo before plating (Fig. 2d). Clearly, the less an embryo is manipulated, the smaller the chance of causing any damage to its cells. However, as trophectoderm cells proliferate very fast, they can suppress the growth of the ICM, and even generate a trophectoderm stem cell line [14].

The technique mostly employed until 2004 for the isolation of human ICM was immunosurgery, a non-specific process based on the embryo susceptibility to complement-dependent antibody cytotoxicity [31]. However, when the trophectoderm is not intact, immunosurgery may damage cells from the ICM [32], decreasing the chances of those cells to yield a new hESC line. In addition, this approach involves the use of animal-derived components which may not be adequate for some applications of the hESC lines. Therefore, from 2005 on, an increasing number of groups have been using alternative methods, namely mechanical or laser-assisted isolation of the ICM (Fig. 2d), although the latter is less widely adopted probably due to the limited availability of the laser equipment. Finally, lines of hESC have been established from whole plated embryos, mostly when embryo quality is poor hampering isolation of the ICM. Nevertheless, this strategy was also employed with 10.6% of the good quality embryos, indicating a lack of requirement for ICM isolation.

Human blastocysts are not usually abundantly available, and thus researchers have been trying to bypass the need of high-quality blastocysts for hESC derivation. Since the first report of hESC lines derived from blastomeres in 2004 [29, 30], there has been an increase in the use of alternative sources of pluripotent cells (e.g., morulae or single blastomeres) and also of poor quality embryos, usually not employed for reproduction. These developments may be related to the growing number of hESC lines that have been established without ICM isolation between 2006 and 2008.

Besides a trend not to employ immunosurgery, there is a tendency to use mechanical dissection of the ICM, but the best methodology to isolate ICM from human embryos has not yet been consolidated. Thus, the morphology and

quality of the embryo play important roles in the choice of the method for ICM isolation. Nevertheless, since many lines have been established from whole plated embryos, the main novelty in the last 12 years has been the lack of requirement for isolation of ICM for hESC derivation, simplifying the procedure.

Culture Media

Four main components are generally used during the derivation and maintenance in culture of a hESC line: the basic medium (e.g. DMEM, DMEM-F12, KO-DMEM), the protein source (e.g. FBS, KSR), the factor(s) used to stimulate self-renewal (e.g. bFGF, LIF, insulin, different small molecules, and others), and the matrix/cell support (e.g. MEF, STO, HFF, Matrigel, and others). Tens of different combinations of the various types of these four components have been shown to be adequate for maintenance of the hESC lines in culture [33], and our analysis shows that many of them are also suitable for the derivation of new cell lines (Fig. 3).

Regarding the basal culture medium and protein source, KO-DMEM and KSR have been the most used reagents for the derivation of hESC lines since 2003 (Fig. 3a, b). In fact, 59.9% (212 of 354 of informative

lines) of all the hESC considered in this review were cultured in these two components at least during some stage of the derivation (Supplementary Table 1). Thus, it comprises the most employed medium basis not only during the maintenance of the hESC lines in culture [33] but also during their derivation.

KO-DMEM is a "DMEM-based" medium with osmolarity reduced in order to improve the growth of the undifferentiated ESC, while the KSR, developed in 1998, is more suitable for culturing ESC than the regular fetal bovine serum because it does not contain undefined growth or differentiation promoting factors [34]. In agreement to this, FBS has been shown to promote differentiation of the ESC at a higher rate [35]. Furthermore, although containing animal-derived components, KSR is a defined supplement, avoiding the lot-to-lot composition variation found in FBS [36]. Nevertheless, even in the last 2 years, a significant number of hESC lines (21 lines out of 91 informative lines—23.1%) were still established in a combination of KSR/FBS, which apparently improves the initial outgrowth of the ICM, and then are subsequently transitioned into KSR alone for long term culture [17] (Fig. 3b and Supplementary Table 1).

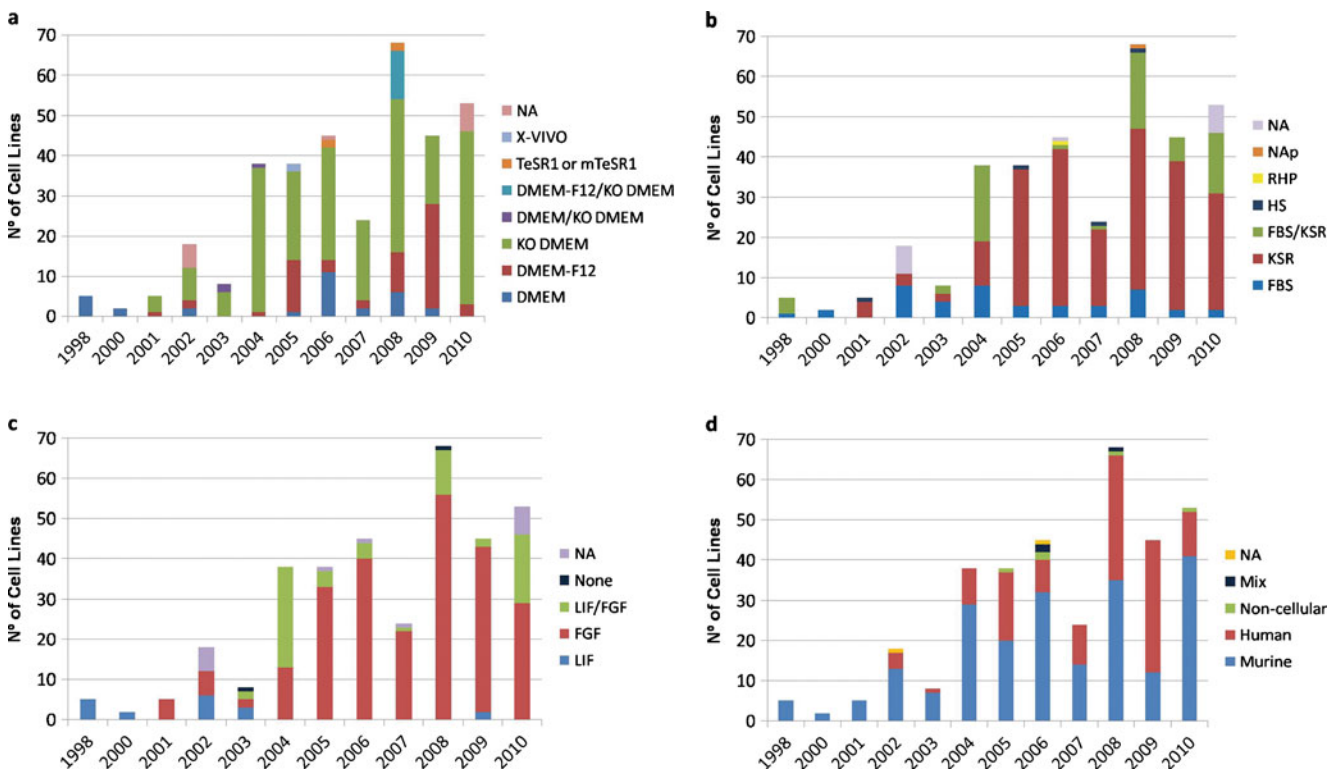The use of animal-derived components in hESC cultures has became a major concern after the discovery, in 2005, of

Fig. 3 Media composition. Human embryonic stem cell lines derived each year showing **a** basal medium; **b** protein supplement—*RHP* recombinant human protein, *HS* human serum, *KSR* knockout serum replacement, *FBS* fetal calf serum or fetal bovine serum, *NAp* do not apply (lines derived in complete medium); **c** factor for maintenance of pluripotency; **d** Type of cell support. *Mix* different combinations of support; *NA* information not available

potentially immunogenic nonhuman sialic acid, Neu5Gc, on hESC lines cultured on animal cells or in animal serum products [37] Although this is not a limitation for lines established for research purposes, different groups, interested in deriving hESC lines for therapy, have been working on alternative animal-free conditions, using xeno-free media including the use of TeSR1 and X-VIVO, and recombinant human proteins as serum substitutes.

Maintenance of pluripotency of murine ES cells is achieved by culturing them on mitotically inactivated mouse embryonic fibroblasts (MEF)—feeder layers, and in the presence of LIF. Although hLIF was used in the establishment of the first lines of hESCs [4, 9], it has been shown that it is not capable of promoting long term maintenance of these cells in an undifferentiated state [38]. From 2002 on, bFGF, either alone or in combination with hLIF, has been consolidated as the major factor for the maintenance of pluripotency of hESC during derivation and long term culture [33] (Fig. 3c).

Similar to murine ES cells, the first hESC lines were established in MEFs. However, in order to avoid using xeno-components, since 2003 the use of different types of alternative human support cells and acellular matrices for the establishment of hESCs is observed (Fig. 3d). In spite of that, a significant number of cell lines continue to be derived in the presence of MEFs, indicating that support cells have a greater capacity of maintaining pluripotency of hESCs, and/or their availability and cost are more advantageous than the other alternatives.

We listed other parameters of the methodology for hESC derivation, including time of first passage, method of passage (enzymatic versus mechanical), frequency of media change, and efficiency of derivation (Supplementary Table 1). However, these parameters varied widely among cell lines, hampering their analysis, which was not conclusive. One important information would be the efficiency of the different protocols, regarding the number of cell lines obtained using that particular methodology per embryo manipulated. Nevertheless, this data was not readily available in several publications, and therefore could not be evaluated in this survey.

Conclusions

When starting a project involving the derivation of new lines of hESC, how to choose the best strategy? This will depend primarily on the quality of the available embryos, and on the use one wants to make of the lines. If they are meant exclusively for research, there may be no concerns about the use of animal-derived products. In contrast, if the new lines are to be used for therapy, although not a requirement, defined and animal-free conditions are more adequate. Nevertheless, it is important to note that hESC

lines established in the presence of xeno-components can be transitioned into culture conditions adequate for clinical use, as it is the case of the very first hESC line established, H1, differentiated into oligodendrocytes for the treatment of spinal cord injury (www.geron.com).

Due to the lack of homogeneity on data presented for hESCs derivation, meta-analysis and reviews like our own are hampered. One of the most striking difficulties we have found concerns the definition of embryo quality, as researchers use different embryo classification systems, and there is little information available on the proportion of embryos of different quality used to derive each line. Another issue refers to the efficiency of derivation: when available, which is not the rule, rates are based on the number of hESCs versus either the initial number of embryos or, alternatively, the number of embryos that reached blastocyst stage, thus being heterogeneous. Although scientific advisory boards define and monitor eligibility criteria for the listing of hESCs in the registries, there is an urge to standardize the information deposited.

In summary, our data shows a great heterogeneity in the methodologies for hESC derivation, indicating that the optimum conditions have not been consolidated yet. Nevertheless, the strategies have significantly evolved since 1998: MEFs are still the most used support cells, but KO-DMEM/KSR with bFGF has been recognized as a more adequate culture medium. In addition, the initial requirement for isolation of ICM is not observed anymore, and lines can be established also from whole plated and low quality embryos. Finally, the concern about the emergence of commerce of embryos created for research purposes [39] has not materialized, since the vast majority of the available lines were established from embryos generated for reproductive purposes. However, it is important to notice that all the listed methodologies yield lines of hESC that still differ significantly from the murine counterpart in morphology and epigenetic stability, among other characteristics [40]. More recent findings on the effects of physiological levels of oxygen on the epigenetic stability of hESC [41], and on the role of kinase inhibitors and hLIF in the maintenance of human induced pluripotent stem cell (hiPSC) in a mouse ESC (mESC)-like state [40], promise to revolutionize the field. Human ESCs may never be as homogeneous as mESCs, after all, humans are outbred. Nevertheless, for the next years we expect important improvements in the conditions for establishing and culturing new lines of hESC more amenable for research and therapy than the currently available ones.

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