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Kursad Turksen *Editor*

Human Embryonic Stem Cells Handbook

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Human Embryonic Stem Cells Handbook

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Preface

Considerable advances have taken place since the initial isolation and characterization of human embryonic stem cells (HES). Nevertheless, significant challenges remain before their potential for restoration and regeneration processes in patients can be realized. Understanding the diversity among HES lines and isolating lines with robust differentiation potential remain difficult. To this end, I have attempted to collect the many protocols that have been used by various laboratories around the world so as to allow both novices and experienced investigators to compare and contrast different approaches to HES isolation and characterization, with the hope that from these protocols we might standardize approaches for HES biology. I am grateful to all the contributors to this volume for taking the time to describe their methods in great detail. I am confident that this volume will become a valuable reference for future research studies in the field. It is also expected that the protocols will provide a useful teaching tool for graduate students and postdoctoral fellows as they launch their research careers.

I am grateful to Dr. John Walker, the Editor in Chief of the *Methods in Molecular Biology* series, for supporting the idea for this volume. His suggestions and insights were invaluable during the editing of the volume.

Patrick Marton was, as always, available for addressing my questions and concerns during the course of putting together the volume.

David Casey remains an indispensable support, pointing out details that I might have missed in the chapters and helping to make the volume complete.

Ottawa, ON, Canada ***Kursad Turksen, Ph.D.***

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Chapter 1

Establishment of New Lines of Human Embryonic Stem Cells: Evolution of the Methodology

Ana Maria Fraga, Érica Sara Souza de Araújo, Raquel Stabellini, Naja Vergani, and Lygia V. Pereira

Abstract

Although since 1998 more than 1,200 different hESC lines have been established worldwide, there is still a recognized interest in the establishment of new lines of hESC, particularly from HLA types and ethnic groups underrepresented among the currently available lines. The methodology of hESC derivation has evolved significantly since the initial derivations using human LIF (hLIF) for maintenance of pluripotency. However, there are still a number of alternative strategies for the different steps involved in establishing a new line of hESC. We have analyzed the different strategies/parameters used between 1998 and 2010 for the derivation of the 375 hESC lines able to form teratomas in immunocompromised mice deposited in two international stem cell registries. Here we describe some trends in the methodology for establishing hESC lines, discussing the developments in the field. Nevertheless, we describe a much greater heterogeneity of strategies for hESCs derivation than what is used for murine ESC lines, indicating that optimum conditions have not been identified yet, and thus, hESC establishment is still an evolving field of research.

Key words: Embryonic stem cells, Pluripotency, Derivation, Defined media

1. Introduction

Pluripotent embryonic stem cells (ESC) were first derived from preimplantation mouse embryos in the early 1980s (1). Since then, hundreds of lines of murine ESCs have been established worldwide, following basically the same protocol of plating whole embryos free of the zona pelucida in mitotically inactivated mouse embryonic fibroblasts (MEFs), in the presence of leukemia inhibiting

Ana Maria Fraga, Érica Sara Souza de Araújo, Raquel Stabellini, and Naja Vergani have contributed equally to this work.

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factor (LIF) for the maintenance of pluripotency (reviewed by Guasch and Fuchs (2)). Despite continuous efforts from many laboratories, it was only in 1998 that the first lines of human ESC (hESC) were described (3). After that, many other groups reported on the derivation of hESCs, and the number of different lines registered on the available databases rapidly increased.

The conditions described for the establishment of the first hESC lines (3, 4) were essentially the same as those used for the derivation of mouse ESCs (1). However, 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 (5). 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.

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

We analyzed parameters regarding the embryo [source (generated for reproduction or research), fresh or frozen, stage of development, quality, and method for ICM isolation], and the culture conditions (basic medium, protein source, factors for maintenance of pluripotency, type of support, and 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.

2. Evolution of Numbers of HESC Derivation

From the 1,291 different hESC lines registered in the two stem cell registries (hESCReg and UMass ISCR), 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 (6). Twenty-six hESC lines lacked information regarding the year of derivation and were therefore excluded from the analysis.

Figure 1 shows the increase in the total number of hESC lines derived from 1998 until 2010. It is interesting to notice that it took 2–4 years since the first derivations by Thomson et al. (3) and by Reubinoff et al. (4) for other groups to establish new lines of

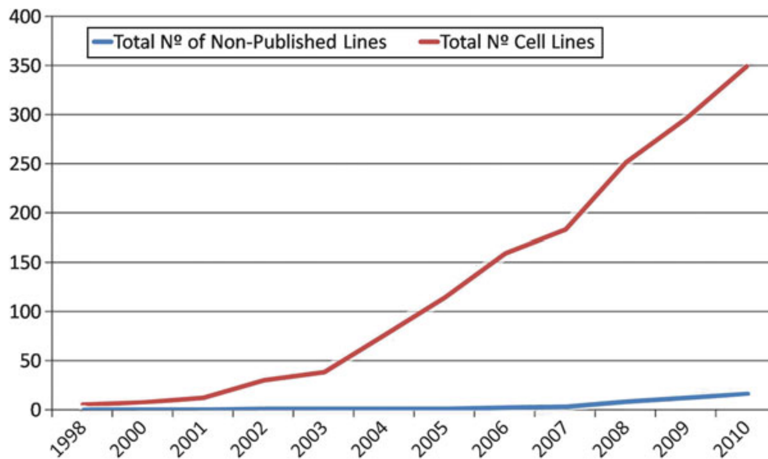

Fig. 1. Increase in the number of new hESC lines derived from 1998 through 2010 (*red line*), and number of hESC lines established but not published in the period (*blue line*).

hESC. Those two groups described derivation with hLIF (human leukemia inhibitor factor) and MEFs (mouse embryonic fibroblasts) as factors for maintenance of pluripotency. However, in 2000 the use of bFGF (basic fibroblast growth factor) was described as a requirement for the prolonged culture of hESC in serum-free medium (7), and from then on, this growth factor has been consolidated as the major factor for maintenance of hESCs' pluripotency. This finding may have had a role in the raise in the number of derivations observed thereafter. In fact, the number of new hESC lines as well as the number of research groups and laboratories engaged in their establishment greatly increased from 2004 to the end of 2010.

Most of the derivations of those hESC lines were described in peer-reviewed journals, even in the last 2 years (Fig. 1). However, 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. Nevertheless, given the interest of increasing the diversity of available hESC lines, it is important to have a venue for publishing the new lines and their specific characteristics, a role that can be performed by human stem cell registries.

3. Methods for Derivation of HESC Lines

3.1. Source of the Embryo

The vast majority (362 or 98.4% of the informative lines) of embryos used for the derivation of hESC lines was surplus from reproductive cycles (Table 1). In contrast, only six lines of hESCs (1.6%)

Table 1
Main characteristics of embryos and procedures used for the derivation of hES cell lines

		Number of cell lines from 1998 to 2010 (%[a])	Number of cell lines from 2008 to 2010 (%[a])
Source of the embryo	Reproduction	362 (98,4)	166 (100)
	Research	6 (1,6)	0
	NA	7	0
Fresh/frozen embryo	Fresh	111 (43,5)	52 (45,2)
	Frozen	144 (56,5)	63 (54,8)
	NA	120	51
Stage of development	Blastocysts	349 (95,4)	156 (94)
	Pre-blastocysts	7 (1,9)	4 (2,4)
	Blastomere	10 (2,7)	6 (3,6)
	NA	9	0
Quality of the embryo	Good	69 (48,6)	22 (32,8) ($p=0.04$)
	Intermediate	21 (14,8)	5 (7,5)
	Poor	52 (36,6)	40 (59,7) ($p=0.002$)
	NA/not classifiable	233	99
Method of ICM isolation	Immunosurgery	148 (46,8)	26 (19,4) ($p<0.001$)
	Mechanical	89 (28,2)	63 (47) ($p<0.001$)
	Whole embryo plated	62 (19,6)	34 (25,4)
	Laser	12 (3,8)	10 (7,5)
	Hypotonic lysis	5 (1,6)	1 (0,7)
	NA/non applicable	59	32

[a]Percentage of cell lines, excluding those missing information. Significant differences between percentages of each parameter between 1998–2010 and 2008–2010 are shown with the corresponding *p*-value (two-sided test of equal proportions). NA: information not available

were derived from embryos generated specifically for research—four in China and two in Belgium (Table 1), countries with permissive legislations concerning stem cell research and somatic cell nuclear transfer for therapeutic purposes (8). 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. Finally, between 2008 and 2010, all hESC lines were derived from embryos produced for reproductive reasons (Table 1). Therefore, creating embryos exclusively to generate hESC lines is not a common practice, probably due to the difficulties in obtaining donors of human oocytes.

One implication of the use of surplus embryos for the establishment of new lines of hESC is the possible limited genetic diversity of these embryos. Most of them are generated in private clinics of assisted reproduction that, due to the high cost of the procedure, may not attend the whole ethnic admixture of a given population (9). In fact, recent articles have described that most of the hESC lines established worldwide are mainly of European and East-Asian

ethnicity (10–12). Thus, an effort must be made 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, a trend that has not changed in the last 2 years (Table 1). While fresh embryos, if supernumerary from reproduction cycles, tend to be available in smaller numbers and at shorter notice for each experiment, the use of frozen embryos allows working with larger batches of embryos at the same time. Nevertheless, some researchers reported better efficiencies of hESC line derivation with fresh rather than frozen embryos (13).

3.2. Embryo Quality

Embryo quality is an important parameter for the derivation of hESC lines. Earlier reports suggested the requirement for good quality blastocyst-stage embryos for that end. From the 375 cell lines analyzed here, only 216 (57.6%) were informative for the quality of the embryo from which they were derived (Table 1). Earlier-staged embryos (6-cell up to morula staged embryos) and blastomere-derived lines were excluded from this analysis because the information on embryo quality was usually not available.

Since diverse methodologies for embryo scoring were employed by the different laboratories, we used the classification provided on the hESC databases (or in the corresponding articles), where the quality of the embryos was defined 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. 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" (Table 1). When looking at the lines derived more recently, a significantly higher proportion (59.7%) was derived from "poor" embryos (Table 1, Fig. 2). Nevertheless, although "good" embryos appear to be more adequate for hESC derivation, a high proportion of lines was established from "poor" embryos, not suited for reproduction—perhaps due to a greater number of this kind of human embryo donated for research. Note that 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.

3.3. Developmental Stage of the Embryo

The great majority (349 or 95.4% of the informative lines) of the hESC lines with potential of teratoma formation were originated from embryos at the blastocyst stage, whereas only 1.9% (7 lines) were established from earlier-staged embryos, and 2.7% (10 lines) from single blastomeres, a distribution that has not changed significantly in the last 2 years (Table 1). Note that the derivations of hESC lines from earlier-staged embryos and from single blastomeres,

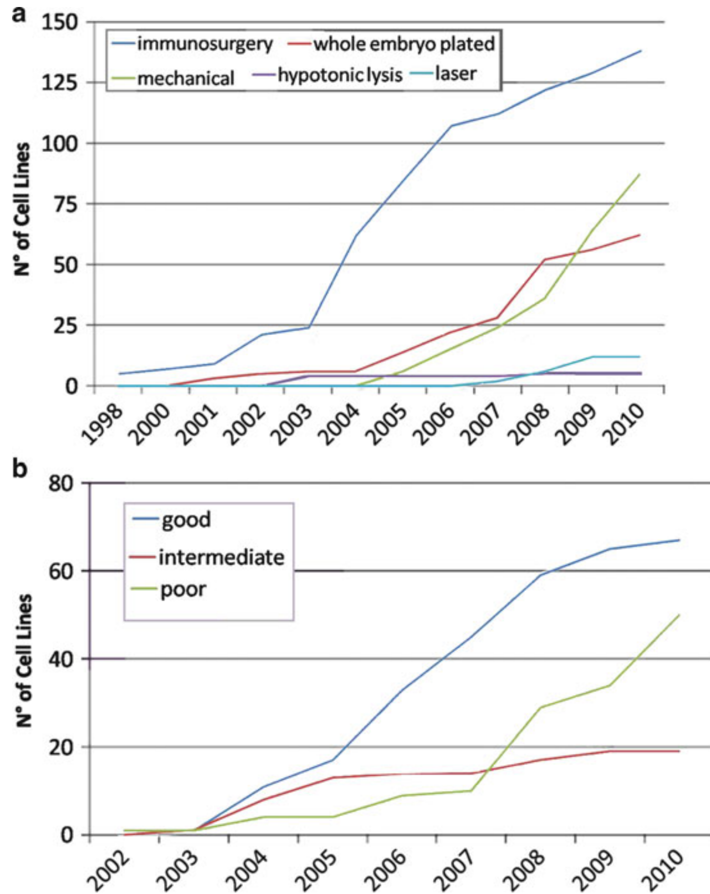

Fig. 2. Embryos used for establishment of hESC lines. Increase in the total number of cell lines derived from 1998 through 2010 according to (**a**) method employed for extraction of pluripotent cells; and (**b**) embryo quality.

although not frequent, have been achieved by different independent groups (14, 15), demonstrating the feasibility of these strategies, which in turn increases the number of embryos adequate for hESC derivation. Nevertheless, the derivation of hESC lines from single blastomeres was generally performed to avoid the controversies involved in the destruction of human embryos, rather than to obtain a better source of cells for hESC derivation.

3.4. Methods of ICM Isolation

Mouse ES cells are established by plating whole blastocyst and mechanically isolating cells derived from the ICM from the surrounding trophectoderm within few days. In contrast, the procedures adopted until 2004 for the derivation of hESC lines usually included the isolation of the ICM from the embryo before plating (Fig. 2). The lesser an embryo is manipulated, the smaller is the chance of causing any damage to its cells. However, as trophectoderm cells proliferate very fast, they can suppress the overgrowth of the ICM and even generate a trophectoderm stem cell line (16).

From 1998 until 2010, the technique mostly employed for the isolation of human ICM was immunosurgery (Table 1), a nonspecific process based on the embryo susceptibility to complement-dependent antibody cytotoxicity (17). The main disadvantage of this method is the risk of damaging cells from the ICM (18), thus 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, alternative methods were developed, mainly mechanical or laser-assisted isolation of the ICM. In fact, from 2008 until 2010, mechanical dissection of the ICM became the most commonly used strategy (Table 1). In addition, a large proportion of hESC lines have been established from whole plated embryos (Table 1), mostly poor-quality embryos without a clear ICM, but also good-quality embryos.

In conclusion, we identified a trend not to employ immunosurgery and to use mechanical dissection of the ICM. However, the best methodology to isolate ICM from human embryos has not been consolidated yet. Therefore, the morphology and quality of the embryo, together with the availability or not of laser and micromanipulation equipments, play important roles in the choice of the method for ICM isolation. Finally, since several lines of hESC have been established from whole plated embryos, mostly in the last 2 years, the main novelty has been the lack of requirement for isolation of ICM for hESC derivation, simplifying the procedure.

3.5. Culture Conditions

We divided the culture media for derivation of a hESC line into four main components: (1) basic medium (e.g., DMEM, DMEM-F12, KO-DMEM), (2) protein source (e.g., FBS, KSR), (3) factor(s) used to stimulate self-renewal (e.g., bFGF, LIF, insulin, different small molecules), and (4) matrix/cell support (e.g., MEF, STO, HFF, Matrigel). Tens of different combinations of the various types of these four components have been shown to be able to maintain pluripotent hESCs in culture (reviewed in ref. (19)), and our analysis shows that many of them are also suitable for the establishment of new cell lines (Table 2).

Since 2003, KO-DMEM and KSR (knockout serum replacement) have been the most used basal culture medium and protein source for the derivation of hESC lines, respectively (Fig. 3a). In fact, 65.0% of all the hESC considered in this review were cultured in these two components at least during some stage of the derivation (Table 2). Thus, it comprises the most employed medium basis not only during the maintenance of the hESC lines in culture (19) but also during their derivation.

KO-DMEM is a “DMEM-based” medium with reduced osmolarity, which improves the growth of the undifferentiated ESC, while the KSR, developed in 1998, is more suitable for culturing ESC than the regular fetal bovine serum (FBS) because it does not contain

Table 2
Culture condition for the derivation of hES cell lines

		Number of cell lines from 1998 to 2010 (%[a])	Number of cell lines from 2008 to 2010 (%[a])
Culture medium	KO DMEM	230 (65)	98 (61,6)
	DMEM-F12	66 (18,6)	39 (24,5)
	DMEM	37 (10,5)	8 (5)
	DMEM-F12/KO DMEM	12 (3,4)	12 (7,6)
	TeSR1 or mTeSR1	4 (1,1)	2 (1,3)
	DMEM/KO DMEM	3 (0,8)	0
	X-VIVO	2 (0,6)	0
	NA	21	7
Protein source	KSR	229 (65,1)	106 (67,1)
	FBS/KSR	67 (19)	40 (25,3)
	FBS	51 (14,5)	11 (7) ($p = 0.02$)
	HS	4 (1,1)	1 (0,6)
	RHP	1 (0,3)	0
	NA/NAp	23	8
Growth factors	FGF	262 (74,9)	126 (79,2)
	LIF/FGF	66 (18,8)	30 (18,9)
	LIF	20 (5,7)	2 (1,3) ($p = 0.04$)
	No growth factors	2 (0,6)	1 (0,6)
	NA	25	7
Surface coating/ matrix	Murine	232 (62,9)	88 (53) ($p = 0.04$)
	Human	126 (34,1)	75 (45,2) (p = 0.02)
	Mix	6 (1,6)	1 (0,6)
	Non-cellular	5 (1,4)	2 (1,2)
	NA	6	0

[a]Percentage of cell lines, excluding those missing information. Significant differences between percentages of each parameter between 1998–2010 and 2008–2010 are shown with the corresponding p value (two-sided test of equal proportions). NA: information not available

undefined growth or differentiation promoting factors (20). Furthermore, although containing animal-derived components, KSR is a defined supplement, avoiding the lot-to-lot composition variation found in FBS (21). Nevertheless, even in the last 2 years, a significant number of hESC lines (40 lines—25.3%) were still established in a combination of KSR/FBS, which apparently improves the initial attachment and outgrowth of the ICM (22) and were subsequently transitioned into KSR alone for long-term culture. In contrast, the use of FBS as a single protein source for derivations has significantly decreased in the last 2 years (Table 2).

An additional concern regarding the use of animal derived components in hESC cultures has been the discovery, in 2005, of potentially immunogenic nonhuman sialic acid on hESC lines cultured on animal cells or in animal serum products (18). Although this is not a limitation for lines established for research purposes,

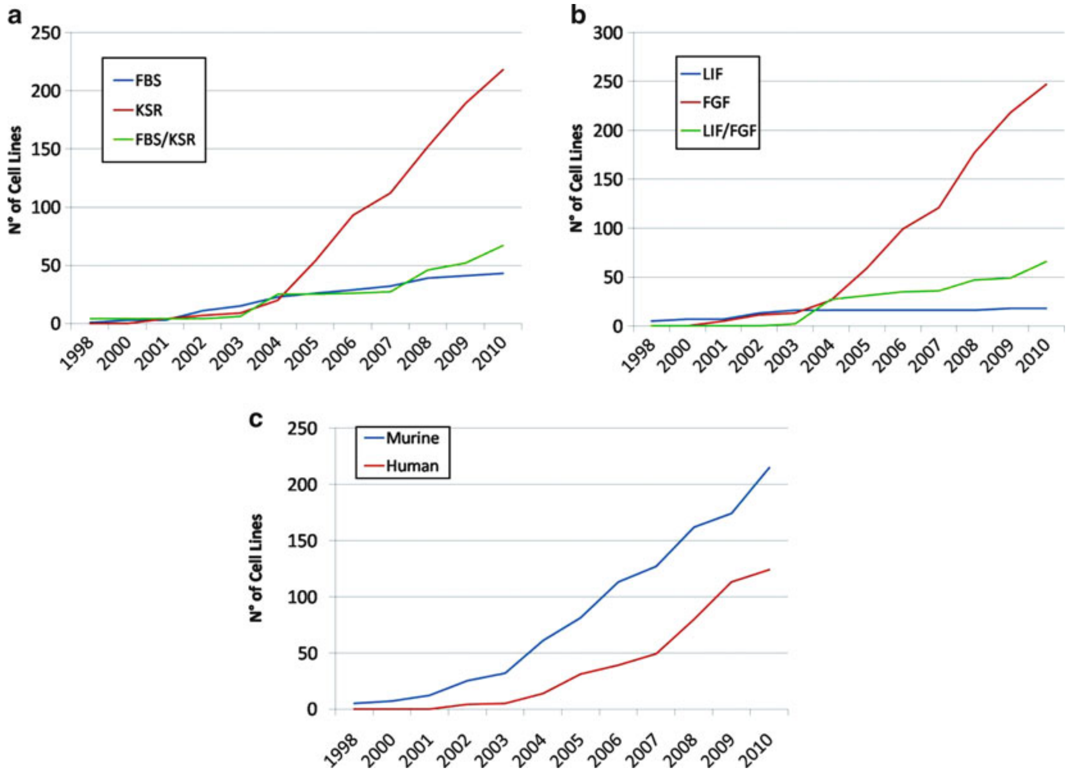

Fig. 3. Media composition. Increase in the total number of cell lines derived from 1998 through 2010 according to (**a**) protein supplement—*KSR* knockout serum replacement, *FBS* fetal calf serum or fetal bovine serum; (**b**) factor for pluripotency maintenance; (**c**) Type of cell support—murine or human feeder cells.

different groups deriving hESC lines for therapy started working on alternative animal-free conditions, using defined xeno-free media like TeSR1 and X-VIVO, and recombinant human proteins as serum substitutes (Table 2).

Leukemia inhibiting factor (LIF) and mitotically inactivated mouse embryonic feeder layers (MEFs) are responsible for maintenance of pluripotency of murine ES cells. Although human LIF (hLIF) was used in the establishment of the first lines of hESCs (3, 4), it has been shown not to be capable of promoting long-term maintenance of these cells in an undifferentiated state (23). Thus, the use of hLIF alone has significantly decreased in the last 2 years, whereas from 2008 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 (19) (Table 2, Fig. 3b).

Although MEFs are the most commonly used support for the establishment of hESC lines (62.9%), since 2003 we observe the use of different types of alternative human support cells and acellular matrices, probably in order to avoid contamination with animal-derived products (Table 2). From 2008 until 2010, we observed a

significant decrease in the use of MEF and significant increase in the use of human feeders (Table 2). In spite of that, a large proportion (53%) of hESC lines continue to be derived in the presence of MEFs, indicating that these support cells have a greater capacity of maintaining pluripotency of hESCs, and/or their availability and cost are more advantageous than the other alternatives.

Other parameters of the methodology for hESC derivation, including time of first passage, frequency of media change, and efficiency of derivation were also analyzed. However, they varied widely among cell lines, and therefore their analysis was not conclusive. It would be particularly important to have information on the efficiency of the different protocols, i.e., the number of hESC lines obtained per embryo manipulated using a particular methodology. Regrettably, in addition to this data not being readily available in several publications, the relatively small number of cell lines established in each specific condition hampers a significant statistical analysis of efficiency.

4. Conclusions

When starting a project involving the derivation of new lines of hESC, the choice of the best strategy will depend mainly on the quality of the available embryos and on the use one wants to make of the lines. For lines used for basic research, there may be no concerns about the use of animal-derived products. However, if the new lines are to be used for therapy, defined and animal-free conditions are more adequate. Nevertheless, it is important to note that this is not a requirement—hESC lines established in the presence of animal products can be transitioned into culture conditions adequate for clinical use. In fact, that was the case for the very first hESC line established, H1, differentiated into oligodendrocytes for the treatment of spinal cord injury—the very first hESC-derived product to be injected in humans (http://www.geron.com).

In summary, the great heterogeneity in the methodologies for hESC derivation described here indicates that optimum conditions have not been identified yet. Even so, we can identify significant improvements since 1998: MEFs are still the most used support cells, but KO-DMEM/KSR with bFGF has been consolidated as a more adequate culture medium. In addition, isolation of ICM is not required anymore, and lines can be established also from whole plated and low-quality embryos. However, all the listed methodologies still yield lines of hESC that differ significantly from the murine counterpart in morphology, epigenetic stability and growth kinetics, among other characteristics (24).

More recently, the positive effects of physiological levels of oxygen on the epigenetic stability of hESC have been described (25).

In addition, human induced pluripotent stem cell (hiPSC) derived in the presence of kinase inhibitors and hLIF have been shown to be more similar to mESC (24). These studies must still be consolidated by other groups, but have the potential to promote great changes in the field. Finally, we must consider the genetic heterogeneity of humans, which add another level of complexity to hESCs when compared to the murine counterpart, derived from isogenic animals, mostly the 129/Sv strain. Nevertheless, for the next years we anticipate important improvements in the conditions for establishing and culturing new lines of hESC more amenable for research and therapy.

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Chapter 2

Human Embryonic Stem Cells Derived in Xeno-Free Conditions

Amparo Galán and Carlos Simón

Abstract

In this chapter, we describe the derivation and characterization of nine human embryonic stem cells (hESC) (VAL-3 to -11B) from different developmental embryo stages (inner cell mass from a blastocyst, morula, and blastomere from a 3-day embryo) under xeno-free conditions providing the necessary protocols and techniques to carry out their derivation, characterization, and propagation.

Key words: Human embryonic stem cells, Derivation, Culture conditions, Molecular characterization, Immunophenotyping

1. Introduction

Human embryonic stem cells (hESC) are regarded as an unlimited cell source for replacement therapy in regenerative medicine for their properties of undifferentiation, pluripotentiality, and unlimited proliferation in vitro. Due to their clinical potential, the scientific community must provide proper derivation and propagation in clinical-grade conditions, characterization, and registration for worldwide applications. In this chapter, we report the derivation in xeno-free conditions of 9 hESC lines from different developmental embryo sources, from inner cell masses (ICM) from blastocysts (VAL-3, -4, -5, -6M, -7, -8) (1, 2), morula (VAL-9) (2), and from single biopsied blastomeres at 6- and 8-cell stage embryos (VAL-10B, -11B) (3) with further cryopreservation of the resulting blastocyst confirming its potential use as a derivation source preserving embryo viability (4) (Fig. 1). All hESC lines were derived after approval by the institutional review board of the Prince Felipe Research Centre and granted permission by the National Spanish Authority, Instituto de Salud Carlos III (ISCIII) (December 13, 2006). Human embryos frozen at different stages were donated

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Cell Line	Embryo			Colony Morphology		Last passage Criovials
VAL-3			Whole normal blastocyst stage embryo (46,XY) derivation in human foreskin		10x, P42	80 29 CV
VAL-4			Whole normal blastocyst stage embryo (46,XX) derivation in human foreskin		10x, P40	54 38 CV
VAL-5			Whole normal blastocyst stage embryo (46,XX) derivation in human foreskin		10x, P43	49 37 CV
VAL-6M			Whole monogenetic affected embryo derivation (DM Type1) (46,XY) in human foreskin		10x, P45	49 29 CV
VAL-7			Derivation from ICM isolated by laser (46,XY) in human foreskin		10x, P22	65 18 CV
VAL-8			Whole normal blastocyst stage embryo (46,XX) derivation in human foreskin		10x, F6	22 30 CV
VAL-9			Whole normal morula stage embryo (46,XY) derivation in human foreskin		10x, F6	55 43 CV
VAL-10B			Derivation from single biopsied blastomere(46,XY) in human foreskin		10x, P13	25 39 CV
VAL-11B			Derivation from single biopsied blastomere(46,XX) in human foreskin		10x, P15	25 46 CV

Fig. 1. Derivation sources for each VAL hESC line derived including embryo karyotype origin. 10× magnification of each hESC line morphology, and last passage registered and available criovials.

for this work at the Instituto Valenciano de Infertilidad (IVI) according to Spanish law 45/2003. Progenitors were asked to sign a specific consent form for stem cell derivation as indicated in Royal Decree 2132/2004. All the blastocysts were scored grade A-B (5), and blastomere biopsies were obtained from each embryo following the single-cell biopsy procedure similar to that used in the preimplantation genetic diagnosis (6).

Derivation and culture conditions were performed on microbiologically tested and irradiated human foreskin fibroblasts designed to minimize contact with xeno-components. Cryopreservation of the hESC lines was also successfully performed following a slow freezing and rapid thawing method in serum-free conditions (7).

In this chapter, we also describe all necessary techniques for hESC characterization, including fingerprinting to allow their identification and traceability, the HLA haplotype, and all required processes to assess their undifferentiated molecular profile and immunophenotype by current techniques carried out in our

laboratory, and following the International Stem Cell Initiative (ISCI) recommendations (8). *In vitro* and *in vivo* pluripotency assessment of VAL lines is also shown. For the *in vitro* differentiation study, embryoid bodies (EBs) were generated as described (9), and *in vivo* differentiation was carried out by the generation of teratomas after intratesticular injection of hESC in NOD-SCID animals. In both cases, the three embryonic germ layers formation were confirmed by immunostaining. VAL-3 to -11B lines are registered and available upon request to the Spanish Stem Cell Bank (http://www.isciii/htdocs/terapia/terapia_bancocelular.jsp) and to the hESC European Registry (http://www.hescreg.eu).

2. Materials

2.1. Equipment for Derivation and Culture Material

1. Equipment.

   Gamma irradiator Biobin 8000.

   Holding micropipettes (Humagen Inc).

   Micromanipulator (Nikon, Narishige).

   Inverted Microscope (Nikon).

   Micromanipulation plate (Beckton & Dickinson).

   Infrared laser (Zilos-tk™, Hamilton Thorne Biosciences).

   50 µm biopsy pipette (HumaGene Inc).

2. Culture material.

   6-well cell culture plates (Beckton & Dickinson).

   Multiwell cell culture plates (Beckton & Dickinson).

   Low-attachment 6-well culture plates (Corning).

   4-well adherent culture plates (Nunc).

   30 mL conical tubes.

   Sharp flame-pulled Pasteur Pipettes hand made for mechanical dissection.

2.2. Cell Lines and Culture Media

1. Human foreskin fibroblasts CCD 1112Sk (Catalogue CRL-2429) (American Type Culture Collection, (ATCC)).
2. Culture media.
   (a) Feeders.

   Iscoves' modified Dulbecco's medium (IMDM) (ATTC) plus 10% fetal bovine serum (FBS) (PAA).

   (b) Embryo thawing, blastomere biopsy, and samples processing.

   Embryo Thaw Kit (Vitrolife).

IVF (Vitrolife).

CCM (Vitrolife).

Tyrode's acid (Sigma).

GPGD medium (Vitrolife) supplemented with 5% Human Serum Albumin (Vitrolife).

Mineral oil (Sigma).

Human laminin (Sigma).

3. Derivation and undifferentiation culture medium.
HES culture medium: 80% Knockout DMEM (Gibco), 20% Knockout SR (Gibco), 2 mM GlutaMAX (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 1% nonessential amino acids stock (Gibco), with variable concentrations of human basic Fibroblast Growth Factor (hbFGF) (Invitrogen) and 0.5% Penicillin-Streptomycin (Sigma).

4. *In vitro* differentiation.
Differentiation medium: 80% Knockout DMEM, 20% FBS (Gibco), 2 mM GlutaMAX (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 1% nonessential amino acids stock (Gibco).
PBS.
4% paraformaldehyde.

2.3. Freezing and Thawing

Dimethylsulfoxide (DMSO) solutions at a final concentration of 1.0 M, 1.2 M, 1.5 M, and 2.0 M prepared in HES medium.

Isopropanol.

Liquid nitrogen (LN_2).

Bench centrifuge.

2.4. Karyotype Samples Preparation

Colcemid (Gibco).

Accumax (Chemicon).

Potassium chloride solution (KCl, 0.075 M).

Carnoy fixative solution (Methanol (3)/Acetic acid (1)).

Bench centrifuge.

2.5. Telomerase Activity Detection

$TRAP_{EZE}$® Telomerase Detection Kit (Chemicon).

SYBR® Gold Nucleic Acid Gel Stain (Molecular probes) (1:10,000).

PBS (free of Mg^{2+} y Ca^{2+}).

Taq polymerase.

Ready Gel 15% TBE. Precast gel for polyacrilamide electrophoresis (BioRad).

Tris–Borate–EDTA (TBE) 10×.

Sample loading buffer 5× (BioRad).

Double distilled water (DDW).

Refrigerated bench centrifuge.

Thermal cycler.

Vertical Electrophoresis equipment (BioRad).

CCD lector (GelDoc) (BioRad).

2.6. Genomic DNA Extraction for Fingerprinting and HLA Analysis

FlexiGene DNA Kit (Qiagen).

PBS.

Isopropanol (stored at −20°C).

70% Ethanol (stored at −20°C).

Double Distilled Water (DDW).

Bench centrifuge.

Nanodrop platform (Nanodrop).

2.7. Molecular Profile

1. RNA Purification and cDNA synthesis.

   Quick-RNA™ MicroPrep (Zymo Research).

   PBS (Ca^{2+} and Mg^{2+} free).

   Ethanol.

   DDW.

   Scriptguard RNase Inhibitor 2.500 U @ 40 U/μl (Ecogen).

   Advantage™ RT-for-PCR kit containing MMLV enzyme (Clontech, BD).

   Thermal cycler.

   Nanodrop platform (Nanodrop).

2. PCR and electrophoresis separation.

   PCR primers and amplicons are listed in Table 1.

   Taq polymerase Kit.

   Agarose.

   TAE 1× buffer pH 8.

   Ethidium bromide (1:10,000).

   Loading buffer.

   Thermal cycler.

   Horizontal electrophoresis equipment.

   CCD lector.

2.8. Immunocytochemistry

PBS.

Paraformaldehyde (PFA): 4% in distilled water.

Tris (hydroxymethyl) aminomethane.

HCl.

NaCl.

Tween-20.

Triton X-100 (Sigma): prepare at 0.1% in PBS.

Table 1
List of primers used for molecular characterization of hESC

Gene	Primer sequence	Amplicon	Characteristic
POU5F1	AAGAACATGTGTAAGCTGCGGCCC GGAAAGGCTTCCCCCTCAGGGAAAGG	455 pbs	Undifferentiation
NANOG	CTGATTCTTCCACCAGTCCCAA TGTTCCAGGAGTGGTTGCTCCA	449 pbs	Undifferentiation
CRIPTO	CCATCAGGAATTTGCTCGTCCA GAAAGGCAGATGCCAACTAGCA	453 pbs	Undifferentiation
DNMT3B	TTTGGCCACCTTCAATAAGC GGCAACATCTGAAGCCATTT	412 pbs	Undifferentiation
GABR3	CTTGACAATCGAGTGGCTGA CAACCGAAAGCTCAGTGACA	396 pbs	Undifferentiation
GDF3	CTTCACCCCAGAAGTTCCAA GCAGGTTGAAGTGAACAGCA	435 pbs	Undifferentiation
NFH	TGAACACAGACGCTATGCGCTCAG CACCTTTATGTGAGTGGACACAGAG	400 pbs	Ectoderm differentiation
REN	AGTCGTCTTTGACACTGGTTCGTCC GGTAGAACCTGAGATGTAGGATGC	590 pbs	Mesoderm differentiation
AMI	GCTGGGCTCAGTATTCCCCAAATAC GACGACAATCTCTGACCTGAGTAGC	492 pbs	Endoderm differentiation
RPL19	CGAATGCCAGAGAAGGTCAC CCATGAGAATCCGCTTGTTT	153 pbs	Housekeeping

Primer sequences designed for the International Stem Cell Initiative (ISCI) markers (8) plus differentiation markers characteristic of ectoderm, mesoderm and endoderm

Normal Goat Serum (NGS) (Sigma): prepare at 10% and 4% in PBS.

Normal Donkey Serum (NDS) (Sigma): prepare at 10% in PBS.

Bovine Serum Albumin (BSA) (Sigma): prepare at 0.1% in PBS.

Rinse buffer: 20 mM Tris–HCl + 0.15 NaCl + 0.05% Tween-20+ PBS.

Prolong Gold Antifade with DAPI (Molecular Probes).

Xylen.

Absolute ethanol.

Trisodium citrate dihydrate for citrate buffer 0.05 M pH = 6.

LSAB + System-HRP (Dakocytomation).

Harris hematoxilin solution modified (Sigma).

Entellan (Merck).

Fluorescence microscope.

Antibodies used for immunocytochemistry are described in Table 2.

Table 2
Antibodies used for immunophenotyping

First antibody	Working solution	Company	Second antibody	Working solution	Company
Mouse anti-human SSEA-4	1:50	Chemicon	Goat anti-mouse IgG-Alexa Fluor® 488	1:500	Molecular Probes
Mouse anti-human SSEA-1	1:50	Chemicon	Goat anti-mouse IgM-FITC	1:128	Sigma
Mouse anti-human TRA-1-60	1:50	Chemicon	Goat anti-mouse IgM-FITC	1:128	Molecular Probes
Mouse anti-human TRA-1-81	1:50	Chemicon	Goat anti-mouse IgM-FITC	1:128	Molecular Probes
Mouse anti-human TRA-1-254	1:200	Chemicon	Goat anti-mouse IgG_1- Goat anti-mouse Alexa Fluor® 568	1:500	Molecular Probes
Anti-human Nanog	1:40	R&D	Anti-goat IgG-Alexa Fluor® 568	1:500	Molecular Probes
Mouse anti-OCT-4 monoclonal	1:200	Abcam	Anti-mouse IgG_1-Alexa Fluor® 568	1:500	Molecular Probes
Monoclonal anti-α-fetoprotein	1:500	Sigma	Alexa Fluor®[a] Goat Anti-mouse IgG_1	1:500	Molecular Probes
Monoclonal mouse anti-human muscle actin	1:50	DakoCytomation	Alexa Fluor®[a] Goat anti-mouse IgG_1	1:500	Molecular Probes
Mouse anti-tubulin β-III isoform monoclonal antibody	1:250	Chemicon	Alexa Fluor®[a] Goat anti-mouse IgG_{2A}	1:500	Molecular Probes

SSEA- and TRA-antibodies are diluted in NGS at 4%. Nanog and Oct4 antibodies are diluted in 0.1% Triton and 1% BSA in PBS. Anti-α-fetoprotein, anti-human muscle actin and anti-tubulin β-III isoform antibodies are diluted in BSA 1%. Secondary antibodies are diluted in PBS

[a]Both Alexa Fluor 488 (Green) and 568 (Red) can be used

2.9. In Vivo Differentiation

Non Obese Diabetic (NOD) severe combined immunodeficient (SCID) mice (Charles River).

HES medium.

PBS (free of Mg^{2+} and Ca^{2+}).

Formaldehyde: 4% in distilled water.

Insulin syringe 30 G needle (Beckton & Dickinson).

Petri dishes.

3. Methods

3.1. Feeder Cells Preparation

1. Culture microbiologically tested human feeder cells (1) in plates with medium at a cell density of 6,500 cells/cm^2 at 37°C in 5% CO_2 atmosphere.
2. Change medium every 2–3 days.
3. Gamma irradiate cells at 55 Gy for 5 min in 6-well cell culture plates at a final concentration of 21,500 cells/cm^2.

3.2. Derivation

3.2.1. Derivation from Whole Embryos

1. Thaw donated frozen embryos using Embryo Thaw Kit (see Note 1).
2. Transfer pronuclear stage and day-2 embryos to IVF and CCM 1:1. Transfer thawed day-3 embryos to CCM medium and culture for additional 2–3 days in standard culture conditions (37°C in 5% CO_2 atmosphere).
3. Remove zona pellucida from blastocysts by treatment with a 30 μL drop of acid Tyrode's solution for 30 s to 1 min at 37°C in the micromanipulation plate.
4. Wash embryos sequentially in CCM and HES medium for few seconds.
5. Culture zona-free blastocysts on irradiated human foreskin fibroblasts in multiwell cell culture plates in HES medium containing 20 ng/mL of hbFGF.
6. Incubate the plate in standard conditions with no manipulation for 3 days.
7. Change HES medium every 48 h and maintain the culture for 2–3 weeks, until outgrowth with hESC appears.
8. Dissociate mechanically the outgrowth avoiding the areas corresponding to trophectoderm.
9. Replate the isolated fragments in a new well containing new irradiated feeder and fresh HES medium.
10. Change medium every 48 h and check the growth of colonies with hESC morphology under microscope.
11. This process was followed for VAL-3, -4, -5, -6M, -8, and -9 derivation (1, 2) (Fig. 1).

3.2.2. Derivation from Isolated Inner Cell Mass

1. Put the holding micropipettes in the micromanipulator of the inverted Microscope.
2. Place the blastocyst in a drop of GPGD medium with HSA in a micromanipulation plate.
3. Hold the blastocyst with holding pipettes from both sides, trying to localize the ICM at 9 o'clock position.
4. Separate ICM and trophectoderm by 20–30 infrared laser pulses (200 mW × 0.5 ms) cutting perpendicularly to the pipettes, from up to down as near as possible to ICM but avoiding its damage.
5. Separate the the zona pellucida by carefully pipetting.
6. Seed the ICM on irradiated human foreskin fibroblasts and follow the above protocol.
7. This protocol was followed for VAL-7 derivation (2, 6) (Fig. 1).

3.2.3. Derivation from Single Biopsied Blastomeres

1. Thaw donated frozen day-3 stage human embryos and incubate in CCM medium for at least 3 h under standard culture conditions.
2. Place the embryo in the micromanipulation plate in a drop of GPGD medium with 5% HSA.
3. Hold the embryo with a holding pipette and make an approximately 50 μm diameter hole in the zona pellucida with a specific 50 μm biopsy pipette.
4. Transfer the biopsied embryo to CCM medium, culture it for additional 2–3 days under standard culture conditions and cryopreserve it at blastocyst stage.
5. Transfer the isolated blastomere onto irradiated human foreskin fibroblasts in drops of CCM medium supplemented with 10 μg/mL human laminin, cover it with mineral oil, and culture it under standard culture conditions.
6. From day 3, refresh daily the medium drop containing the attached blastomere by replacing 1/3 of the volume with CCM medium supplemented with human laminin (10 μg/mL) and 25 ng/mL of hbFGF.
7. From day 5, replace CCM medium by HES medium containing 25 ng/mL hbFGF and 10% FCS.
8. Replace drops on a daily basis.
9. When an initial hESC colony is detected, make a dissection in the same drop.
10. Repeat the procedure in approximately 5 days.
11. Transfer small hESC clumps into 4-well dish with freshly seeded irradiated human feeders and incubate under standard conditions for 24 h.

12. Withdraw the FCS from the medium and replace it with the standard serum-free HES medium.
13. This process was followed for the derivation of VAL-10B and VAL-11B (3, 6) (Fig. 1).

3.2.4. Culture and Maintenance of Undifferentiated hESCs

1. Culture hESC lines on irradiated human foreskin fibroblasts in 6-well cell culture plates with 3 mL of HES medium containing hbFGF at 10 ng/mL at standard culture conditions.
2. Change HES medium every 48 h.
3. Dissect colonies mechanically in clumps every 4–5 days.
4. Transfer dissected colonies to dishes containing new inactivated human foreskin feeder cells.

3.3. Freezing and Thawing of hESC Lines

3.3.1. Freezing

1. Collect 30 colonies per vial at 163 × *g* for 3 min.
2. Add to the pellet 300 μL of HES medium plus increasing concentrations of DMSO.

   75 μL of DMSO 1.0 M incubated at RT for 10 min.

   75 μL of DMSO 1.2 M incubated at RT for 10 min.

   150 μL of DMSO 1.5 M incubated at RT for 10 min.

   600 μL of DMSO 2.0 M incubated at RT for 15 min to make a final volume of 1.2 mL.
3. Slow cooling down by immersion in Isopropanol at −80°C overnight.
4. Plunge and store the vials in LN_2 (7) (Fig. 1).

3.3.2. Thawing

1. Remove the cryovials from LN_2 and plunge them immediately into a water bath at 37 °C.
2. Transfer the contents of each thawed cryovial to a new tube and add equilibrated HES medium at 37 °C as follows.

   1 mL of HES during 2 min.

   2 mL of HES during 2 min.

   4 mL of HES during 2 min (6 min total).
3. Centrifuge the tubes at 163 × *g* for 3 min at RT.
4. Discard the supernatant and resuspend the pellet in 1 mL of HES. This final suspension is used for hESC culture with the routine protocol (7).

3.4. Characterization of hESC Lines

3.4.1. Genomic Purification for Fingerprinting and HLA Analysis

1. Collect 200 colonies in a 1.5 mL microcentrifuge tube.
2. Centrifuge for 5 min at 300 × *g* at 4°C. Remove the supernatant completely and discard, taking care not to disturb the pellet.
3. Wash with 500 mL of PBS. Centrifuge at 300 × *g* for 5 min at 4°C and remove supernatant.

4. Add 300 μL of Buffer FG1 to the cell pellet and mix by pipetting up and down until the cells are resuspended.
5. Add 300 μL Buffer FG2 and 3 μL of Qiagen Protease, and mix by inversion for three times. Place the tube in a heating block and incubate at 65°C for 10 min.
6. Add 600 μL of Isopropanol and mix thoroughly by inversion until the DNA precipitate becomes visible as threads or a clump.
7. Centrifuge for 10 min at 12,000 × *g* at 4°C. Discard the supernatant and briefly invert the tube onto a clean piece of absorbent paper for 2–3 min, taking care that the pellet remains in the tube.
8. Add 600 μL of 70% ethanol and vortex for 5 s.
9. Centrifuge for 5 min at 10,000 × *g* at 4°C. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for 8 min, taking care that the pellet remains in the tube (see Note 2).
10. Add 200 μL Buffer FG3, vortex for 5 s at low speed, and dissolve the DNA by incubating for 40 min at 65°C in a heating block with 300 rpm of agitation.
11. Measure on the NanoDrop and store the sample at –20°C.

3.4.2. Samples Preparation for Karyotype Analysis

1. Dissect a minimum of 150 colonies mechanically on HES medium from the feeder layer.
2. Incubate hESC in HES medium, supplemented with 0.4 μg/mL of Colcemid at 37°C for 30 min.
3. Centrifuge at 130 × *g* for 15 min.
4. Collect hESC in Accumax and incubate at 37°C for 15–20 min.
5. Centrifuge at 160 × *g* for 5 min.
6. Resuspend cell pellet in 1 mL on prewarmed KCl for 15 min at 37°C.
7. Prefix cells with 1 mL of Carnoy fixative solution at –20°C, and immediately spin at 160 × *g* for 5 min.
8. Discard the supernatant and resuspend in Carnoy three times centrifuging at 160 × *g* for 5 min each time.
9. Analyze at least 20 metaphase spreads using the GTG-banding method.

3.4.3. Telomerase Activity Detection

(a) Preparation of cells

1. Collect cells from 30 colonies at 370 × *g* for 8 min at 4°C.
2. Wash them with 0.5 mL PBS and collect them at 370 × *g* for 5 min at 4 °C.

3. Freeze cell pellet at –80°C or follow the experiment in ice.
4. Resuspend cell pellet immediately in 20 μL lysis CHAPS buffer.
5. Incubate suspension in ice for 30 min.
6. Centrifuge samples in a microcentrifuge at 13,000 × *g* for 20 min at 4°C.
7. Transfer supernatant to a new tube (see Note 3).

(b) PCR.

1. Prepare a master mix with all the components except the cell extract (see Note 4).

10× TRAP reaction buffer	5 μL
50× dNTP mix	1 μL
TS primer	1 μL
TRAP primer mix	1 μL
Taq polymerase (5 U/μl)	0.5 μL
DDW	csp 50 μL
Cell extract	2 μL

2. Include in each lot of reactions.

   Positive control: 2 μL of a hESC line previously characterized.

   Negative control of the technique: 2 μL of a hESC line previously heat inactivated at 85°C for 10 min.

   Negative control: 2 μL of foreskin fibroblasts (somatic cells).

3. PCR reaction.

30°C	30 min (Telomerase extension)	
94°C 59°C 72°C	30″ 30″ 1′	33 cycles
4°C	∞	

(c) PAGE and analysis.

1. Add loading buffer in each reaction tube and load 15 μL in each polyacrilamide gel well.
2. Run the gel in 0.5× fresh TBE for 1.5–3 h at 150 V until the upper band of the loading buffer exits the gel.
3. Thaw and dilute SYBR green to 1:10,000 in 1× TBE (pH 8).
4. Transfer the gel to a plastic tray and incubate with the staining solution for 20 min at RT with gentle agitation and covered from light.

Table 3
Molecular profile and immunophenotype of undifferentiated VAL hESC lines derived

	VAL-3	VAL-4	VAL-5	VAL-6M	VAL-7	VAL-8	VAL-9	VAL-10B	VAL-11B
Telomerase detection									
Activity	+	+	+	+	+	+	+	+	+
Molecular profiling									
POU5F1	+	+	+	+	+	+	+	+	+
NANOG	+	+	+	+	+	+	+	+	+
CRIPTO	+	+	+	+	+	+	+	+	+
DNMT3B	+	+	+	+	+	+	+	+	+
GABR3	+	+	+	+	+	+	+	+	+
GDF3	+	+	+	+	+	+	+	+	+
NFH	–	–	–	–	–	–	–	–	–
REN	–	–	–	–	–	–	–	–	–
AMI	–	–	–	–	–	–	–	–	–
RPL19	+	+	+	+	+	+	+	+	+
Immunophenotyping									
SSEA-4	+	+	+	+	+	+	+	+	+
SSEA-1	–	–	–	–	–	–	–	–	–
TRA-1-60	+	+	+	+	+	+	+	+	+
TRA-1-81	+	+	+	+	+	+	+	+	+
TRA-1-254	+	+	+	+	+	+	+	+	+
Oct4	+	+	+	+	+	+	+	+	+
Nanog	+	+	+	+	+	+	+	+	+

5. Visualize the gel in a CCD lector.
6. Check results in Table 3.

3.4.4. Molecular Profile

(a) RNA extraction.

1. Pellet 30 colonies (1×10^5 cells) by centrifugation at $500 \times g$ for 5 min. Remove the supernatant completely and resuspend the cell pellet in 600 μL of ZR RNA buffer. Vortex briefly.
2. Transfer lysate into the Zymo-Spin™ IC Column in a collection tube and centrifuge at $\geq 12{,}000 \times g$ for 1 min. Discard the flow-through.
3. Add 400 μL of RNA prewash buffer to the column and centrifuge at $\geq 12{,}000 \times g$ for 1 min. Discard the flow-through.
4. Add 700 μL RNA wash buffer and centrifuge at $\geq 12{,}000 \times g$ for 1 min. Discard the flow-through. Repeat step 4 with 400 μL RNA wash buffer.
5. Centrifuge the Zymo-Spin™ IC Column at $\geq 12{,}000 \times g$ for 2 min in the emptied collection tube to ensure complete removal of the wash buffer.

6. Place the Zymo-Spin™ IC Column into an RNase-free tube. Add ≥6 μL of DDW directly to the column matrix and let stand at RT for 1 min. Centrifuge at top speed for 30 s.
7. Add RNase inhibitor at 1 U/10 μL of RNA before storage at −80°C.

(b) cDNA synthesis.

1. Take enough μL to make 1 μg of RNA and dilute in DDW to make 11 μL in 0.2 mL sterile tubes.
2. Thaw reactive tubes in ice. Perform all reactions in ice.
3. Slightly centrifuge reactive tubes and keep them in ice.
4. Add 1 μL of primer oligo$(dT)_{18}$.
5. Heat RNA at 72°C for 5 min in a thermal cycler. Transfer tubes to ice to add the rest of components.
6. Add Kit components as follows (see Note 4):

5× Reaction buffer	4.0 μL
dNTP mix (10 mM each)	1.0 μL
RNase inhibitor	0.5 μL
Reverse transcriptase MMLV	1.0 μL
DDW	1.5 μL

7. Incubate the reaction for 1 h at 42°C in a thermal cycler.
8. Inactivate MMLV at 72°C for 10 min in a thermal cycler.
9. Dilute reaction in a final volume of 100 μL adding 80 μL of DDW.

   cDNA is now ready for use or storage at −20°C or −70°C.
10. Quantify in a NanoDrop with 1–1.5 μL.
11. Use 5–10 μL of diluted cDNA for each PCR reaction.

(c) PCR.

1. Prepare for each reaction (see Note 4):

	Vol/reaction
10× NH4 reaction buffer	5 μL
$MgCl_2$ 50 mM	2 μL
dNTPs (10 mM each)	1 μL
Primer 5′ Forward (10 μM)	1.5 μL
Primer 3′ Reverse (10 μM)	1.5 μL
Taq pol. 5 (U/μl)	0.5 μL
cDNA	100 ng to 1 μg
H_2O	Final volume 50 μL

2. Program (see Note 5)

94°C	5′	
94°C 50–60°C 72°C	30″ 30″ 1′	30–35 cycles
72°C	10′	
4°C	∞	

3. Run 15 μL of each PCR reaction in an agarose gel 1–2% (1.5%).

(d) Agarose electrophoresis

1. Prepare agarose at 1–2% in TAE 1×
2. Melt agarose in the microwave and add ethidium bromide before solidification
3. Put the gel in the tank and cover it with TAE 1× buffer
4. Load the gel with 15–20 μL of PCR product and loading buffer
5. Run it at 80–120 V for approximately 45 min to 1 h
6. Watch the gel in a CCD lector
7. Check results in Table 3

3.4.5. Immunophenotype

(a) For SSEA-1, SEA-4, TRA-1-60, TRA-1-81, TRA-2-54 (Alkaline fosfatase), anti-muscle actin, anti-tubulin β-III-isoform, and anti-α-fetoprotein.

1. Discard growth medium from culture wells.
2. Wash with PBS to discard the rest of the culture medium.
3. Fix with PFA for 20 min at RT.
4. Wash with rinse buffer for 5 min for two or three times.
5. Make cells permeable with Triton for 10–15 min at RT.
6. Block with NGS (4%) for 30 min at RT.
7. Incubate colonies for 1 h at RT with the first antibody at the corresponding dilution (Table 2).
8. Wash with rinse buffer 5 min for three times.
9. Incubate colonies for 1 h with the secondary antibody at the corresponding dilution (Table 2) in darkness.
10. Wash with rinse buffer 5 min for three times and cover cells with PBS until mounting.
11. Mount cells with DAPI solution and look in the fluorescence microscope.
12. Check results in Table 3 and Fig. 2.

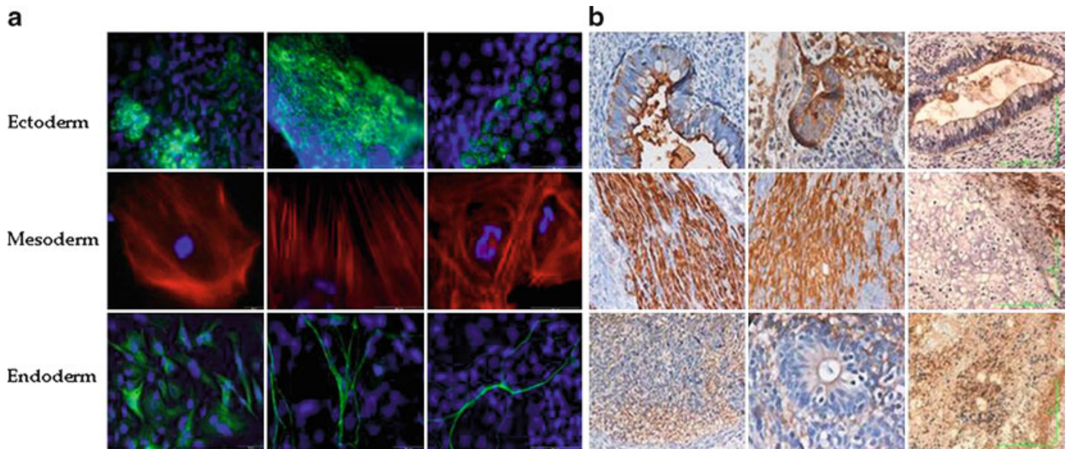

Fig. 2. *In vitro* and *in vivo* differentiation assessment by immunophenotyping. (**a**) In vitro cultured EBs and (**b**) in vivo induced teratomas sections were immunostained for anti-tubulin β-III (*Ectoderm*), anti-human muscle actin (*Mesoderm*) and anti-α-fetoprotein (*Endoderm*).

(b) For Nanog and Oct-4.

1. Discard growth medium from culture wells.
2. Wash with PBS to discard the rest of the culture medium.
3. Fix with PFA for 20 min at RT.
4. Wash with BSA solution for 5 min for two or three times.
5. Make cells permeable and block for 45 min at RT with:
   Triton, NDS, and BSA for Nanog.
   Triton, NGS (10%), and BSA for Oct-4.
6. Wash with BSA solution for 5 min for two or three times.
7. Incubate overnight at 4°C.
8. Wash with BSA solution for 5 min for two or three times.
9. Incubate colonies for 1 h with the secondary antibody at the corresponding dilution (Table 2) in darkness.
10. Wash with BSA solution for 5 min for three times and cover cells with PBS until mounting.
11. Mount cells with DAPI solution and look in the fluorescence microscope.
12. Check results in Table 3.

(c) For anti-muscle actin, anti-tubulin β-III-isoform, and anti-α-fetoprotein from paraffin-embedded sections.

1. Leave paraffin preparations overnight at 37°C to improve tissue adherence to the slide.
2. Incubate at 60°C for 1 h to allow paraffin to melt.

3. To allow dewaxing and hydration of the slides:

   Embed three times each with xylen.

   Embed three times each with ethanol.

   Hydrate by: 5 min in ethanol 96°C, 5 min in ethanol 70°C, 5 min in ethanol 50°C.
4. Wash with water.
5. Surround the tissues with an hydrophobic pen avoiding the slides to get dried (see Note 6).
6. Demask the slides with citrate buffer at 95°C for 20 min.
7. Add Peroxidase Block (Contained in the LSAB Kit) for 5 min.
8. Wash with PBS.
9. Block with BSA 4% for 30 min at 37°C in a wet chamber.
10. Add the first Antibody in the corresponding dilution (Table 2) for 1 h at RT (see Note 6).
11. Wash three times with PBS for 5 min.
12. Add Biotinylated link (contained in the LSAB Kit) and incubate for 20 min at RT (see Note 6).
13. Wash three times with PBS for 5 min.
14. Add Streptavidin-HRP (contained in the LSAB Kit) for 20 min (see Note 6).
15. Wash three times with PBS for 5 min.
16. Add one drop of cromogen per substrate buffer mL (contained in the LSAB Kit) and incubate until brown color appears.
17. Wash with distilled water to stop the reaction.
18. Stain the slides immersing and getting out in hematoxylin solution several times.
19. Dehydrate by washing in:

    Ethanol 50°C for 5 min.

    Ethanol 70°C for 5 min.

    Ethanol 96°C for 5 min.

    Absolute ethanol for 5 min.

    Xylol for 5 min.
20. Add Entellan mounting solution and cover slides.
21. Look in a phase contrast microscope (Fig. 2).

3.4.6. In Vitro Differentiation

1. Grow colonies routinely.
2. Separate colonies from feeders mechanically and cut in two or three pieces each.

3. Collect fragment colonies in suspension and transfer to a sterile tube, where 12 mL of prewarmed (37°C) differentiation medium is added.
4. Distribute cell suspension on low-attachment 6-well culture plates with a final volume of 3 mL each well.
5. After 4–7 days in standard culture conditions, embryoid bodies (EBs) must be observed (see Note 7). Transfer EBs mechanically to a conical tube and allow them to get deposited at the bottom.
6. Discard culture medium carefully not to touch the EBs.
7. Add differentiation medium to the tube and resuspend EBs.
8. Grow EBs in adherent 4-well culture plates.
9. Incubate at standard conditions for 10–14 days to allow differentiation, changing medium every 2–3 days.
10. Perform immunocytochemistry for differentiation markers as specified above.

3.4.7. In Vivo Differentiation

1. Collect mechanically 30 colonies per testicle to be injected, which account approximately 3×10^4 undifferentiated hESCs into four SCID mice per each cell line to be tested.
2. Transfer cells and medium in a 1.5 mL tube.
3. Centrifuge at $100 \times g$ for 3 min at RT.
4. Discard the supernatant.
5. Resuspend in PBS (30 μL/testicle).
6. Perform 30 μL drops in a Petri dish.
7. Load in a syringe in three phases as follows: 0.1 mL of air, 30 μL of cell suspension, 0.1 mL of air.
8. Leave the syringe in horizontal position on ice and correctly marked.
9. Transfer syringes to SPF area where SCID mice are correctly anesthetized (see Note 8).
10. Mark properly each animal and inject 30 μL in testicular lumen with a 30 G needle.
11. Assess adequate animal reanimation (see Note 8) and follow its recuperation.
12. Detect tumors by palpation after 8 weeks from the injection.
13. Sacrifice SCID mice (see Note 8) after 12 weeks from the injection.
14. Extract tumors, fix them with formaldehyde and embed them in paraffin for morphological analysis (Fig. 2).

3.5. Availability

hESC lines VAL-3, -4, -5, -6M, -7, -8, -9, -10B, and -11B are deposited at the National Stem Cell Bank and available upon request for the scientific community at the Spanish Stem Cell Bank at http://www.isciii/htdocs/terapia/terapia_bancocelular.jsp.

4. Notes

1. Derivation processes require special training and skills with human embryos that must be taken into account before starting with derivation protocols.
2. In genomic DNA extraction, the DNA pellet must be air-dried until all the liquid has evaporated. Avoid overdrying the DNA pellet, since overdried DNA is very difficult to dissolve.
3. In the telomerase assay, cell extracts can be aliquoted and stored at −80°C. In these conditions, telomerase activity is kept for over 1 year.
4. In cDNA synthesis and PCR reactions, for more than one reaction calculate a master mix for $n+1$ (being $n=$number of samples).
5. In PCR reactions, temperature and number of cycles may vary according to primers and amplicons.
6. In paraffin-embedded preparations, it is of high importance not to allow the slides to get dried.
7. In vitro differentiation process is improved by changing differentiation medium every 3–4 days following the same process of transferring carefully embryoid bodies, add new medium and deal in low-attachment culture plates.
8. Anesthetic, reanimation, and sacrifice procedures are distinctive from each laboratory, and must always follow good ethical animal procedures

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Chapter 3

Procedures for Derivation and Characterisation of Human Embryonic Stem Cells from Odense, Denmark

Linda Harkness and Moustapha Kassem

Abstract

In 1998, a development occurred in stem cell biology with the first report of the derivation of a human embryonic stem cell (hESC) line. Since then a number of techniques have been used to derive and characterise hESCs. Here, we describe the derivation methods used by our laboratory for isolation of the ICM by immunosurgery and outgrowth of the whole blastocyst. We have added protocols for routine culture, passaging and cryopreservation of our hESC lines as well as the methods we have used for characterisation (flow cytometry, karyotyping, immunocytochemistry, in vitro and in vivo differentiation). Additionally, we have included gene sequences for PCR and an antibody list for immunocytochemistry.

Key words: Human embryonic stem cells, Derivation, Characterisation

1. Introduction

Human embryonic stem cells (hESCs) offer a unique potential to generate cells from the three basic cell lineages (ectoderm, endoderm, and mesoderm) and are regarded as a promising cell source for clinical applications, toxicity testing, drug discovery and, additionally, can offer insights into the early stages of developmental differentiation. Since the derivation of the first human embryonic stem cell line in 1998 (1) differing methodologies and procedures have been utilised by laboratories worldwide with differing results in success (1–7). Derivation of hESC lines involves the outgrowth of the inner cell mass either by isolation using enzymatic techniques (8) or excision by laser (9) or by outgrowth of the whole blastocyst (3, 4). The differences in the success rates from different labs revisit the quality of the initial embryo and it has been shown that the number of embryos which can successfully produce a good quality expanded blastocyst is small (4). All methodologies have their pros

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and cons and the method chosen by a laboratory is often due to the availability of equipment, and experience and techniques of the laboratory. However, those attempting to derive hESC lines, of necessity, need a working knowledge of routine culture of hESC and an understanding of the differences between the way individual cell lines grow. Traceability of the cell lines, ethical approval, compliance with governmental regulations governing derivation of hESC lines, patient consent, assessment of developmental status of the embryos (10), and good record keeping during derivation are all required to be able to fully utilise derivations and also to enable banking of cells for worldwide usage. It continues to be necessary for work to be performed on a variety of stem cell lines to facilitate enough coverage for population diversity. Although it has been estimated that 150 hESC lines will cover the majority of donors (11) two points should be taken into account (1) differences in regional variability (i.e. Asian, African, European populations (12)) and (2) cell lines can have a tendency to have a preferential differentiation capacity in vitro (13). Utilising locally sourced embryos from two IVF clinics, we describe the procedures and materials utilised for the derivation and characterisation of seven cell lines in Odense, Denmark.

2. Materials

	Component	Supplier	Cat. no.
MEF Media	DMEM (high glucose)	Invitrogen	31966
	10% FBS	PAA	A15-101
	1% penicillin/ streptomycin	Invitrogen	15140122
106 Media	Medium 106	Cascade biologics	M-106-500
	Low serum growth supplement	Cascade biologics	S-003-10
	Human dermal fibroblasts, neonatal	Cascade biologics	C-004-5 C
KO Media	Knockout DMEM	Invitrogen	10829018
	15% Knockout serum replacement	Invitrogen	10828010
	1% Glutamax I	Invitrogen	35050038
	1% NEAA	Invitrogen	11140035
	0.5% HSA	CSL behring	vnr:10 96 97
	0.1 μM 2 mercaptoethanol	Sigma-Aldrich	M6250
	1% penicillin/ streptomycin (opt)	Invitrogen	15140122
	Human basic FGF (5–10 ng/ml)	Invitrogen	PHG0021

(continued)

	Component	Supplier	Cat. no.
EB Media	Knockout DMEM	Invitrogen	10829018
	15% Knockout serum replacement	Invitrogen	10828010
	1% Glutamax I	Invitrogen	35050038
	1% NEAA	Invitrogen	11140035
	0.5% HSA	CSL behring	vnr:10 96 97
Cell Culture	0.05% trypsin/EDTA	Invitrogen	25300054
	PBS^{2-}	Invitrogen	14190094
	Reduced growth factor Matrigel	Becton Dickinson	354234
ICM	Acid tyrodes	Origio	10600002
isolation	Anti-human antiserum	Sigma-Aldrich	I2011
	Guinea pig complement	Sigma-Aldrich	S1639
	Portable embryo transporter	Minitub	19180/0000
Inactivation	Mitomycin C	Sigma	M4287
Karyotyping	KaryoMAX® Colcemid	Invitrogen	15210040
	Difco Bacto trypsin	Becton Dickinson	215310
	KaryoMAX® Giemsa Stain	Invitrogen	10092013
	Gurr's Buffer pH 6.8	Invitrogen	10582013
Staining	PBS^{2+}	Qiagen	19131
	0.5% Triton-X100	Bie & Bernstein	LAB46205.0500
	Tris buffered saline (TBS)	VWR	95059-270
	4% PFA	Bie & Bernstein	LAB46002.0001
	Histostain-SP Kit (mouse)	Zymed/ Invitrogen	95-6543B
	Histostain-SP Kit (rabbit)	Zymed/ Invitrogen	95-6143B
	ACE (red) Kit	Zymed/ Invitrogen	00-2007
	Chemate Antibody diluent	Dako	S2022
Facs Buffer	0.5 g BSA Fraction V	Sigma-Aldrich	A4503
	500 ml PBS2-	Invitrogen	14190094
Facs Blocking buffer	2.5 ml serum		
	47.5 ml Facs buffer		
	Cytofix/Cytoperm Kit	Becton Dickinson	554714
Cryopreservation	Dimethyl Sulphoxide	Sigma-Aldrich	D2650
	Nalgene 5100 cryo 1°C freezing container	Thermo Fisher Scientific	5100-0001

3. Methods

3.1. Preparation of Feeders

Two different types of feeders were used to grow the blastocysts on (1) E13.5 isolated mouse embryonic fibroblasts (MEFs) (2) human, neonatal, dermal fibroblasts (HDFn).

3.1.1. Isolation of MEFs

The mouse strain we used were CD1 E13.5 pregnant females.

1. Use sterile instruments for all steps.
2. Kill the adult mice and dissect out the gestational sacs into a 90 mm Petri dish.
3. Remove deciduas and placenta transferring each embryo into a new dish.
4. Eviscerate and decapitate the foetuses. Transfer the body tissue into a collecting tube with PBS^{2-} + 2× penicillin/streptomycin.
5. All steps from this point are to be carried out in a tissue culture hood using aseptic techniques.
6. Wash the embryos twice with fresh PBS^{2-} + 2× pen/strep.
7. Transfer three embryos into a 60 mm dish and finely mince the embryos using sterile single edge razor or scalpel blades. Add 1 ml of trypsin/EDTA and incubate for 5 min at 37°C.
8. Inactivate the trypsin/EDTA by adding 5 ml of MEF media. Transfer all the contents to a sterile 15 ml centrifuge tube. Triturate, using a 1 ml pipette with filter tip, to increase the number of single cells. Centrifuge the tubes at 200 × *g* for 5 min.
9. Aspirate the media off, resuspend the pellet in 5 ml of MEF media, and leave to stand for a few minutes.
10. Once the larger pieces have settled to the bottom of the tube transfer the cell suspension from one tube to two T80 flasks and add MEF media to each flask making the total volume to 15 ml, incubate at 37°C, 5% CO_2.
11. When finished the initial plating, combine the larger pieces from five to ten tubes and triturate again, allowing the larger pieces to settle to the bottom of the tube. Transfer the supernatant layer to a T80 flask, add MEF media to a final volume of 15 ml and incubate at 37°C, 5% CO_2.
12. The following day examine flasks for cell density and any sign of possible contamination.
13. Eliminate any flasks that show any signs of contamination.
14. If the flasks are confluent: passage at 1:6 (1 flask to 6 flasks) or cryopreserve the cells (10% DMSO in MEF media, see Subheading 3.4 below). If they are not confluent refeed the flasks (if there is a lot of cell death), reincubate the flasks

overnight and check them again the following day. Continue checking, freezing, or passaging until all flasks are either passaged or cryopreserved.

3.1.2. Use of HDFn Cells

HDFn cell line was bought from Cascade Biologics, grown in their recommended media (106 + LSGS) and cryopreserved (106 media + 10% KOSR). Cells were revived and centrifuged (200 × *g*, 5 min) in Media 106 + LSGS before plating in 1× 80 flask. Procedures used for HDFn cells were similar to those used for MEF expansion and cryopreservation.

3.1.3. Inactivation of Feeder Cells

MEFs/HDFn cells must be actively dividing prior to inactivation for feeder layers.

Inactivation by Irradiation

1. Between MEF passage 3 and 7 trypsinise the cells to a single cell suspension (see Note 1). For commercially bought cell lines, trypsinised to a single cell suspension when enough flasks have been created for inactivation. Add media and centrifuge at 200 × *g* for 5 min.
2. Combine all the feeders into a 50 ml centrifuge tube, count the cells using a haemocytometer and calculate the total number of cells. Transport the tubes to the irradiator.
3. Calculate the amount of time needed to irradiate the cells (the amount of time is dependent on the age/status of the irradiator). 40 Gy is needed for irradiation (MEF feeders) and 50 Gy is necessary (HDF feeders) (see Note 2).
4. Following irradiation centrifuge the cells at 350 × *g* for 5 min, aspirate the supernatant and resuspend the cells in an appropriate volume of MEF or 106 media for use as feeder layers, or for cryopreservation.
5. Cells can be cryopreserved and stored in liquid nitrogen post-irradiation at specific cell counts for use as feeder layers. As we work at a concentration of 20–25,000/cm^2; we cryopreserved at a concentrations of 1.5×10^6 (revives to 1 × 6 well plate) and 5×10^6 (4 × 6 well plates); this allowed for some cell death following revival from cryopreservation.

Inactivation by Mitomycin C

1. Remove 50% of the medium from confluent cells (MEF or HDFn) between passages 3 and 7 and add mitomycin C at a final concentration of 10 μg/ml (stock is prepared in H_2O).
2. Return the media with mitomycin C to the cells and incubate at 37°C, 5% CO_2 for 2–3 h.
3. Aspirate off the media and wash the cells with PBS 3× disposing of both the media and PBS carefully as mitomycin is toxic (see Note 3).

4. Trypsinise the cells to a single cell suspension and count the number of cells on a haemocytometer.
5. Centrifuge the cells at $200 \times g$ for 5 min and resuspend in an appropriate volume of Media 106 + LSGS or MEF media to give a plating density of 20–25,000 cells/cm^2.

3.1.4. Plating of Inactivated Feeders

1. Following inactivation cells are plated on wells/flasks coated with 0.2% gelatin in H_2O for 30 min at room temperature. Gelatin was prepared by allowing it to equilibrate at room temperature until the solution cleared, then diluted in sterile water and filter sterilised before use. Cells were left overnight to plate in MEF or HDFn media before being used. If cells were not used within 3 days of plating, they were discarded.

3.1.5. Creation of Conditioned Media

1. Inactivated feeders are plated on 0.2% gelatin-coated flasks at a concentration of 125,000 cells/ml of collected media. Feeders are plated overnight in their normal media (DMEM + 10% FBS or media 106 + LSGS) (see Note 4).
2. The following day the media is removed and media for conditioning (i.e. Knockout media) added. For each of the next 7 days media is collected and replaced, on the 8th day media is collected and the cells discarded. Media can be stored at –20°C until needed.

3.2. Isolation of the ICM

1. Two differing methods of ICM isolation were carried out in our laboratory (a) through removal of the zona pellucida (ZP) and isolation of the inner cell mass (ICM); or (b) outgrowth of the whole embryo. Feeders should be plated the day before and media changed to conditioned media + 10 ng/ml hbFGF 2–3 h prior to the addition of the blastocysts.
2. Fresh blastocysts from the IVF clinic were collected in the embryo transporter (Minitube, Portable ET Incubator, cat no: 19180/0000) in 1.5–2.0 ml of embryo culture medium (see Note 5).
3. Under sterile conditions pipette the culture medium into a 30 mm culture dish, kept on a heated stage at 37°C, leave a little media in the bottom of the tube.
4. Place the dish under the stereo microscope and search for the blastocyst(s). Assess and record the stage of development (10) and, if possible, photographically record each embryo (see Note 6).

3.2.1. Isolation of the ICM by Immunosurgery

1. Transfer the embryo into acid tyrodes solution and watch under the microscope for the ZP to begin to dissolve.
2. Wash 8× in KO media.

3. Briefly transfer to a 20 μl drop of diluted anti-human antiserum diluted 1:10 in KO DMEM to wash the embryo.
4. Transfer to a fresh 20 μl drop of antiserum and leave at 37°C for 30 min.
5. Wash the embryo through 8× 20 μl drops of KO media.
6. Briefly transfer to a 20 μl drop of guinea pig complement diluted 1:10 in KO DMEM, then transfer to a second 20 μl drop of complement (see Note 7).
7. Leave at 37°C for up to 20 min, observing at regular intervals for signs of blebbing of the surface of the blastocyst.
8. Wash through 8× 20 μl washes of KO media. In the final wash, pipette through a very fine tipped pipette. The lysed trophoblast cells should come off leaving the ICM intact.
9. Transfer the ICM to the plates with feeders and conditioned media + 10 ng/ml hbFGF, and incubate at 37°C, 5% CO_2 and change one-half media and monitor every 2 days. Continue as from 3.2.2.7.

3.2.2. Outgrowth of the Whole Embryo

1. Following assessment of the embryo, transfer each embryo to 1 well of a 4 well nunc dish (1 embryo/well) with conditioned media supplemented with 10 ng/ml hbFGF.
2. Incubate at 37°C, 5% CO_2.
3. If the blastocyst has not hatched within a period of 2 days, transfer it to 50 μL of acid tyrodes solution briefly and gently pipette the embryo up and down to assist hatching while observing using a dissecting microscope.
4. Wash through 3×500 μl drops of the appropriate media and return the embryo to its original well. Reincubate at 37°C, 5% CO_2.
5. Every 2 days remove one-half of the medium and add one-half of fresh conditioned media + 10 ng/ml hbFGF. Photograph the individual embryos to record growth rate patterns and initial colony formation.
6. Once the embryos have hatched or had the ZP removed the blastocysts may take 24–48 h to attach. Colonies can appear within a few days to several weeks.
7. In the initial passages as the outgrowths appear, use a stem cell cutter to mechanically remove any differentiation and cut any colonies. Using the stem cell cutting tool transfer (Vitrolife, Cat no. 14602) isolated colonies into a fresh well, with the appropriate feeders/media, allow the clumps to resettle and continue growing (see Note 8).
8. Re-incubate at 37°C, 5% CO_2 and monitor every 2–3 days.

3.3. Passaging of Cells

3.3.1. Mechanical Passaging

1. Feed the well to be passaged with fresh conditioned medium supplemented with 10 ng/ml hbFGF.
2. Using the dissecting microscope at low magnification identify any differentiated areas.
3. Using the stem cell cutting tool (Vitrolife, Cat no. 14602) or single use needle cut away any differentiated areas.
4. Discard these cells into an empty well.
5. Using the dissecting microscope at low magnification identify the colony areas to be transferred. In initial stages of derivation, the colonies should be able to fill (or almost fill) the field of view at 10× magnification prior to passaging.
6. Using the stem cell tool or single use needle score across the colonies in several directions to mark the colony into smaller areas.
7. Using a plate scraper gently scrape the dissected colonies off the culture dish.
8. Gently pipette the cells up and down through a P1000 tip five to ten times depending on the size of the clumps, cells should remain in small discrete clumps and not be single cells.
9. Transfer the required proportion of the cells (usually 1:1 in the first couple of passages or if enough cells 1:2 splits are performed at the early stages of derivation) to a new well with feeders.
10. Repeat dissection until the colony number and size is enough to transfer to a 6-well plate with feeders. Continue to mechanically passage the cells until there are enough to perform an initial freeze in liquid nitrogen (see Note 9).

3.3.2. Enzymatic Passaging

1. All steps to be carried out in the tissue culture hood under aseptic conditions.
2. Aspirate off the media and wash the cells with PBS (volume is dependent on size of area treated).
3. Add trypsin/EDTA
4. Incubate at RT until cells are starting to round up and the edges of the colony is lifting (approximately 1 min).
5. Carefully aspirate the trypsin off.
6. Add conditioned media (if the cells are cultured on Matrigel) or KO Media (if the cells are cultured with inactivated feeders) to the flask and scrap the cells off using a plate or flask scraper.
7. Using a wide bore serological pipette, triturate to break the colonies into small clumps.
8. Transfer the required amount of cells into the appropriate flask or well on feeders or Matrigel.

3.4. Cryopreservation and Cell Revival

1. Trypsinise cells as for routine passaging (either mechanical or enzymatic).
2. Transfer all cells to a 15 ml tube combining all wells to one tube.
3. Centrifuge at 200 × *g* for 5 min, aspirate the supernatant from the tube.
4. Resuspend the cells in 50% of the final freezing volume of the appropriate media.
5. Slowly add a mixture of 80% media with 20% DMSO (10% of the final volume) and mix thoroughly.
6. Using pre labelled cryo tubes (date, cell line information), transfer the cell suspension to cryo-vials 0.5 ml per vial.
7. Place the vials into a Nalgene freezing container and transfer the container to –80°C overnight.
8. The following day transfer the vials to liquid nitrogen for long-term storage.
9. To revive cells from LN_2. Thaw the ampoule of cells in a water-bath at 37°C until the ice crystals are just disappearing.
10. Transfer cells to a 15 ml conical bottom tube add 10 ml of media. Rinse the ampoule with 1 ml of the appropriate medium and add to the tube.
11. Centrifuge the cells at 200 × *g* for 5 min, aspirate the supernatant, and resuspend the cell pellet in the appropriate volume of media supplemented with an appropriate amount of hbFGF for the well/flask (see Note 10).
12. Transfer to a well or flask of inactive feeders.
13. Incubate the flask at 37°C, 5% CO_2.

3.5. Routine Culture

1. Once the hESC cultures have been established the conditioned media can be changed to KO media by slowly transitioning dilutions of conditioned media:KO media (80:20; 50:50; 20:80). Each stage needs a minimum of 3–4 days in each media before the ratio is changed (see Note 11).
2. Once the media has been changed to 100% KO media the amount of hbFGF can be reduced to 5 ng/ml—again reduction in the amount of FGF should be performed slowly, 1 or 2 ng/ml per passage (see Note 11).
3. Routine culture conditions are either on feeders (as described above) or on a matrix, such as Matrigel, in conditioned media. Any matrix used should be in accordance with manufacturer's instructions. Cells grown on feeders require a one-half change of media (KO media supplemented with hbFGF) and cells grown on a matrix need a full change of media (conditioned media supplemented with hbFGF) on a daily basis. Changing media less frequently can lead to the cells not growing optimally.

3.6. Characterisation

3.6.1. Immunocyto-chemistry (see Table 1 for Antibody List and Dilutions, see Note 12)

1. Cells were grown on either chambered slides (Nunc), or in 6-well plates until 50% confluent before being fixed for 5 min at RT in 4% PFA and washed, 3× 5 min in PBS^{2+} before storage at 4°C.
2. Wells were permeabilised on 0.5% Triton X-100 in TBS (tris-buffered saline) for 10 min at RT, rinsed 3× in TBS (5 min each wash), and blocked for 10 min in 5% normal serum in TBS (species dependent on species primary antibody was raised in).
3. Primary antibodies are diluted in ChemMate Antibody diluent (Dako), and then added to each well and left at RT for a minimum of 1 h.
4. Wells were washed 3× (5 min each) in TBS. The secondary antibody is from a Zymed kit and is biotinylated and used according to manufacturer's instructions.
5. Visualisation is through a Zymed ACE red (chromatogen) kit and used according to manufacturer's instructions.

Table 1
List of antibodies and dilutions used in immunochemistry staining

Immunocytochemistry	Company	Cat. no.	Dilution
Oct3/4	SantaCruz Biotechnology	SC-5279	1:100
Sox2	R&D Systems	MAB2018	1:100
Nanog	R&D Systems	AF719	1:100
CD44	Dako	M7082	1:100
Tra1-81	Chemicon/Millipore	MAB 4381	1:100
Tra1-60	Chemicon/Millipore	MAB 4360	1:100
SSEA4	R&D Systems	MAB1435	1:100
β-III-Tubulin	R&D Systems	MAB1195	1:200
NeuroD1	Chemicon/Millipore	AB15580	1:500
HNF4α	Santa Cruz Biotechnology	sc-6556	1:300
α-1-antitrypsin	Dako	A0012	1:80,000
CD31	Dako	M0823	1:100
CD34	NovoCastra/Leica	END-L-CE	1:20
CD166	NovoCastra/Leica	NCL-CD166	1:100
Tra1-85	Chemicon/Millipore	MAB4385	1:100

6. For tissue/cell pellets processed and embedded in wax, sections were cut at a 5 μM thickness, dewaxed and rehydrated though a graded series of alcohols on an automatic slide processor (Techmate500, Dako, Glostrup, Denmark). Primary antibodies were used at the dilutions stated in the table above.
7. Immunocytochemical staining was performed on sections using DAKO En Vision+ and PowerVision according to manufacturer's instructions.
8. All analysis was carried out on an IX50 Olympus microscope using Olympus DP Software v3.1 (Olympus, Essex, UK) or a Leica DM4500 (Leica, Wetzlar, Germany) using the Surveyor Turboscan Mosaic acquisition imaging analysis system v5.04.01 (Objective Imaging Ltd, Cambridge, UK).

3.6.2. Karyotyping

1. To ensure there will be enough cells to perform a karyotypic analysis passage a sub-confluent flask of the hESC line of interest at a ratio of 1:1 or 1:2.
2. Approximately 18 h (on MEFs) or 20 h (MG) after passage add KaryoMAX (10 μl per 1 ml of culture medium).
3. Between 1 and 2 h later harvest the cells to a single cell suspension using trypsin.
4. Transfer the resulting cell suspension to a centrifuge tube and spin at $350 \times g$ for 5 min.
5. Decant the supernatant and resuspend the cell pellet in 10 ml of freshly made 0.56% KCl (hypotonic solution) while mixing on the vortex mixer. Incubate at room temperature for 10 min (see Note 13).
6. Centrifuge the cells at $350 \times g$ for 5 min then decant the supernatant into a waste beaker.
7. Resuspend the cell pellet using the vortex mixer, add 10 ml of freshly made fixative (3:1 Methanol:Glacial Acetic Acid) dropwise to the tube while mixing continuously on a vortex mixer (see Note 13).
8. Spin the cells at $350 \times g$ for 5 min then decant the supernatant into a waste beaker.
9. Repeat steps 7 and 8 of Subheading 3.6.2 twice more.
10. Tap the cell pellet to resuspend the cells and add a small volume of fresh fixative, the resulting suspension should be just cloudy.
11. Polish a cleaned microsope slide (slides are cleaned in ethanol + 5% hydrochloric acid to ensure the slide surface is clean and free from grease). Breath on the slide and drop one drop of the cell suspension, using a fine tipped pastette onto the surface of the slide while holding it at an angle, thus allowing

the cell suspension to run down the slide. Watch to ensure the drop spreads evenly over the slide surface, leave on the bench to air dry.

12. Stain with haemotoxylin and examine the slide on the microscope to check for the mitotic index, spreading and fixation.
13. After making the required number of slides for G banding transfer, the remaining suspension to a 1.5 ml Eppendorf tube and store at −20°C until the banding has been done and scored on the cell line. Cells can be stored in this way indefinitely.

3.6.3. Giemsa Banding

1. Allow slides of chromosome preparations on slides to age at room temperature for 3–5 days.
2. Fill a coplin jar with 2× SSC and incubate at 60°C for 20–30 min to allow the solution to warm up.
3. Incubate the slides in the 2× SSC at 60°C for 2–4 h (timing is not critical).
4. To 50 ml of sterile distilled water in a coplin jar, add 50 μL of Bacto trypsin (made according to manufacturer's instructions).
5. Make up 5% Giemsa solution in Gurrs buffer pH 6.8.
6. Remove the slides from 2×SSC and wash the slides individually with running tap water.
7. Incubate the slides individually in 1% trypsin for 20–30 s. (NB: timing is critical, the older the slides the longer they need in trypsin; however, the time in trypsin should not exceed 90 s; see Note 14).
8. Wash the slides individually with running tap water then stain the slides in 5% giemsa for 8–10 min.
9. Wash the slides individually with running tap water, dry and add mounting media and cover with a cleaned coverslip.
10. Leave to dry and allow the coverslip to set completely. Examine using 100× oil immersion objective to count and analyse the chromosomes (see Note 15).

3.6.4. Flow Cytometry (see Table 2 for Antibody List and Dilutions)

1. Harvest cells using the trypsin/EDTA to a single cell suspension and perform a cell count.
2. Spin at 350 × *g* for 5 min, aspirate off the supernatant and resuspend cells in FACS buffer.
3. Centrifuge at 350 × *g* for 5 min resuspend cells in FACS blocking buffer (volume dependent on the number of antibodies being stained for—50 μl/antibody).
4. Dispense 50 μl of the cell suspension into Facs tubes, and incubate on ice for 10 min.
5. Add the primary antibody to the appropriate tube and store on ice for 45 min (in the dark if using preconjugated antibodies).

Table 2
List of antibodies with catalogue numbers and dilutions used in flow cytometry

Oct3/4	Santa Cruz Biotechnology	sc-5279	1 μg/10^6 cells
IgG2b (Oct isotype)	Southern Biotech	0103-09	10 μl/10^6 cells
Tra1-60	eBiosciences	12-8863-82	1 μg/10^6 cells
Tra1-81	eBiosciences	12-8883-82	1 μg/10^6 cells
SSEA1	Developmental Hybridoma Studies Bank	MC-480	10 μl/10^5 cells
IgM (SSEA1) secondary	Southern Biotech	1022-09	10 μl/10^6 cells
IgM Isotype	eBiosciences	12-4752-73	1 μg/10^6 cells
SSEA4	R&D Systems	FAB1435P	10 μl/10^5 cells
IgG3a (SSEA4 isotype)	Southern Biotech	0105-09	10 μl/10^6 cells

6. Add 3 ml of Facs buffer and spin at 350 × *g* for 5 min. Decant the supernatant; if using preconjugated antibodies resuspend the cells in 0.5 ml Facs buffer and store on ice. If using unconjugated antibodies resuspend in 100 μL of appropriately diluted secondary antibody in Facs buffer and incubate on ice for 45 min in the dark.
7. Add 3.0 ml of Facs buffer to each tube to wash out excess antibody. Spin at 350 × *g* for 5 min. Decant the supernatant and resuspend in 0.5 ml of Facs buffer.
8. If storing for any length of time before running on the FACS machine, add 0.1% PFA in PBS instead of Facs buffer, to preserve the samples. Store at 4°C in the dark.
9. Samples are now ready to run on the FACS machine.
10. If using intracellular markers cells must be permeabilised prior to addition of the primary antibody. Using the BD Cytoperm/Cytofix kit, add 250 μl Cytoperm/Cytofix to each tube instead of 50 μl Facs blocking buffer.
11. Store on ice for 20 min. Wash 3× in 2 ml 1×BD wash buffer centrifuging between washes for 5 min at 350 × *g* and decanting supernatant. Following this primary antibody can be added as above (3.6.4.5); however, all washes need to be carried out 3× in 1×BD wash buffer for each step.

3.6.5. In Vitro Differentiation

1. Trypsinise the cells as for routine passaging so small clumps of cells are achieved. Centrifuge at 200 × *g* for 5 min and discard the supernatant.
2. Add EB medium and transfer to a low attachment plate (Corning), label with cell line information date and your initials and incubate at 37°C, 5% CO_2.

3. 48 h later transfer the EBs to a 15 ml conical bottom tube and allow the clumps to settle by gravity sedimentation.
4. Aspirate the supernatant and add 10 ml of fresh EB media to the tube.
5. Transfer the EBs back into the low cluster plate, and incubate at 37°C, 5% CO_2.
6. Change media in this way every 2–3 days.
7. For routine examination of differentiation capabilities, we took samples at day 5, 10, 15, and 20 for immunocytochemistry and qRT-PCR. Cells were pelleted in a 1.5 ml Eppendorf and stored dry at −80°C until RNA extraction (see Table 3 for gene list and primer sequences), or fixed for 5–10 min in 4% PFA and stored in PBS^{2+} until histological processing and staining (see Table 2 for antibodies and dilutions).

3.6.6. In Vivo Teratoma Formation

Using the method previously reported (14) and described, in detail, below we achieve a 94% teratoma formation rate (see Note 16).

1. Trypsinise the cells as for routine passaging so small clumps of cells are achieved. We use 3 wells of a confluent 6-well plate per injection site. All following details are for one injection site.
2. Centrifuge the cells at 200 × *g* for 5 min and aspirate off the supernatant.
3. Using a 1:2 dilution of Matrigel in KODMEM, make the total volume of cells to 150 μl. Aliquot into a 1.5 ml sterile Eppendorf, place on ice and take to the animal facility.
4. Sedate the immunodeficient mouse with ketamine and xylazine for easier handling. The normal dose for operations is 100 mg/kg ketamine and 10 mg/kg xylazine, approx 2/3 of this dose will sedate the mouse enough to inject the cells.
5. Do not draw up the cell suspension into the syringe until just before you are ready to perform the injection. The solution is small and slightly viscous and will gel when it reaches 37°C.
6. Put a cooled 21 G needle on a 1 ml syringe (25 mm length, diameter 0.8 mm), use the tip of the needle to gently resuspend the cells, and then draw the fluid into the syringe though the needle. When this is done carefully, with the tip of the needle submerged during the whole step, the cell suspension is not "broken up" by air bubbles—there is only one volume of air between the fluid and the piston.
7. Lift up the skin of the sedated mouse, in the dorsolateral area of one side, insert the needle about 1–2 cm and inject in the subcutaneous space. A maximum of four sites in the dorsolateral area can be used for each mouse, however, only one cell line should be used per mouse.

Table 3
List of markers used in PCR to detect self renewal and differentiation

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size	T (°C)
Oct3/4	GAAGGTATTCAGCCAAAC	CTTAATCCCAAAAACCCTG	650	60
Sox2	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT	448	60
Nodal	AGACATCATCCGCAGCCTACA	GACCTGGGACAAAGTGACAGTGAA	268	55
ALP	ACGTGGCTAAGAATGTCATC	CTGGTAGGCGATGTCCTTA	475	60
β-III-Tubulin	CAACAGCACGGCCATCCAGG	CTTGGGGCCTGGGCCTCCGA	244	55
NeuroD1	CTTGGCCAAGAACTACATCTGG	GGAGTAGGGATGCACCGGGAA	229	60
α-1-antitrypsin	AGACCCTTTGAAGTCAAGGACACCG	CCATTGCTGAAGACCTTAGTGATGC	360	60
HNF4a	CGTGGTGGACAAAGACAAGA	CACTCAACGAGAACCAGCAG	297	55
Sox17	CAGCAGAATCCAGACCTGCA	GTCAGCGCCTTCCACGACT	69	
FoxF1	CAGCCTCTCCACGCACTC	CCTTTCGGTCACACATGCT	122	60
Msx2	CGGTCAAGTCGGAAAATTCA	GCATAGGTTTTGCAGCCATT	400	60
Tbx6	CAGCTCTGTGGGAACAGAAA	CGGAATCACATCCAGAAGAAA	124	60
βActin	ATTGGCAATGAGCGGTTCCG	AGGGCAGTGATCTCCTTCTG	211	60
B2M	GAGTATGCCTGCCGTGTG	AATCCAAATGCGGCATCT	111	60
GUS	GAAAATATGTGGTTGGAGAGCTCAT	CCGAGTGAAGATCCCCTTTTTA	102	60

8. In general, tumours can be observed between 4 and 8 weeks post-injection. Although solid tumours are formed fluid-filled vacuolated areas are also formed. Once areas have started to vacuolate they will expand quickly. An attempt to remove fluid, using a syringe and needle in the tumour of a sedated mouse, from the vacuolated area can be made—this has usually given a maximum of a week more of tumour growth.
9. Once tumour formation has been achieved the mice are sacrificed and tumours removed, fixed in 10% formalin overnight, and processed for histology.

4. Notes

1. The details for the amount of gamma irradiation that will inactivate the feeders used in these protocols is given; however, other cell lines used as feeders may need a different amount of Gy to inactivate them. It is best to check that any new cell line is adequately inactivated before proceeding to use them.
2. It is recommended that primary cell lines, such as MEFS are used as feeder layers between p3 and p7. Prior to p3 cell types other than fibroblasts may be persistent within the cell cultures, however, it is thought that by p3 the majority of these cells will not be contributing to the cell population. Over p7 primary cell lines can start to enter cellular senescence and will not support the hESC at an optimal level.
3. Mitomycin C is toxic and a carcinogen with the possibility of very serious non-reversible effects and should therefore be handled with great care. Disposal of media or PBS that has come into contact with it, following use for inactivation of cells, should be in accordance with local health and safety regulations.
4. Conditioned media can be created from any feeder source simply by exposing the culture media to inactivated feeders for 24 h. The feeders should be plated to look confluent in the flask/well, i.e. 18.8 million cells in T225 flask (collect 150 ml daily), 12.5 million cells in T175 flask (collect 100 ml daily), etc.
5. The media used to grow the blastocysts should be specific to the supplier used by the IVF clinic. In Denmark (as with a number of other European countries) only embryos surplus to IVF requirements are allowed to be used for the creation of hESC lines. Our local IVF clinics used embryo culture media (not specific blastocyst media) to culture the embryos for us. Methods for collection of blastocysts other than the one described (i.e. Minitub portable incubator) can be used, although we found this the most versatile.

6. Evaluation of the development of blastocysts can be useful retrospectively and we would encourage all details of methods, materials, and photographs of different stages of development during blastocyst development and creation of stem cell lines to be recorded in detail.
7. Guinea Pig complement should be made up on ice, using ice cold KO DMEM and pipettes/tips. Complement can be made up in advance and stored at −20°C until just prior to usage or it can be freshly used. However, once thawed or at room temperature it should be discarded.
8. Although it has been stated in the text that a stem cell cutting tool should be used for mechanical passaging or removal of differentiation a small gauge needle can be used instead (with a bend in the needle of 30–45°). There are drawbacks in using needles as they tend to heavily score the plastic and leave "strings" of plastic in the passaged cells which will cause differentiation.
9. It is of importance to freeze the initial bank from low passage cells which have not been exposed to enzymatic passaging as increased passage number and use of enzymes can increase the chances of random chromosomal or epigenetic changes. Freezing sequential aliquots from early passages onward offers the best chances for maintaining cell lines for future usage.
10. The concentration of hbFGF after cell revival should be according to the concentration used prior to freeze, i.e. if the cells are very early passage and were grown in 10 ng/ml FGF, then revival should use 10 ng/ml FGF. If reviving cells from a general use cell bank and the amount of FGF used before cryopreservation is 5 ng/ml, then this concentration should be used after revival.
11. Changing conditions in cell culture of hESC needs to be performed in a gradual manner to accustomise the cells to the new conditions, i.e. changing media from CM to KO Media is carried out over a couple of passages where the amount of CM added is lowered and the amount of KO media increased and then left for a few days before changing the concentrations again. This also applies to changing the concentrations of hbFGF. We have found a tendency for colonies to start to differentiate if changes in media composition occur too rapidly.
12. All antibody dilutions given here were tested on control tissue blocks to give dilutions for optimal staining.
13. Both the hypotonic solution (0.56% KCl) and the fixative (3:1 Methanol:acetic acid) need to be prepared freshly. The hypotonic changes pH on storage and the fixative esterifies over time and will affect fixation of the preparation. Fixative should not be used after it has been prepared for more than a couple of hours.

14. Timing in the Bacto-Trypsin is critical: if the chromosomes appear to have a flattened appearance the amount of time in trypsin needs to be decreased; if they are not sharp and look "fuzzy" the time in trypsin may need to be increased—however, increases/decreases in time are only in seconds.
15. Although analysis of gross karyotyes can easily be assessed and any gross abnormalities (such as triploidy, trisomy, monosomy) identified, detailed analysis of Giemsa-banded chromosomes needs to be performed by expert as smaller (but just as important) abnormalities (such as inversions, translocations, deletions) may get missed.
16. Teratoma formation is still, currently, considered as a "gold standard" test to assess the pluripotency of cell lines. Although in vivo differentiation, such as EB formation, can show all three lineages it does not give any terminal differentiation, such as cartilage, bone, endocrine formation, etc., which can be achieved during teratoma formation.

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Chapter 4

Principles for Derivation of Human Embryonic Stem Cells

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Karin Noaksson, and Johan Hyllner

Abstract

This chapter describes the principles for derivation and maintenance of human embryonic stem cells. Detailed protocols are outlined and researchers who are generally skilled in mammalian cell culture should be able to repeat the processes successfully. Further, the protocols are intended for scientists who do not have access to advanced IVF equipment and therefore cannot perform, e.g. assisted hatching. In addition to derivation, we also discuss characterisation and banking of hES cells.

Key words: Human embryonic stem cell, Derivation, Blastocyst, Feeder cells, Xeno-free

1. Introduction

The first reports of successful culture of blastomere cells derived from human blastocyst inner cell masses (ICM), were published in the 1990s (1, 2). Cultured in vitro, these proliferating cells are referred to as human embryonic stem (hES) cells. Individual ICMs gives rise to populations of cells commonly referred to as hES cell lines. Today, the number of established hES lines can be counted in 1,000+ worldwide (3).

The hES cell technology has given us access to new tools to study early human development and disease pathology. Obvious applications are the generation of cell-based therapies for degenerative disease, such as juvenile diabetes or traumas such as spinal cord injuries. For example, as this chapter is written, the first clinical trials for a treatment for spinal cord injuries have been given the go ahead by the regulators in the USA (4).

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The use of stem cells is not limited to basic research or regenerative medicine development. There is a large potential interest for human specialised cells derived from hES cells in the pharmaceutical industry. Currently, there is a limited source of high quality primary human cells for drug discovery and toxicity testing. For example, cardiomyocytes and hepatocytes derived from pluripotent stem cells could replace other less relevant experimental models and thus increase efficiency and decrease the cost of the drug discovery process.

This chapter describes the principles of derivation and propagation of hES cell lines. An outline of the steps involved is shown in Fig. 1. The methods described should be able to perform in any well-operated cell culture facility, without the need of complicated or expensive equipment, other than you expect a cell culture facility to possess. Over the years, there have been a large number of hES cells established and reported. Naturally, the establishment processes are coloured by the experiences and skills of the performing researchers and there has been technical development over the years with many new protocols, and permutations of existing protocols published. In addition, the need for novel approaches, such as animal component-free, or xeno-free, derivation methods have been driving the development. The development of clinical grade hES cell lines also has the regulatory requirement of having the end point cell type being manufactured according to good manufacturing practice (GMP). This means that the original hES cell line either has to be derived in concert with GMP guidelines, or at a later date be qualified as GMP compliant.

However, for the vast majority of hES cell research and development today, the derivation and culture processes can be conducted out with a GMP regime, however, having a quality system implemented is a mean of generating a reproducible quality of hES cells which is important for achieving high quality research.

For the purpose of assisted conception, or in vitro fertilisation, it is important to culture embryos under supportive conditions that mimic the in vivo development. The transfer of blastocysts rather than early cleavage stage embryos to the patients is physiologically more relevant, and successful protocols for this have been established (5). This also have benefits for the derivation of hES cells, since the surplus blastocysts will be of appropriate age.

An isolated ICM is delicate and survival and proliferation is dependent on optimised conditions. Success rates for hES cell line establishment from isolated ICMs have been reported to be between 5 and 30% (6, 7). The initial quality of the blastocyst is a contributing factor and there is a correlation between blastocyst quality and successful derivation; however, hES cell lines has been established from blastocysts graded as of low quality (8–12). It is possible to receive donated surplus frozen blastocysts in addition to fresh material, and derivation of hES cell lines has the potential to be equally successful from either source (7).

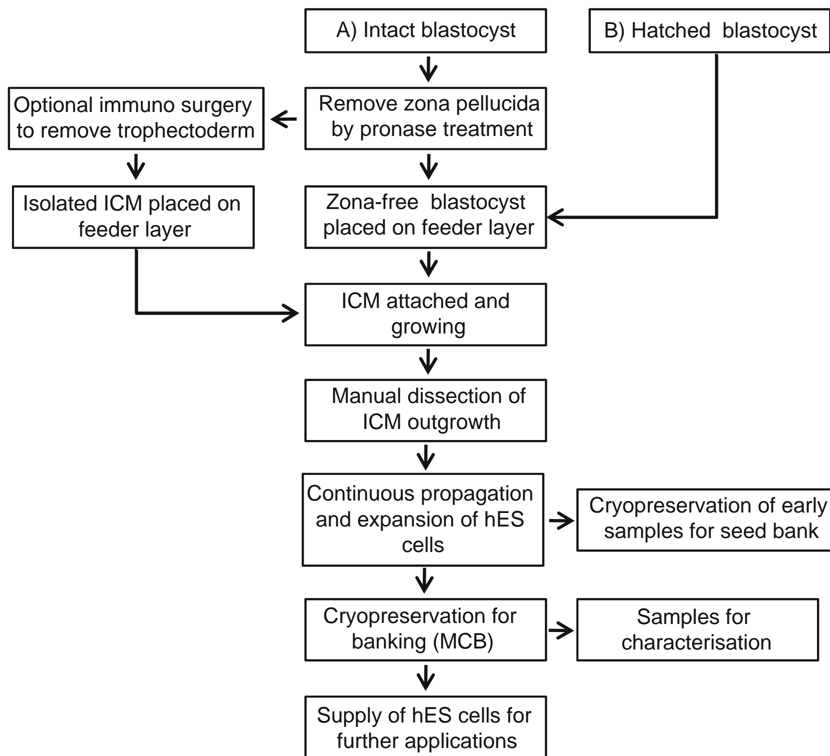

Fig. 1. This diagram illustrates the principle steps for the establishment and the further process of an hES cell line. The starting point can be either (A), an intact blastocyst with the zona pellucida still present and visible, or (B) a hatched blastocyst which has escaped out of the zona pellucida. In the latter case (B), the outer cellular layer of the blastocyst, the trophectoderm cells are exposed. In (A), the zona pellucida is digested by enzymatic treatment (pronase). We have included an optimal step, the process of immuno surgery, where the trophectoderm cells are actively targeted for destruction by specific antibodies and complement factors. The zona-free blastocyst or the isolated ICM are placed on a layer of mEF feeder cells. In the optimal case, the ICM attaches and proliferates. When considered suitable, the ICM outgrowth is micro-dissected and passaged onto a fresh layer of feeder cells and eventually, the cells start to appear with a typical hES cell colony morphology. At this stage, freezing down samples for a seed bank is advisable. The newly established potential hES cells can be expanded to generate an MCB, comprising of a larger amount of units, which will then undergo characterisation to confirm the hES cell phenotype. MCB units can subsequently be used for expansion of hES cells for further use, such as differentiation experiments.

2. Materials

The blastocysts to be used for stem cell derivation are typically 4–6 days old and have a clearly visible inner cell mass (ICM). At this stage, the ICM contains less than a hundred cells (13). Consequently, the success rate is closely connected to the ability of the provided culture environment to support survival and growth of the newly isolated ICMs. The absolute majority of hES cell lines have been derived by the support of feeder cells. Traditionally, mouse embryonic fibroblasts (mEFs) have been employed for hES cell derivations but also cells of human origin, such as human

foreskin fibroblasts (hFF) (14, 15) have been employed with good results. Any feeder cells need to be mitotically inactivated, either by irradiation or with Mitomycin C treatment. mEFs should preferentially be used the day after they have been mitotically inactivated while hFFs should be used not earlier than 48 h after inactivation.

2.1. Sourcing of Cells

1. mEFs can either be prepared using standard procedures (16) or be purchased from commercial suppliers, such as GIBCO/Life Technologies or from American Type Culture Collection (ATCC, Manassas, VA, http://www.atcc.org), (see Note 1).
2. hFFs, e.g. CRL-2429 can be obtained from the ATCC. (ATCC, Manassas, VA, http://www.atcc.org) (see Note 2).

2.2. Materials

1. mEF medium: 10% foetal bovine serum (FBS, Gibco) and 1% of penicillin-streptomycin (Invitrogen) in DMEM (Invitrogen).
2. hFF medium: Iscove's modified Dulbecco's medium (IMDM) (Invitrogen), supplemented with 10% FBS (Invitrogen), and 1% penicillin-streptomycin (Invitrogen).
3. 1× PBS -Ca^{2+}/-Mg^{2+} (Invitrogen).
4. 1× TrypLE Select (Invitrogen) or 1× Trypsin-EDTA (Invitrogen).
5. Mitomycin C (Sigma-Aldrich): Dilute according to manufacturer's instruction. Final concentration should be 10 μg/ml in the medium (see Note 3).
6. Gelatine (Sigma-Aldrich). The final concentration of the gelatine should be 0.1% when diluted in cell culture grade water. Autoclave the gelatine solution promptly (see Note 4).
7. Appropriate sterile cell culture plastic ware.
8. hES cell basal medium: DMEM knock out, 20% knock out serum replacement, 1% of penicillin-streptomycin, 1% non-essential amino acid, 1% GlutaMAX, and 0.2% beta-mercaptoethanol. All reagents are from Invitrogen.
9. Human recombinant basic fibroblast growth factor (hrbFGF, Invitrogen). Final concentration should be 10 ng/ml culture medium. Dilute in Vitro-PBS (Vitrolife) and store aliquots in −20°C.
10. hES cell 10 medium: hES cell basal medium with 10 ng hrbFGF/ml medium. Prepare just prior use.
11. Stem Cell cutting tool (Vitrolife, Gothenburg, Sweden).
12. Transfer pipettes (Vitrolife).
13. Fresh Mitomycin C treated feeders in hESC 10 medium.
14. CCM-30 (Vitrolife).
15. Hyaluronic acid (Ioltech, La Rochelle, France).
16. Ovoil (Vitrolife).

17. Pronase (Sigma-Aldrich).
18. α-human serum antibody (Sigma-Aldrich).
19. Guinea pig complement (Sigma-Aldrich).
20. Appropriate sterile cell culture plastic ware.

3. Methods

3.1. Culture of Feeder Cells: mEF

1. When the mEFs are to be passaged: Remove the media and rinse the cells with 1× PBS -Ca^{2+}/-Mg^{2+}. Add an appropriate volume of 1× Trypsin-EDTA to the culture flask. Leave the Trypsin-EDTA on the cells for 2–5 min.
2. As the cells starts to detach from the surface rinse with mEF medium and resuspend the cell suspension.
3. Centrifuge the cells for 5 min at $400 \times g$. Resuspend the cells in mEF medium and plate at appropriate density (20×10^3–30×10^3 cells/cm^2).
4. In contrast to the hFFs, the mEFs should only be used as feeders at the second and third passage.

3.2. Culture of Feeder Cells: hFF

1. When the hFFs are to be passaged: Remove the media and rinse the cells with 1× PBS -Ca^{2+}/-Mg^{2+}. Add an appropriate volume of 1× TrypLE Select. Return the culture vessel to the incubator, but visually inspect the cells every 2–3 min (see Notes 5 and 6).
2. As the cells starts to detach from the surface, add some 1× PBS -Ca^{2+}/-Mg^{2+} and then gently aspirate and resuspend the cell suspension.
3. Centrifuge the cells for 5 min at $400 \times g$. Resuspend the cells in fresh hFF medium and plate the cells at 4,000 cells per cm^2.
4. Perform a complete medium change twice a week. Before each medium change, equilibrate the required amount of hFF medium at least to room temperature, preferably to 37°C.

3.3. Mitomycin C Treatment of Feeder Cells (mEFs and hFFs)

Preferentially, mEF should be Mitomycin C treated the day before the ICM isolation and it is not recommended to use older mEF feeders then 2 days for derivation work.

hFFs on the other hand should be Mitomycin C treated at least 2 days prior ICM isolation and these feeders can be used within 2–6 days after Mitomycin C treatment.

1. Remove the culture medium from the cell culture flasks with the feeder cells to be treated with Mitomycin C and determine the volume of medium.

2. Add Mitomycin C stock solution to the medium to a final concentration of 10 µg/ml and return the medium to the cells to be inactivated.
3. Return the cell culture flasks into the incubator for 2–3 h.

3.4. Gelatine Coating of Culture Dishes

1. Coat the cell culture wells to be used with 0.1% gelatine solution. Make sure that the surface is completely covered with gelatine solution.
2. Place the cell culture units in the LAF-bench for 0.5–24 h.
3. Aspirate the excess gelatine solution just prior to the seeding of the Mitomycin C treated feeders.

3.5. Seeding of Mitomycin C Treated Feeder Cells

1. Transfer the cell suspension to a tube and centrifuge at $400 \times g$ for 5 min.
2. After the centrifugation step, remove the supernatant and re-suspend the cells in hESC 10 medium.
3. Count the cells in a haemocytometer and calculate the number of cells per ml.
4. Dilute the cells with hESC 10 medium to the required volume to be able to seed the inactivated cells at a density of:

   mEF: $40–70 \times 10^3$ cells/cm^2.

   hFF: 70×10^3 cells/cm^2.
5. Add the prepared cell suspension to the dishes and place them in the incubator at 37°C, 5% CO_2, and 95% humidity.
6. Visually inspect the cells 24 h after seeding in order to confirm that they have attached and that they are evenly distributed within the culture vessel.

3.6. ICM Isolation

Below is a practical description on how blastocyst ICMs can be isolated for further maintenance in culture. There are other methods described for ICM isolation, such as microsurgery and also single blastomere isolation, which is not further discussed here. The ultimate goal of an ICM derivation is to be able to continuously propagate the resulting cells, i.e. the stem cells. The fertilised embryos are further cultured to the blastocyst stage (5, 17). Often at this stage, 4–6 day old post-fertilisation, the blastocyst is still surrounded by the zona pellucida, a layer of glyco proteins. In order to access the blastocyst for further manipulations, the zona pellucida needs to be removed, typically enzymatic treatment by pronase. Furthermore, the trophectoderm cells surrounding the cavity with the ICM are blocking access to the ICM and can be removed by a method denoted "immunosurgery", creating a humoral immune response destroying the trophecoderm cell layer. In principle, the blastocyst cells are exposed to a mix of antibodies raised against trophectoderm cells and complement system components, which

will trigger an immune response destroying the trophectoderm cells. The isolated clump of ICM is placed on a layer of mitotically inactivated fibroblasts, typically mEFs, and allowed to attach and grow out to colonies of cells. Alternatively, the whole blastocyst, without the zona pellucida, can be placed out on an inactivated feeder cell layer. If the ICM cells are able to be continuously propagated, they can be expected to be stem cells; however, further characterisation is necessary to establish this. Below is a step-by-step description of the principles of ICM derivation starting with the feeder cell preparation and ending with the passage of hES cell.

3.6.1. ICM Isolation and Plating: Method

1. At least 2 h before ICM isolation, CCM-30 should be added to an appropriate number of IVF-culture dishes (i.e. non-feeder containing dishes). One needs one culture dish per blastocyst. Place the dishes in the incubator so that the solution equilibrates. An appropriate aliquot of Ovoil should also be heated to 37°C.
2. Add 0.125 mg/ml hyaluronic acid to your newly inactivated feeder dishes. One culture dish/blastocyst is needed. Return the culture dishes to the incubator.
3. Move one of the culture dishes containing equilibrated CCM-30 to the heated stage of the stereomicroscope and add the blastocyst to the culture dish.
4. Confirm via microscopy that the blastocyst now is in the culture dish.
5. Assess the status of the blastocyst microscopically. Depending on whether the blastocyst has hatched or not, different action is needed:

3.6.2. Ongoing Hatching

If the blastocyst is hatching, i.e. zona pellucida is disrupted and the blastocyst is bulging out from the rigid envelope, we recommended that the blastocyst is moved into the incubator. Monitor the hatching process every hour. When the blastocyst has hatched, continue as in Subheading 3.6.3.

3.6.3. The Blastocyst Has Hatched

If the blastocyst has hatched and is separated from zona pellucida (see Fig. 2b), there are two options: One can transfer the blastocyst immediately to an IVF dish with the preferred feeders. Use a transfer pipette to carefully move the blastocyst between the two culture dishes. It is recommended to photo document the blastocyst. The other option is to perform immunosurgery on the blastocyst (see below).

3.6.4. Intact Zona Pellucida

If the blastocyst still is enclosed by the zona pellucida (see Fig. 3), it has to be released. Since most laboratories lack the equipment to physically open up and remove zona pellucida, we describe below a chemical method that easily can be employed:

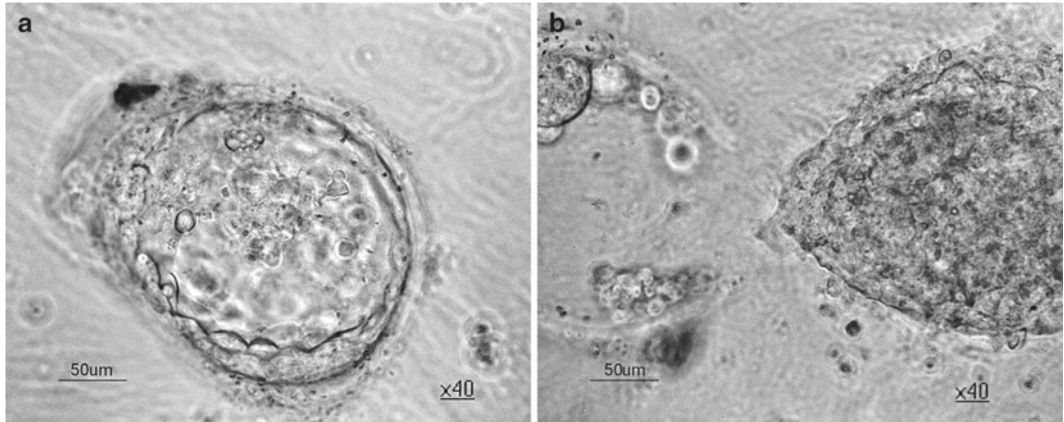

Fig. 2. (**a**) A blastocyst undergoing spontaneous hatching. The zona pellucida has been disrupted and a substantial part of the blastocyst is bulging out. Scale bar has been added, indicating 50 μm. (**b**) The end result, a hatched blastocyst and the remaining, now empty "shell"—zona pellucida. Scale bar has been added, indicating 50 μm.

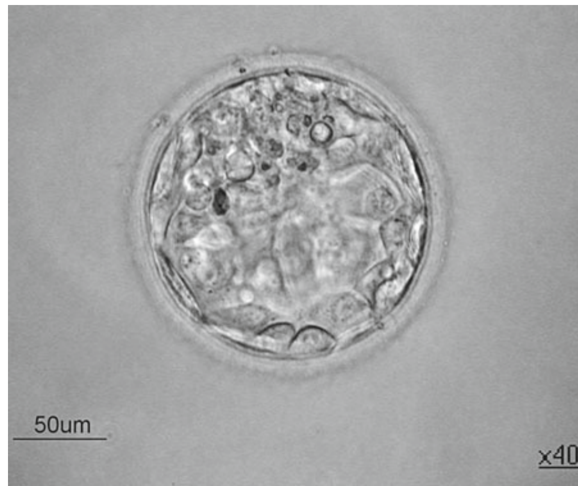

Fig. 3. Blastocyst with intact zona pellucida. Scale bar has been added, indicating 50 μm.

1. Use a sterile culture dish (Ø 6 cm) and place three, separated droplets, of pronase (each droplet is 30 μl from a stock solution of 10 U/ml).
2. Then, add each three droplets of 30 μl of equilibrated CCM-30.
3. Cover everything with 3–5 ml of Ovoil that has been preheated to 37°C.
4. Store the dish in the incubator for at least 15 min to ensure an optimal temperature for the pronase treatment of the blastocyst.
5. During the pronase treatment, the culture dish containing the pronase droplets should be on a heated stage on an inverted microscope. Use a transfer pipette and move the blastocyst into the first pronase droplet.

6. Proceed then directly by moving the blastocysts to the second and finally to the third droplet of pronase.
7. Now, monitor how the zona pellucida will dissolve; one can see how a halo is formed around the blastocysts during this procedure. This is due to swelling/expansion of the zona pellucida.
8. Remove the blastocysts as soon as the halo disappears. The whole pronase process will take 1–3 min.
9. The pronase is removed from the blastocysts by adding the blastocysts consecutively to the three CCM-30 droplets.
10. At this stage, the zona-free blastocysts (for an example, see Fig. 2b) can be transferred to an IVF dish with the chosen feeders or one can perform immunosurgery on the blastocysts.

3.6.5. Immunosurgery

The purpose with the immunosurgery procedure is to destroy the outer cells of the blastocysts, the trophoblasts, since they are not contributing to the formation of embryonic stem cells but may instead overgrow and dominate the culture:

1. Dilute the α-human serum antibody in CCM-30 (12 mg/ml).
2. In a sterile culture dish (Ø 6 cm), place three separate droplets (30 μl/droplet) of the freshly prepared solution and then add three droplets of equilibrated CCM-30 to the dish.
3. Cover with 3–5 ml of Ovoil (37°C).
4. Use a transfer pipette and move the blastocyst into the first droplet of the anti-body solution.
5. Proceed then directly by moving the blastocysts to the second and finally to the third droplet of antibody solution.
6. The blastocyst shall remain in the third droplet for approximately 30 min. In order to ensure optimal temperature during this incubation, move the dish into the incubator.
7. While the blastocyst is incubated with the antibody, the next solution should be prepared: Dilute the Guinea Pig (GP) complement 1:5 with CCM-30.
8. In a sterile culture dish (Ø 6 cm), place three separate droplets (30 μl/droplet) of the freshly prepared GP solution and then add three droplets of equilibrated CCM-30 to the dish. Cover the droplets with 3-5 ml of Ovoil (37°C).
9. After approximately 30 min the antibody solution should be washed away. This is accomplished by transferring the blastocysts over to the droplets of CCM-30.
10. The blastocysts should be in each of the three droplets for 2 min. Return the dish to the incubator for each washing period.
11. After the third wash, move the blastocyst into the first droplet with GP complement.

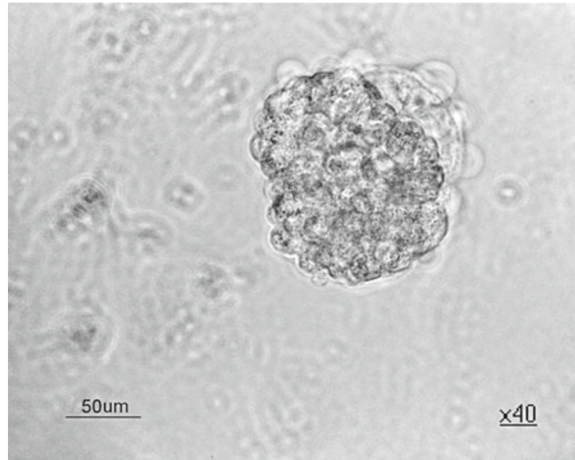

Fig. 4. Isolated ICM after immunosurgery. Scale bar has been added, indicating 50 μm.

12. Proceed then directly by moving the blastocysts to the second and finally to the third droplet of GP complement. The blastocysts shall remain in the third droplet for 5 min (in the incubator).
13. During the GP complement procedure, the trophoblast cells will break up and detach from the blastocysts.
14. Move the blastocyst over to the first CCM-30 droplet. In order to facilitate any further destruction/removal of trophoblast cells, one can flush the blastocysts with the transfer pipette every time it is moved to the three CCM-30 droplets (see Note 7).
15. Now, it is time to transfer the ICM (see Fig. 4) to an IVF dish containing the preferred feeder cells and with the freshly added hyaluronic acid (see Note 8).
16. Move the ICM to the feeder dish with the transfer pipette. Try to place the ICM as central as possible in the IVF dish and as close as possible to the feeders. Label the culture dish accordingly and place the culture dish in the incubator.

3.6.6. From a Plated Blastocyst to Passaging of Human ES Cells

1. The blastocyst need to attach to the feeder cells so avoid any unnecessary movements of the culture dish.
2. Change medium of the blastocysts culture every second day. After medium change, inspect the blastocyst in the inverted microscope (see Note 9).
3. When you have an outgrowth of cells with stem cell morphology, small cells, tightly packed together (see Fig. 5) you should monitor the culture every day. Ideally, you will only have an outgrowth of hES cells, but remaining trophoblast cells may also have increased in number. The trophoblast cells are by far bigger than the stem cells and may prevent stem cells to grow out further.

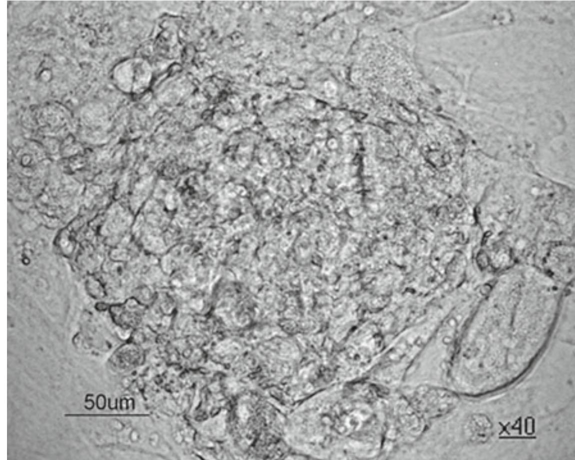

Fig. 5. Outgrowth of plated ICM on feeder cells. Scale bar has been added, indicating 50 μm.

4. At this very first passage, a mechanical passage with a stem cell knife is the preferred choice. It will allow you to make a positive selection (the stem cells), and avoid the majority of any remaining trophoblasts.
5. First, test the edge of the stem cell knife on the feeders to identify the preferred angle, then with the stem cell knife; cut lines through the areas that you want to transfer to a new culture dish (see Note 10).
6. Turn the culture dish 90° so that it enables you to cut new lines which makes a grid pattern (for an example of microdissected hES cells, see Fig. 6).
7. By using a transfer pipette, move the small stem cell squares into a new culture dish with new feeders. Just as with the blastocyst, try to position the pieces as close to the feeders as possible and spread the pieces within the culture dish (see Note 11).
8. Continue to change medium every 2nd day and passage the stem cells to new feeders. The passage interval is very much dependent upon choice of feeders and the individual growth rate of the stem cells (see Note 12).
9. Slowly expand your new potential stem cell line and make an early banking as soon as you have material to do so. Vitrification is a preferred method since it allows freezing and thawing of very few cells in contrast to standard bulk methods (see Note 13).

3.7. Some Notes About Going Xeno-Free

The very first clinical trial has been initiated by Geron Corporation in the USA, where hES cell-derived oligodendrocyte progenitor cells (GRNOPC1) are being transplanted to patients with complete thoracic spinal cord injuries, and more trials will most likely follow (4).

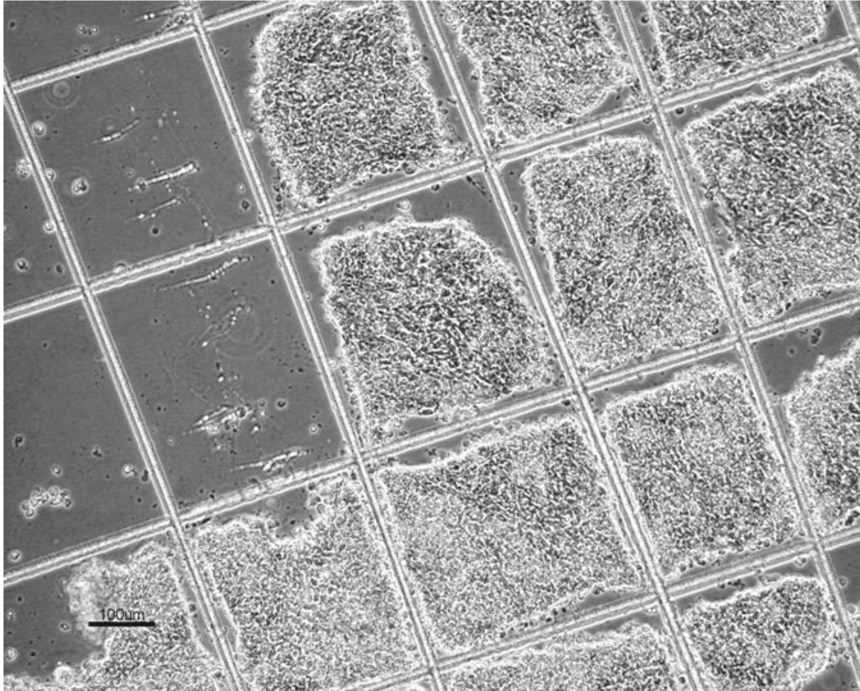

Fig. 6. Micro-dissected hES cell colony. Some pieces have been removed for transfer. Scale bar has been added, indicating 100 μm.

Consequently, increased demand for xeno-free and cGMP-derived hES cells can be expected. Today, there are several defined and xeno-free hES cell media commercially available, but they are all for feeder-free culture of hES cells. By having feeder cells present during the derivation steps, one optimises the cell culture environment for the ICM cells and the outgrowth of hES cells. To derive hES cells under xeno-free conditions, one has to meticulously replace all animal containing products with either human-derived or recombinant/synthetic substances: xeno-free hFFs can either be in house derived, such as previously described (14) according to appropriate ethical regulation or purchased. Several companies offer xeno-free media that can be used. The porcine gelatine, used for the coating of the culture dish, can be replaced by human recombinant collagen. Also the above described hES medium can nowadays be made xeno-free since Invitrogen offer a xeno-free knock out serum replacement. Sources of bFGF are most likely recombinant, but one have to make sure that it is diluted in a buffer containing human albumin. For the establishment procedure, either pronase or Tyrodes solution (14) can be used to remove the zona pellucida, but the immunosurgery has to be excluded since it involves reagents sourced from animals. When it comes to passage of feeder cells and later, for the established hES cell line, e.g. TrypLE Select is an excellent xeno-free alternative.

3.8. Characterization and Banking

The hallmark of a stem cell is really its ability to form derivatives of the three embryonic germ layers ecto-, endo-, and mesoderm. This ability is referred to as pluripotency. In addition, a stem cell should be able to propagate indefinitely in an undifferentiated state, assuming culture techniques are optimal. There is no unanimous marker for pluripotency, the stem cell research community relies on a panel of different markers, spanning from the expression of transcription factors and cell surface molecules to telomerase activity and in vitro and in vivo experimental models for demonstrating the formation of the three germ layers discussed above (2, 11). Below is a brief description of the different components of the characterisation of a hES cell line. In addition, the hES cell should be tested for safety, e.g. the absence of common human pathogens and mycoplasma.

Chromosome integrity can be evaluated by karyotype analysis and fluorescence in situ hybridisation (FISH). This gives information regarding sex and chromosome setup and potential major issues, such as deletions, translocations, and duplication of genetic material. In addition, more detailed analyses, such as multiplex ligation-dependent probe amplification (MLPA) can be performed, generating information on for example gene copy numbers and DNA methylation. Telomerase activity, as a measure of the hES cells ability to continuously go through mitosis, is commonly analysed by the expression of the essential subunit hTERT, which can be analysed by commercially available kits.

Cell membrane bound surface markers associated with pluripotency such as the glycol lipids SSEA-1, -3, -4; and the keratin sulphate molecules TRA-1-60, -1-81 are commonly assayed by immunohistochemistry (IHC). It has been discussed whether SSEA-3 and SSEA-4 actually are indicating pluripotency (18). The presence or expression of transcription factors, such as Oct-4, Sox2, and Nanog can be analysed by IHC, quantitative PCR or flow cytometry, the quality of information generated is dependent on the method. Alkaline phosphatase activity is generally considered as a positive marker for undifferentiated hES cells and can be analysed by an enzymatic reaction and a clearly visible colorimetric read out.

In vitro pluripotency can be assayed by markers for endo-, ecto-, and mesoderm on spontaneously differentiated cell material. hES cells that are allowed to form aggregates in suspension culture can be plated out and allowed to spontaneously differentiate into various cell types. Examples of markers used are the transcription factors forkhead box A2 (Foxa2) transcription factor for endoderm, β-III-tubulin for ectoderm, and arterial smooth muscle actin (ASMA) as a marker for mesoderm.

In vivo pluripotency is assayed by xenografting hES cells into an immunodeficient mouse, for example an SCID mouse. The hES cells will, if pluripotent, generate a tumour which can be analysed for human endo-, ecto-, and mesoderm derivatives, thus demonstrating the potential of the hES cell line.

Ideally, a Master Cell Bank (MCB) of hES cells should be generated at as a low passage number as possible, minimising the risk of acquired abnormalities, such as chromosome modifications or epigenetic changes. An appropriate sized sample of the MCB should be resuscitated and propagated and subsequently tested for safety and hES cell characteristics. In contrast to performing characterisation on continuously cultured hES cells, this gives the advantage that after the finalisation and approval of the characterisation process, one can to a high level of certainty assume that the remaining units in the MCB exhibits all the characteristics and has not acquired any abnormalities during culture. This makes the MCB a valuable asset and contributes to a robustness and reproducibility in the hES cell cultures.

Also, if the amount of early hES cell cultures allow, cryopreserving samples in low passages after safety testing is valuable, if the later cultures fail for some reason. From this "seed bank", cells can be brought up for the generation of a larger MCB.

4. Notes

1. Depending on origin, mEF cell preparations can show batch-to-batch variations. The implementation of feeder cell batch testing routines can contribute to minimise the risk of fluctuating hES cell culture quality.
2. *Nota Bene*! There are many different hFF lines available, but all lines are not suitable as feeder cells for stem cells. If you want to use another human feeder cell line for derivation, you need to ensure that the hFF line is capable of supporting stem cells prior to your derivation work is initiated.
3. Mitomycin C is a biohazard and should be handled with care. A commonly used alternative to Mitomycin C treatment of feeders is γ-irradiation.
4. After autoclaving, the gelatine can be aliquoted and stored at 4°C for 1 month.
5. When hFFs are cultured for expansion purposes, the cells should never be allowed to grow confluent. Instead they should be passaged before they reach 90% confluence. Recommended split ratio is 1:4 to 1:8.
6. Do not use the hFF as feeder cells above 25 population doublings.
7. Some trophoblasts may remain at this stage, but they are most likely damaged and will die off.
8. Again—check that your feeder cell plate is OK via your microscope.

9. The speed of outgrowth of stem cells is depending on many different factors. One is the choice of feeders. If you choose mEFs, the stem cells may growth out faster compared with hFFs. On the other hand, the hFFs can support the blastocysts and the stem cells under a longer time period.
10. The optimal distance between the lines is approximately 200 µm (= the diameter of the knife).
11. For mEF feeders, we recommend 12–15 pieces per IVF dish while for hFF feeders we recommend 10–12 pieces per dish.
12. A rough estimate is that the mEF cultured cells should be passaged every 4–5 days while stem cells cultured on hFFs may be passaged ever 7–10 days.
13. Detailed protocols for vitrification can be found at http://www.nationalstemcellbank.org (SA01, LOT CA001, CoA).

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Chapter 5

Derivation and Maintenance of Undifferentiated Human Embryonic Stem Cells

Guoliang Meng and Derrick E. Rancourt

Abstract

Human embryonic stem cells (hESCs) are self-renewing, pluripotent cells derived from the inner cell mass of blastocysts, early-stage embryos, or blastomeres. hESCs can be propagated indefinitely in an undifferentiated state in vitro and have the ability to differentiate into all cell types of the body. Therefore, these cells can potentially provide an unlimited source of cells and hold promise for transplantation therapy, regenerative medicine, drug screening and discovery, and basic scientific research. Surplus human embryos donated for hESC derivation are extremely valuable, and inefficient derivation of hESCs would be a terrible waste of human embryos. Here, we describe a method for isolating hESC lines from human blastocysts with high efficiency. We also describe the methods for excising differentiated areas from partially differentiated hESC colonies and re-isolating undifferentiated hESCs from extremely differentiated hESC colonies.

Key words: Human embryonic stem cells, Cryopreserved embryos, Embryo culture, Embryonic microsurgery, Derivation, Culture, Rederivation, ROCK inhibitor

1. Introduction

The unique properties of human embryonic stem cells (hESCs), unlimited self-renewal and the potential to differentiate into any cell type of the organism, make them hold promise for regenerative medicine, drug screening and discovery, and basic research (1–4). Traditionally, researchers have used an immune-surgical method to isolate human inner cell mass (ICM) cells by selectively killing surrounding trophoblasts within blastocysts (1–7). This method, which requires animal-derived antihuman antibodies and complement factor, runs the risk of contaminating resulting hESC lines with animal pathogens and animal-specific biological molecules. This method cannot be used to isolate human ICM under xeno-free culture conditions. In addition, this technique also wastes human

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embryos by testing suitable concentrations of each batch of antibody and complement used for immunosurgery.

Another method, whole embryo culture, has been used successfully for derivation of hESCs and involves the outgrowth of *zona pellucida*-free embryos onto mitotically inactivated feeder cells (8, 9). Since trophoblasts can interfere with ICM-derived ES-like cells inside the outgrowth, methods have been developed to microdissect the ICM from blastocysts. Such methods use a specially made flexible metal needle, made of tungsten, together with another blunter needle used to hold the blastocysts while cutting out the ICM. The needles were fixed to handpieces of pencil thickness for manual operation under a stereomicroscope (10).

In this chapter, we describe a simple method to isolate human ICM by removing trophoblasts. Here, a 30 G 1/2 needle attached to a 1-ml syringe was substituted for this complicated operational system and was used to remove the majority of trophoblast cells prior to transferring ICM to feeder cells (11). Moreover, this syringe needle approach is also convenient for cutting hESC colonies for subsequent nonenzymatic passaging in the early derivation stage.

Using this technique, we initially derived three hESC lines from 4 expanded blastocysts, suggesting that this method is highly efficient. Unfortunately, the putative hESC line, CC2, differentiated during its expansion before cryopreservation due to a problem associated with a batch of FGF-basic (bFGF). Consequently, two hESC lines, CC1and CC3, were established. Under optimized conditions, these hESCs can infinitely self-renew in an undifferentiated state. However, in some cases, such as expired factors and reagents, inappropriate handling, freezing/thawing, first several passages after derivation of new hESC lines, and selection after gene transfection, can cause partial or extreme differentiation in hESC colonies. Here, we also describe techniques that remove differentiated areas from partially differentiated colonies and purify undifferentiated hESCs from extremely differentiated colonies. Using the methods described in this protocol, it is easy to maintain completely undifferentiated colonies via a single passage.

2. Materials

All solutions are prepared under sterile conditions and reagents are tested whenever possible. It is strongly recommended that each batch of hESC culture reagents, including fetal bovine serum (FBS) and Knockout Serum Replacement (KSR), should be tested.

2.1. Reagents

1. Acidic tyrode solution (Sigma).
2. Albumin from bovine serum (BSA, cell culture tested, Sigma).
3. Collagenase IV (Invitrogen).

4. Dispase in DMEN/F12 (StemCell Technologies).
5. Dulbecco's Modified Eagle Medium (DMEM, Invitrogen).
6. Dulbecco's phosphate-buffered saline (DPBS, Invitrogen).
7. Fetal bovine serum (FBS) (Invitrogen).
8. GlutaMax™ (Invitrogen).
9. Human bFGF (PeproTech Inc.).
10. Knockout (KO)-DMEM medium (Invitrogen).
11. Knockout™ Serum Replacement (KSR) (Invitrogen).
12. Matrigel™ hESC-qualified Matrix (BD Biosciences).
13. ß-mercaptoethanol (Invitrogen).
14. mTeSR1 medium (Basal medium/400 ml; 5× supplement/100 ml) (StemCell Technologies.).
15. Nonessential amino acid solution (NEAA, Invitrogen).
16. Paraffin oil (EMD chemicals).
17. Penicillin–streptomycin (Invitrogen).
18. Propane-1, 2-diol (Sigma).
19. Quinn's blastocyst medium (Sage Biopharma).
20. Quinn's cleavage medium (Sage Biopharma).
21. Rock inhibitor, Y27632 (CALBIOCHEM).
22. Sucrose (Sigma).
23. Synthetic serum substitute (SSS, Irvine Scientific).
24. 0.05% Trypsin–EDTA (Invitrogen).

2.2. Equipment

1. Bottle-top filter system, 0.22 μm, 500 ml (Millipore).
2. Culture vessels: 4-well culture plates and 35 mm culture dishes (Nunc).
3. Cutting tool: A 30 G 1/2 needle (Becton Dickinson CA) attached to a 1-ml syringe (BD).
4. Dissecting stereo-microscope (LEICA MZ12).
5. Glass slide (Fisher Scientific).
6. Microcentrifuge tube, 1.7 ml (National Scientific Supply Co. Inc.) and Centrifuge tube, 15 ml (Corning).
7. 0.2-μm Syringe Driven Filter Unit (Millipore).

2.3. Media and Solutions

1. Solutions for zygote thawing: (1) DPBS containing 1 M propane-1, 2-diol, 0.3% BSA, and 0.2 M sucrose; (2) DPBS containing 0.5 M propane-1,2-diol, 0.3% BSA, and 0.2 M sucrose; (3) DPBS containing 0.3% BSA and 0.2 M sucrose; and (4) DPBS containing 0.3% BSA.
2. Media for human embryo culture: Quinn's cleavage medium supplemented with 10% SSS and Quinn's Blastocyst medium supplemented with 10% SSS.

3. bFGF solution: Spin the lyophilized bFGF vial briefly to bring the contents down. Prepare 0.2% BSA in DPBS in a sterile tube (1:50). Dissolve bFGF in 0.2% BSA to obtain a final concentration of 20 μg/ml. Make 100-μl aliquots in microcentrifuge tubes and store at −80°C.
4. Complete DMEM medium for feeder cells: To prepare 200 ml medium, add 20 ml FBS, 2 ml Glutamax, 2 ml NEAA, and 2 ml penicillin/streptomycin to 174 ml DMEM. Store it at 4°C in the dark and use within 3–4 weeks.
5. Collagenase IV solution (1 mg/ml): Add 20 mg collagenase type IV into 20 ml Knockout DMEM. Filter the solution with a 0.2-μm syringe filter. Store it at 4°C for up to 1 week.
6. Feeder-dependent hESC culture medium: To prepare 200 ml of hESC medium, add 40 ml KSR (20%), 2 ml Glutamax (2 mM), 2 ml NEAA (1 mM), 2 ml penicillin–streptomycin (50 unit penicillin/50 μg streptomycin per ml), 364 μl ß-mercaptoethanol (0.1 mM), and 100 μl bFGF (10 ng/ml) into 153.5 ml of KO-DMEM. Filter the medium with a bottle-top 0.22-μm filter and store at 4°C in the dark and use within 10 days.
7. Feeder-free hESC culture medium: Aliquot 100 ml of mTeSR1 5× Supplement into 50-ml tubes (ten tubes, 10 ml for each); store aliquots at −20°C. When preparing complete medium, thaw an aliquot of 5× Supplement at room temperature, and aseptically add 40 ml basal medium for a total volume of 50 ml. The complete mTeSR1 medium is stable when stored at 4°C for up to 2 weeks.
8. Matrigel reconstitution and use: Follow the instruction of BD Company (http://www.bdbiosciences.com/external_files/dl/doc/manuals/live/web_enabled/354277_pug.pdf).

3. Methods

3.1. Human Embryo Culture

Surplus human zygotes produced by IVF for clinical purposes were donated for hESC derivation.

1. Thaw zygotes by holding the straws in the air at room temperature for 30 s and then in a 30°C water bath for 40–50 s.
2. After thawing, equilibrate the embryos free of cryoprotectants by transferring them into a series of the following solutions for 5-min incubations at room temperature, respectively: (1) DPBS containing 1 M propane-1,2-diol, 0.3% BSA, and 0.2 M sucrose; (2) DPBS containing 0.5 M propane-1,2-diol, 0.3% BSA, and 0.2 M sucrose; (3) DPBS containing 0.3% BSA and 0.2 M sucrose; and (4) DPBS containing 0.3% BSA (see Note 1).

3. After being incubate embryos in DPBS containing 0.3% BSA at 37°C incubator for a further 5 min, transfer each of them to and culture in a 20-μl drop of 10% SSS in Quinn's cleavage medium, which is covered with paraffin oil and preincubated overnight in the CO_2 incubator (5% CO_2, 37°C).
4. After 2–3 days of culture, transfer each embryo to a 20-μl drop of preincubated 10% SSS Quinn's Blastocyst medium under paraffin oil, and culture until the embryos become expanded blastocysts on day 6 (Fig. 1b, f).

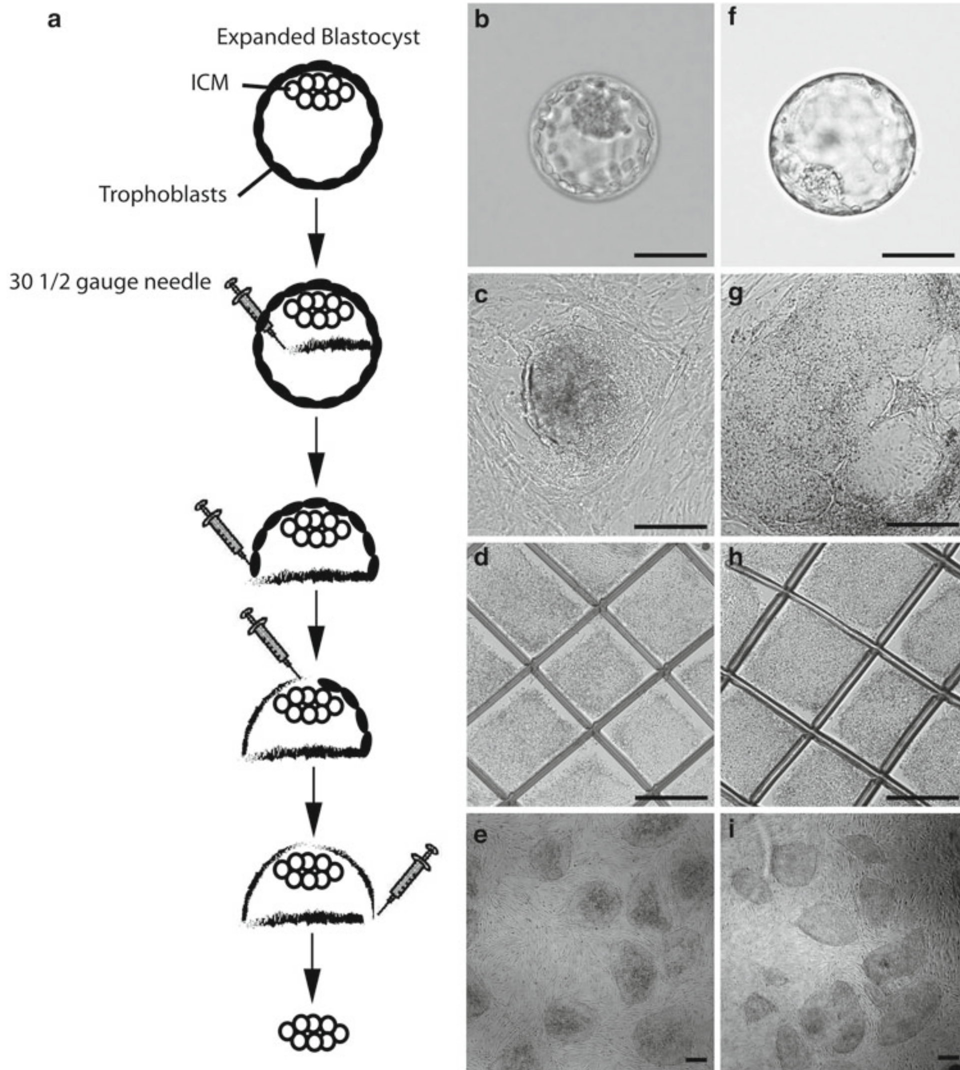

Fig. 1. Derivation of hESC lines using blastocyst microsurgery. (**a**) Our critical method is the mechanical dissection of ICM using a 30 G 1/2 syringe needle. We first cut the blastocysts in half and then dissect the ICM from the remaining trophoblast cells before plating onto feeder cells. (**b**, **f**) Expanded human blastocysts developed from freeze-thawed zygotes used to derive CC1 and CC3, respectively. (**c**, **g**) Outgrowths derived from dissected ICMs. (**d**, **h**) Microsurgical cutting of hESC colonies into cell clumps for replating and expansion; (**e**, **i**) newly isolated hESC lines, CC1 and CC3, respectively.

3.2. Derivation of hESC Lines

A novel microsurgery method is used to isolate intact ICM under a dissecting stereo-microscope (LEICA MZ12) in preparation for outgrowth on feeder cells.

3.2.1. Isolation of Intact ICM (Fig. 1a)

1. Remove the *zona pellucida* with acidic tyrode solution.
2. Wash embryos three times in hESC medium drops.
3. Transfer embryos into prewarmed hESC medium drops prepared on an autoclaved glass slide (see Note 2).
4. Press down embryo with cutting tool held in the left hand: cut it first in half with another cutting tool held in the right hand.
5. Press down the half embryo containing ICM with cutting tool held in the left hand, and then isolate ICM by cutting it free from the trophectoderm using cutting tool held in the right hand (see Note 3).

3.2.2. Culture of ICM (Fig. 1c, g)

Transfer ICM to MEF feeder cells (see Note 4) pre-prepared on 0.1% gelatin-coated 4-well plates containing either.

1. 80% KO-DMEM, 8% FBS, 4% Serum replacement, 4% Plasmanate (Bayer), 2 mM L-GluMax, 1 mM MEM NEAA, 0.1 mM 2-ME, 24 ng/ml hLIF (Chemicon), and 10 ng/ml bFGF or
2. 80% KO-DMEM, 20% Serum replacement, 2 mM L-GluMax, 1 mM MEM NEAA, 0.1 mM 2-ME, and 10 ng/ml bFGF.

3.2.3. Derivation of hESCs (Fig. 1d, h)

1. Eight to ten days later, cut each outgrowth grown from ICM into several cell clumps and replate them into the new MEF-containing culture wells.
2. After 7 days of culture, select hESC-like colonies and expand them continuously using the cutting method in the first three passages.
3. Then, both mechanical and enzymatic methods can be used to expand these new hESC lines (see Note 5).

3.2.4. Passaging of hESCs

Expansion of hESCs with the Mechanical Method (Fig. 1d, h)

1. Before passaging, check hESCs in culture and remove differentiated colonies and differentiated areas from colonies with cutting tool.
2. Aspirate old medium together with differentiated cells and then refeed hESCs with prewarmed hESC culture medium.
3. Cut hESC colonies into clumps containing 100–200 cells with cutting tool under the dissecting stereomicroscope.
4. Detach cell clumps by pipetting up and down with p1000 pipette.
5. Divide cell clumps equally into new culture dishes at 1:6 ratios (see Note 6).
6. After 5–6-day culture, hESCs are ready for further expansion, characterization, cryopreservation, and other experimental use.

Expansion of hESCs with the Enzymatic Method (Fig. 1e, f)

1. Same as step 1 of Subheading "Expansion of hESCs with the Mechanical Method."
2. Aspirate old medium together with differentiated cells and then wash cells in DPBS.
3. Add appropriate volume of collagenase IV (0.7 ml for each 35-mm culture dish) to dissociate cell colonies.
4. Incubate at 37°C for 20–30 min. When the edges of hESC colonies curl up, aspirate collagenase IV from the cells.
5. Wash the cells gently twice with DMEM/F12, and then aspirate it completely.
6. Add 2 ml prewarmed culture medium in each culture dish to dissociate hESC colonies into cell clumps using pipette P1000.
7. Transfer cell clumps to new culture dishes containing MEF feeder cells and KSR medium supplemented with 10 μM Y27632 at 1:6 ratios (see Note 7).
8. The second day after cell replating, replace Y27632-containing medium with fresh medium without it.
9. Observe the cells, remove the differentiated colonies and differentiated areas, and replace medium every day.
10. Repeat the above steps to subculture undifferentiated hESCs or for other experimental purposes.

The mechanical method can be applied to cutting newly established hESC colonies for passaging, as well as removing differentiated regions of hESC colonies. It has also proven to be a valuable technique for improving attachment of hESCs, as this method can generate uniform and large cell clumps in size. However, as this method has certain technical difficulty in technique and is time consuming for hESC passaging, it is a good choice to expand hESCs in bulk with the enzymatic method. Normally, hESC colonies are dissociated into cell clumps of different sizes with the enzymatic method, and small clumps (such as 2–10 cells) have poor attachment capacity. Addition of rock inhibitor, Y27632, can resolve this problem effectively, as Y27632 is safe and efficient at supporting hESC attachment and viability greatly (12–15).

3.3. Maintenance of Undifferentiated hESCs in Culture

3.3.1. Excision of Partially Differentiated Cells from hESC Colonies

hESCs have been cultured on feeder-dependent or feeder-free conditions. No matter what kind of culture system is used, the spontaneous differentiation of hESCs is unavoidable. When partial differentiation occurs, a cutting method can be used to eliminate differentiated regions of colonies. When expanding hESCs, a combined use of mechanical and enzymatic method helps to decrease the amount of differentiated cells in the hESC population. These same protocols also apply to human-induced pluripotent stem cells.

1. Check hESCs in culture every day before changing medium.
2. Excise the differentiated regions with cutting tool inside the margin of undifferentiation, some distance (such as ~50 μm)

away from the border between the differentiated cells and undifferentiated cells (see Note 8).

3. Detach the differentiated areas from the undifferentiated parts using a P200 pipette.
4. Aspirate the old medium together with the differentiated cells, and then refeed the cells with fresh medium.

3.3.2. Isolation of Undifferentiated hESCs from the Highly Differentiated Colonies (Fig. 2)

Various factors (expired factors and reagents, inappropriate handling, freezing/thawing, first several passages after derivation of new hESC lines, and gene transfection/selection) can cause extreme differentiation (more than 80%) in hESC colonies (Fig. 2B: a, d, g). Under these circumstances, it is inefficient to cut away the differentiated areas from the whole colonies, as the undifferentiated areas are too small to be cut out from the extremely differentiated colonies. To avoid the loss of valuable hESCs, we developed a novel method to isolate or purify undifferentiated hESCs from extremely differentiated colonies (16).

1. Excise the completely differentiated areas inside colonies using the cutting tool as much as possible (Fig. 2A: a). Aspirate these differentiated cells using P200 micropipette.
2. Cut the remaining parts containing undifferentiated hESCs into several clumps (Fig. 2A: b). If several more (>3) island-like, undifferentiated cell areas are scattered throughout a colony, directly cut the colony into several clumps.
3. Pick the cell clumps with a P200 micropipette and transfer them into a 1.5-ml sterile centrifuge tube containing 30–40 μl prewarmed collagenase IV solution or dispase solution (Fig. 2A: c) (see Note 9).
4. Dissociate cell clumps with dispase for 5–10 min or with collagenase IV for 15–30 min at 37°C.
5. Add 200 μl culture medium into the tube using P200 micropipette. Pipette cell clumps up and down several times. Add medium up to 1 ml (see Note 10).
6. Centrifuge at 250 × *g* for 1–2 min and then aspirate the supernatant as much as possible.
7. Resuspend with fresh medium. Replate cell pieces into feeder-containing or feeder-free Matrigel-coated culture wells of 4-well plates or 35-mm dishes in media with Y27632 (10 μM) (see Notes 11 and 12).
8. From the second day after seeding, refeed the cells with medium without Y27632 every day (see Note 13). Two to three days after seeding, some undifferentiated, semi-differentiated, and differentiated colonies appeared in culture dishes or culture wells (Fig. 2A: d; B: b, e, h).

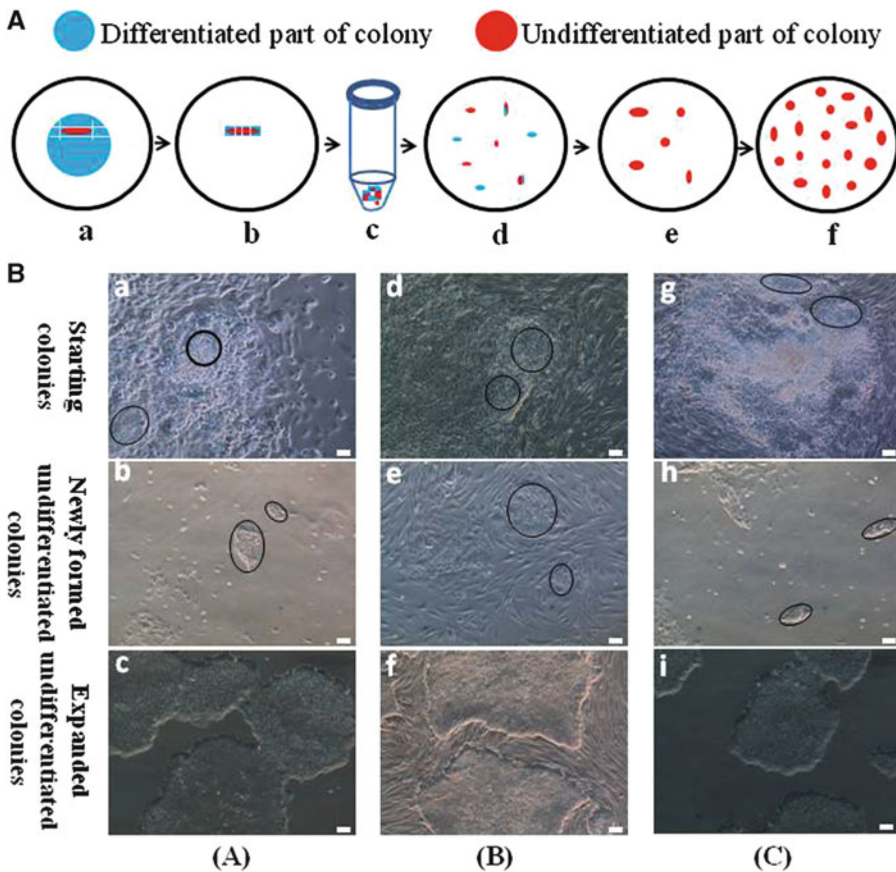

Fig. 2. Re-isolation of undifferentiated hESCs from extremely differentiated colonies. **A**. Flowchart for re-isolation of undifferentiated hESCs. (a) An extremely differentiated hESC colony appears in culture. (b) Excise differentiated area as much as possible, and cut the remaining parts into cell clumps. (c) Dissociate cell clumps into smaller cell clusters by digestion. (d) Newly formed undifferentiated, semi-differentiated, and differentiated colonies apart from each other. (e) After removing differentiated colonies and differentiated areas from semi-differentiated colonies, undifferentiated hESC colonies are ready for expansion. (f) Expand undifferentiated hESC colonies for passaging, cryopreservation, or characterization. **B**. hESC re-isolation using the same or alternative culture system. (A) Isolation of undifferentiated hESCs from extremely differentiated colonies in feeder-free culture: (a) An extremely differentiated hESC colony appears in culture. The area inside *black circle* mainly contains undifferentiated cells. (b) Newly formed undifferentiated colony is marked inside *black circle*. (c) Expanded undifferentiated colonies. (B) Isolation of undifferentiated hESCs from extremely differentiated colonies in feeder-dependent culture: (d) An extremely differentiated hESC colony appears in culture. The area inside *black cycle* mainly contains undifferentiated cells. (e) Newly formed undifferentiated colony is marked inside *black circles*. (f) Expanded undifferentiated colonies. (C) Isolation of undifferentiated hESCs in feeder-free culture from extremely differentiated colonies appearing in feeder-dependent culture: (g) An extremely differentiated hESC colony appears in feeder-dependent culture. The areas inside *black circles* mainly contain undifferentiated cells. (h) Newly formed undifferentiated hESC colonies in feeder-free culture are marked inside *black circles*. (i) Expanded undifferentiated hESC colonies in feeder-free culture.

9. Both in feeder-free and feeder-dependent conditions, 2–3 days after seeding, begin to remove the differentiated colonies and differentiated parts of the semi-differentiated colonies every day before refeeding or passaging (Fig. 2A: e) (see Note 14).

10. Expand undifferentiated hESC colonies (Fig. 2A: f; B: c, f, i). Aspirate old medium from culture wells or dishes. Wash cells in DPBS. Add appropriate volume of dispase or collagenase IV to dissociate cell colonies. Incubate at 37°C for about 10 min (feeder free) or 20 min (feeder dependent) expansion. When the edges of hESC colonies curl up, aspirate the digestion solution from the cells. Wash the cells gently twice with DMEM/F12, and then aspirate it completely. Add appropriate volume of prewarmed culture medium in culture wells or culture dishes. Use pipette P200 (for culture well) or pipette P1000 (for 35-mm culture dish) to dissociate undifferentiated colonies into cell clumps (containing 50–100 cells). Transfer cell clumps to new culture wells or culture dishes covered with Matrigel or feeder cells previously in mTeSR1 medium or KSR medium, respectively. Observe the cells, remove the differentiated colonies, and replace medium every day. Repeat the above steps to subculture undifferentiated hESCs.

3.4. Characterization of hESCs

Characterize the resulting hESC lines or sublines through PCR, karyotype analysis, hESC-specific marker expression, and in vitro and in vivo differentiation (see Note 15).

4. Notes

1. For thawing human zygotes, the two researchers are required to act in close coordination. Especially when thawing of many embryos at one time, it should be made sure that the operation is conducted smoothly without any neglect and confusion.
2. Culture dish surface cannot be used to isolate human ICM, as it is too soft and easy to produce superficial scratches that stick embryo quite strongly to the surface. This easily causes interference to isolation of ICM resulting in the loss of embryos.
3. This operation should be finished as fast as possible, as the vitality of the ICM will be lowered the longer it stays outside an incubator. At the same time, the trophoblasts should be excised from the ICM as much as possible.
4. Prepare MEF feeder cells as described previously (17, 18).
5. CC1 and CC3 were initially derived in FBS and KSR conditions, respectively. CC1 displayed the higher level of spontaneous differentiation in its early passages, although regions of spontaneous differentiation were effectively removed using colony microdissection. After five passages, the CC1 line was transferred to KSR-containing medium, where it continued to propagate with little differentiation. This suggested that the

unknown factors present in FBS induced the spontaneous differentiation of hESCs to a certain degree.

6. It is not necessary to spin down cells during the procedure of cell passaging with the mechanical method. Omitting centrifugation step helps to improve hESC viability.
7. It is not necessary to spin down cells during the procedure of cell passaging with the enzymatic method. Omitting centrifugation step helps to improve hESC viability.
8. If the differentiated regions are excised along the border between the differentiated cells and undifferentiated cells, the differentiated cells are not removed completely from undifferentiated areas. After replating, these differentiated cells can trigger the differentiation of undifferentiated hESCs.
9. According to experience, collagenase IV is suitable for dissociation of cell colonies grown on feeder cells, and dispase is suitable for dissociation of cell colonies grown on feeder-free conditions.
10. It is suitable to add KSR medium or mTeSR1 medium to hESC cells grown on feeder-dependent cells or feeder-free culture system, respectively.
11. We have explored the transfer of rescued colonies from feeder-dependent to feeder-free conditions and vice versa. In some cases, extreme differentiation occurring in one culture system can imply that the culture system is not optimal due to batch-to-batch variability of reagents. Here, the alternative culture system can be used to isolate undifferentiated hESC cells.
12. A suitable density of cell clumps seeded to culture dish or plate is crucial to this method. Based on our experience, seeding cell clumps from single colony or multicolonies (3–4) with extreme differentiation into one well of a 4-well plate (or of 24 well plate) or one 35-mm dish (or one well of 6-well plate), respectively, is practical. Keeping newly formed cell colonies at a distance is helpful to remove differentiated colonies and pick undifferentiated colonies for further expansion.
13. Rock inhibitor, Y27632, can greatly improve hESCs' attachment, but there is no significant effect on their proliferation. So addition of Y27632 is not necessary after cell attachment.
14. The P200 pipette and cutting tool are used for removing differentiated colonies or differentiated parts in colonies grown on feeder-free or feeder-dependent conditions, respectively.
15. References 19–22 describe the commonly used methods to characterize the pluripotency and differentiation potential of hESCs.

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Chapter 6

Establishment of hESC Lines from the Inner Cell Mass of Blastocyst-Stage Embryos and Single Blastomeres of 4-Cell Stage Embryos

Ileana Mateizel, Mieke Geens, Hilde Van de Velde, and Karen Sermon

Abstract

More than 600 human embryonic stem cell (hESC) lines have been reported today at the human European Embryonic Stem Cell Registry (http://www.hescreg.eu/). Despite these high numbers, there are currently no general protocols for derivation, culture, and characterization of hESC. Moreover, data on the culture of the embryo used for the derivation (medium, day of ICM isolation) are usually not available but can have an impact on the derivation rate. We present here the protocols for derivation, culture and characterization as we applied them for the 22 hESC lines (named VUB-hESC) in our laboratory.

Key words: Human ESC, Preimplantation genetic diagnosis, 4-cell stage embryos, Immunosurgery

1. Introduction

Assisted reproduction helps patients with infertility problems (in vitro fertilization; IVF) and/or couples at risk of transmitting genetic diseases (preimplantation genetic diagnosis; PGD) to achieve a healthy pregnancy.

For PGD, the embryos are diagnosed for the presence of a genetic defect prior to their transfer in utero. During this procedure, a blastomere is removed from the embryo and the genetic material is examined for abnormalities, while the rest of the embryo is maintained further in culture. If the test shows the absence of the genetic abnormality, the embryo is transferred to the patient or cryopreserved for later use. PGD is performed by fluorescent in situ hybridization for the diagnosis of aneuploidies, chromosomal aberrations, or sexing for the X-linked pathologies, and by PCR for the diagnosis of single-gene disorders (1).

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The embryos donated for research from infertile patients at different stages of development (blastocyst, morula, or single blastomeres) or from patients that carry a genetic disorder (blastocyst stage) can be used for the production of hESC lines, allowing the derivation of presumably genetically normal hESC lines (2–7) or, in the latter case, hESC lines carrying genetic abnormalities (8–10).

As a result of collaboration between the Vrije Universiteit Brussel and the Centres for Reproductive Medicine and Medical Genetics from the Universitair Ziekenhuis Brussel, 22 hESC lines were derived in the period from 2003 to 2008 (7, 9, 10). All human embryos used were donated for research after obtaining an informed consent from the couple and the approval of the ethical committee at the UZ-Brussel and of the Federal Commission for Medical and Scientific Research on Embryos in vitro that represents the competent authority in Belgium. All the donors are traceable and, as a part of IVF/PGD procedure, were screened for the presence of viral and/or microbiological contamination (10). The embryos from normal IVF cycles were donated for research after the best quality embryos were transferred or cryopreserved for the patient and consequently were not of the best morphological quality. By contrast, PGD embryos were usually of good quality and were donated for research because of the presence of a genetic abnormality. The genetic tests performed on embryos during PGD depend on the mutation(s) present in the parents. The biopsied blastomeres were analyzed by single cell PCR, according to the mutations for which they were tested (10).

Out of the 22 hESC derived, 20 hESC lines were isolated from blastocyst-stage embryos (6 from normal IVF and 14 from PGD embryos) and 2 lines from single blastomeres of two distinct 4-cell stage embryos. From the group of 14 hESC lines carrying mutations for monogenic diseases, seven lines were derived from embryos affected by trinucleotide repeat disorders: Myotonic dystrophy type 1 (DM1) (VUB03_DM1, VUB19_DM1, VUB24_DM1), Huntington's disease (VUB05_HD), Fragile-X syndrome (FXS) (VUB11_FXS, VUB13_FXS), and Spinocerebellar ataxia type 7 (SCA7) (VUB10_SCA7); one hESC line was derived from an embryo affected with both Huntington's disease and Marfan Syndrome (VUB28_HD_MFS); five other hESC lines were derived from embryos diagnosed as carrying Marfan syndrome (MFS) (VUB08_MFS), Fascioscapulohumeral muscular dystrophy (FSHD) (VUB09_FSHD), Charcot-Marie Tooth type 1A (CMT1A) (VUB20_CMT1A), Cystic Fibrosis (CF) (VUB04_CF, VUB22_CF), and Osteogenesis Imperfecta (OI) (VUB23_OI) (10).

The hESC derived from surplus embryos after IVF represent an important source of cells for regenerative medicine, pharmacology, and for the study of early development.

Human ESC lines obtained from PGD embryos, therefore carrying mutations for monogenic diseases, may have great potential in drug screening and in studying the pathology of the diseases, especially in those for which animal models are not fully representative or not available, or for which the culture of the relevant cell type is difficult (10, 11). Indeed, because hESC can differentiate into all different cell types of the body, they could be used as a constant source of cell types affected by the disease. Furthermore, hESC have been suggested as a unique model for early human development, therefore, hESC lines carrying specific mutations (e.g., triple nucleotide repeats) may prove useful for the understanding of the genetic mechanisms during the embryonic stage, and possibly gametogenesis. In a recent report, 56 lines are reported to carry genetic abnormalities (12) and considering that the number of PGD cycles for monogenic diseases is rising (13), their number is expected to increase.

The derivation of hESC from single blastomeres represents a method for isolating pluripotent stem cells without the need of destroying the embryo. This method may also have an important clinical value in cases where HLA-matched embryos are selected with the purpose of obtaining hematopoetic stem cells from the cord blood of the newborn. In cases where the cord blood cells proved to be insufficient, hESCs isolated from the same embryo may provide an alternative source of cells for therapy, by differentiating hESC into hematopoetic stem cells (7).

All the hESC lines derived at our centres, except VUB17, are reported in the European hESC registry (http://www.hescreg.eu) where their characterization and culture conditions are specified. All the lines are available upon request after signing a Material Transfer Agreement.

2. Materials

2.1. Biological Material

1. Mice Embryonic Fibroblasts mitomycin inactivated (Millipore), stored in Liquid Nitrogen tanks.
2. Human 4-cell stage embryos and day-6 blastocysts from IVF lab (CRG, UZBrussel).
3. Mice SCID-beige or NOD-SCID, 4 weeks old (Taconic, Denmark).

2.2. Tissue Culture Plastic and Glassware

Express-Plus vacuum-driven disposable filtration system and accessories, 0.22 μm (Millipore).

Serological pipettes 5 mL, 10 mL, polystiren, non-pyrogenic, individually wrapped (Becton Dickinson).

Kimble/Kontes glass pipettes.

Glass capillaries melting point determine tubes, 150 mm, both ends opened (Hirschmann Laborgerate).

Bunsen burner.

CryoTubes™ 1 mL, polypropylene tubes and screw cap (Nunc).

Conical centrifuge tubes, 15 mL (Becton Dickinson).

Conical centrifuge tubes, 50 mL (Becton Dickinson).

4-well dishes (Nunc).

Centre-well organ culture dish 1 centre well culture dish (Becton Dickinson).

Easy Grip™ tissue culture dishes 35 mm diameter, polystyrene, non-pyrogenic (Becton Dickinson).

Easy Grip™ tissue culture dishes 60 mm diameter, polystyrene, non-pyrogenic (Becton Dickinson).

Easy Grip™ Petri dishes 60 mm diameter, polystyrene, non-pyrogenic (Becton Dickinson).

Eppendorf tubes 1 mL.

Syringe with needle 1 mL, 26Gx1/2″ (Terumo).

Needles 30 G (Terumo).

2.3. Reagents

KnockOut™ DMEM, Optimized for ES Cells 1× (Gibco), stored at 2–8°C.

KnockOut™ SR, Serum Replacement for ESCs/iPSCs (Gibco), aliquots of 50 mL, stored at −20°C.

L-glutamine 200 mM 100× (Gibco), aliquots of 5 mL stored at −20°C.

MEM Non-essential Amino Acids, 100× (Gibco), stored at 2–8°C.

2-Mercaptoethanol (Sigma), stored at 2–8°C.

bFGF, human, recombinant, 10 μg (Invitrogen), alisuots stored at −20°C.

Penicillin-Streptomycin (Pen/Strep), 10,000 units/mL penicillin G sodium and 10,000 μg/mL streptomycin sulphate in 0.85 saline (Gibco), aliquots of 500 μL, stored at −20 °C.

DMEM Dulbecco's Modified Eagle Medium, 1× (Gibco), stored at 2–8°C.

Foetal Bovine Serum, Heat inactivated (Gibco), aliquots of 50 mL stored at −20°C.

Gelatine Type A from porcine Skin (Sigma), stored at room temperature.

Protease type XXIV: bacterial (Sigma), stored at −20°C.

Trypsine-EDTA 0.05% (Gibco), stored at 2–8°C.

Collagenase type IV, lyophilized from *Clostridium hystoliticum* (Gibco), stored at –20°C.

Complement Sera from guinea pig (Sigma), stored at –20°C.

Anti-human serum, antibody produced in goat (Sigma), stored at –20°C.

Phosphate buffer saline (PBS; Sigma), stored at room temperature.

Embryo culture media: EmbryoAssist and BlastAssist (Medicult), stored at 2–8°C.

mTeSR™1, basal medium for maintenance of hESC and iPSC, (Stem Cell Technologies), stored at 2–8°C.

mFreSR®, defined cryopreservation medium for hESCs and hiPSCs, stored at 2–8°C.

Oil for embryo culture (Vitrolife), stored at room temperature.

KaryoMAX® Colcemid® Solution 10 μg/mL (Gibco), stored at 2–8°C.

Methanol, stored at 2–8°C.

Glacial Acetic Acid, stored at room temperature.

KCl, stored at room temperature.

Primary antibodies: mouse anti SSEA4 (IgG), TRA-1-60 (IgM), TRA-1-81 (IgM) and rat SSEA3 (IgM), stored at 2–8°C.

Secondary antibodies: Fluorochrome-conjugated $F(ab)_2$ fragments of goat anti-mouse immunoglobulin IgM or IgG (Invitrogen), stored at 2–8°C.

RNeasy Mini kit (Qiagen), stored at room temperature.

First-Strand cDNA Synthesis Kit (GE Healthcare), stored at –20°C.

Taq polymerase (GE Healthcare), stored at –20°C.

Expand High Fidelity Taq polymerase (Roche Diagnostics), stored at –20°C.

Jumpstart Taq polymerase (Sigma-Aldrich), stored at –20°C.

2.4. Working Solutions and Media Preparation

1. Protease XXIV: Dissolve at 250 U/mL in KO-DMEM and filter; aliquots can be stored at –20°C.
2. Anti-Human Serum antibody; aliquots can be stored at –20°C.
3. Guinea Pig Complement: Reconstitute the lyophilized complement serum 1:10 (v/v) in cold hESC medium; aliquots can be stored at –20°C.
4. Gelatine, 0.1%: Add 0.1 g gelatine per 100 mL distilled water to a bottle and autoclave it (see Note 1).
5. Mouse embryonic fibroblasts (MEF) culture medium: 10% FCS, 2 mM L-glutamine, Non-essential Amino Acids. Filter through 0.22-μm Express-Plus filters.

For 500 mL medium: DMEM, 50 mL FCS, 5 mL L-glutamine, 5 mL Non-essential Amino Acids.

6. 2-Mercaptoethanol 0.5 mM working solution: 7 μL 2-Mercaptoethanol + 10 mL KnockOut™ DMEM.
7. Human Embryonic Stem Cells (hESC) culture medium: KnockOut™ DMEM, 20% KnockOut™ Serum Replacement, 2 mM L-glutamine, 1% Non-essential Amino Acids, 0.1 mM 2-Mercaptothanol (see Note 2), 4 ng/mL bFGF (see Note 3), Pen/Strep (1 μL/mL). Filter through Express-Plus 0.22 μm filters.

   For 500 mL hESC medium: KnockOut™ DMEM, 100 mL KnockOut™ Serum Replacement, 5 mL L-glutamine, 5 mL Non-essential Amino Acids, 5 mL of working solution 2-Mercaptothanol (0.5 mM), 200 bFGF, 500 μL Pen/Strep.
8. Human Embryoid Body culture medium: KnockOut™ DMEM, 20% 20% FBS, 2 mM L-glutamine, 1% Non-essential Amino Acids, 0.1 mM 2-Mercaptothanol. Filter through Express-Plus 0.22-μm filters.

   For 500 mL hESC medium: KnockOut™ DMEM, 100 mL FBS, 5 mL L-glutamine, 5 mL Non-essential Amino Acids, 5 mL of working solution 2-Mercaptothanol (0.5 mM).
9. Collagenase stock solution: 50 mg/mL in KODMEM. Working solution: 1 mg/mL in KO-DMEM, kept at −20°C.
10. KCL, 0.075 M: dissolve KCL at 5.6 g /1 L water, kept at room temperature.
11. 0.1% Triton-X: 1 μL Triton X-100 in 10 mL PBS. Homogenize.

3. Methods

3.1. Preparation of Feeder Layer Dishes

In our laboratory we are using mitomycin-inactivated mouse embryonic fibroblasts (MEF) as feeder layers (FL). Initially, we were producing our own fibroblasts from mice of the CF1 strain (see Note 4). However, this is a long and labour-intensive process requiring an animal house. Therefore, we are now using commercially available mitomycin-inactivated MEFs (Millipore).

1. Cover the surface of the culture dishes with 0.1% gelatine for at least 1 h at room temperature in the flow hood (see Note 5).
2. Take out a vial of inactivated MEFs from the liquid nitrogen storage and thaw it in the water bath at 37°C by gentle swirling.
3. When the cell suspension is almost thawed, wipe the cryovial with 70% alcohol and transfer the cell suspension drop by drop into a 15-mL centrifuge tube containing 10 mL MEF medium (see Note 6).

4. Centrifuge the cell suspension at $180 \times g$ for 5 min.
5. Meanwhile, remove the excess gelatine solution from the culture dishes and add MEF medium to each dish (see Note 7).
6. After centrifugation of the MEF suspension, discard the supernatant and resuspend the cell pellet in MEF medium. Add as much medium as to reach the desired concentration of cells/mL and distribute the cell suspension over the gelatine-coated dishes in order to reach a final concentration of $1.5–2 \times 10^4$ cells/cm^2 (see Note 8).
7. Gently shake each dish well, so the cells are homogenously distributed over the culture dish surface (see Note 9).
8. Note the day of plating on the cover of the dish.
9. Place the dishes in an incubator in 5% CO_2 at 37°C overnight.
10. The next day, check the dishes to evaluate the concentration and the distribution of MEFs and possible microbiological contamination (see Subheading 3.4; see Note 10).
11. MEF medium is refreshed every 2 days (see Note 11).

3.2. Derivation of hESC from Blastocyst-Stage Embryos

At day 6 of development, the human embryo (blastocyst stage) consists of an outer layer (trophectoderm) that after implantation will form the placenta, an inner group of cells called inner cell mass (ICM) that will form the embryo proper and a fluid-filled cavity (blastocoel). The zona pellucida (ZP) surrounds the trophectoderm layer and protects the embryo throughout its preimplantation development. To be able to implant, the embryo should extrude from the zona pellucida, a process called hatching. Human ESCs are derived from the ICM of the blastocyst-stage embryo. To be able to isolate the ICM, first the ZP and then the trophectoderm cells should be removed.

We will describe the two methods applied in our laboratory to isolate ICM from blastocyst-stage embryos, either from normal IVF or from PGD embryos. The embryos are cultured in 25 μL sequential Medicult media M1 (EmbryoAssist) and M2 (BlastAssist) under embryo culture oil at 37°C in 6%CO_2, 5%O_2, and 89%N_2.

3.2.1. Isolation of ICM Using Immunosurgery

Immunosurgery is based on a nonspecific immunological reaction leading to the lysis of the trophectoderm cells (14).

Three steps are necessary. In the first step the ZP is dissolved using a protease solution. In the second step rabbit anti-human whole serum antibody will bind to trophectoderm cells, and in the third step the trophectoderm cells to which the antibody attached will be lysed after incubation with guinea pig complement (GPC).

Prepare three 60-mm diameter dishes: one for protease treatment, one for antibody and one for GPC incubation. Each dish contains three drops of 25 μL each with hESC medium and three drops of 25 μL with either protease, antibody or GPC solution

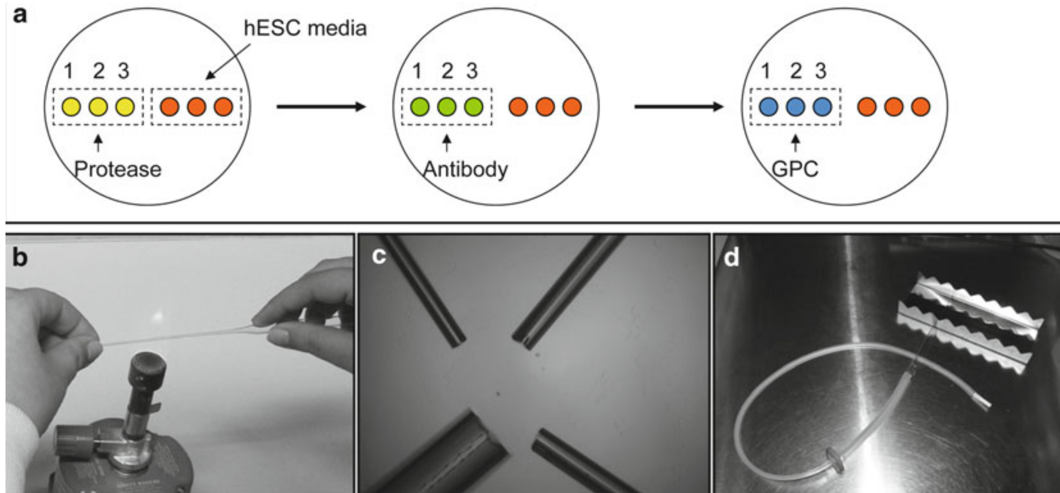

Fig. 1. Isolation of ICM from blastocyst-stage embryos. (**a**) Three dishes prepared with hESC medium and protease, antibody and GPC solutions, respectively. (**b–d**) Preparation of the glass pipettes for their use during isolation of ICM: (**b**) Keep the dish in the flame while rotating the pipette and pull out when start to melt, (**c**) pipettes with different diameters (**d**) Mouth aspiration system provided with a 0.22-μm filter.

(Fig. 1a) (see Notes 12 and 13). Place the dishes in the incubator at 37°C in 5%CO_2 for equilibration of the solutions.

Prepare the pipettes for embryo manipulation:

1. Glass Kimble pipettes and/or glass capillaries (see Note 14) may be used for immunosurgery and further for passaging of hESC.
2. Take the pipette and turn on a Bunsen burner (low flame) (Fig. 1b).
3. Keep the pipette/capillary from both ends and start heating the middle of the pipette while keeping the pipette rolling in the flame.
4. When the glass is almost melted, pull the pipette from both ends until it breaks and two smaller pipettes are obtained. The longer the pipette is kept in the flame, the smaller the diameter. Try to prepare more pipettes, with different diameters (Fig. 1c).
5. Using a diamond pen cut the end part (that was in the flame) in order to get clear sharp edges (see Note 15).
6. Place the fine-drawn pipettes in a holder in the flow until use.
7. The pipettes can be used with a mouth pipette system (Fig. 1d) provided with a 0.22-μm filter or, if available, a pipette holder used in IVF practice.

To proceed with immunosurgery, follow the steps below:

1. Replace the MEF medium from a dish with FL with hESC medium.

2. Take the protease dish out of incubator and bring it in the flow on the heating stage of the stereo microscope (see Note 16).
3. Fill the tip of the pipette with the biggest diameter with protease solution (see Note 17) from drop 1 and transfer the blastocyst in the second drop with protease.
4. Empty the pipette, aspirate again protease solution from the next drop and transfer the blastocyst to the third drop with protease.
5. Keep monitoring the ZP in the stereomicroscope. The ZP should get thinner and thinner until it dissolves completely. Normally the process takes up to 1 min (Fig. 2a, b; see Note 18).
6. To wash off the protease, fill the tip of the pipette with hESC medium and transfer the embryo to the three consecutive droplets of hESC.
7. Take the dish with antibody solution out of the incubator.

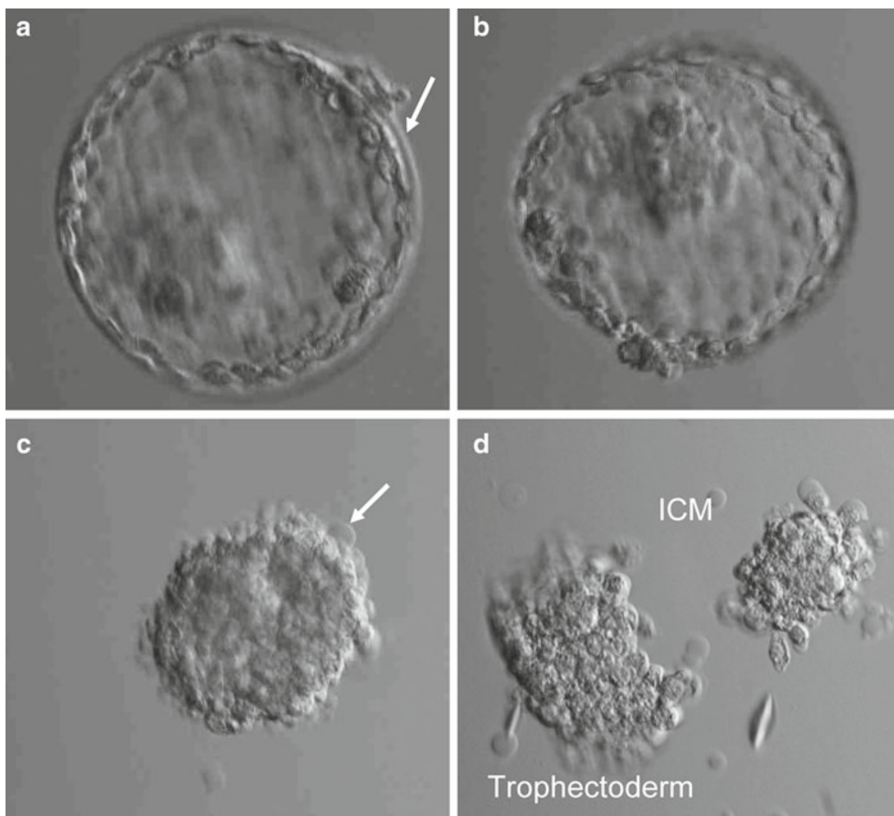

Fig. 2. Immunosurgery procedure. (**a**) Expanded day 6 blastocyst; the arrow points out the thin zona pellucida. (**b**) Expanded blastocyst after protease treatment. The zona pellucida is dissolved and no longer visible. (**c**) Collapsed blastocyst after complement reaction; the arrow points out the lysing trophectoderm cells. (**d**) Trophectoderm and ICM cells separated after manipulation of the collapsed embryo.

8. Fill in the tip of the pipette with antibody solution from the first drop and transfer the embryo into the second drop with antibody.
9. Empty the pipette, aspirate again antibody solution from the next drop and transfer the blastocyst to the third drop with antibody solution.
10. Incubate for 30 min at 37°C in 5%CO_2.
11. To wash off the antibody solution, proceed as described above in step 6.
12. Take the dish with GPC solution out of the incubator.
13. Fill in the tip of the pipette with GPC solution from the first drop and transfer the embryo into the second drop with GPC.
14. Empty the pipette, aspirate again GPC solution from the next drop and transfer the blastocyst to the third drop with GPC solution.
15. Place the dish back into the incubator.
16. After 15 min check under the microscope if the immunological reaction took place. When trophectoderm cells start to swell which is a sign of the beginning of cell lysis (Fig. 2c), monitor the cells continuously under the stereo microscope.
17. When most of the cells are swollen, take a new pipette with an opening slightly bigger than the ICM and vigorously but carefully aspirate the embryo in and out so that the ICM will separate from the rest of the trophectoderm (Fig. 2d; see Note 19).
18. Transfer the ICM to a pre-prepared MEF-coated culture dish containing hESC medium and place the dish in the incubator at 5%CO_2, 37°C. To allow the ICM to attach to FL, do not change the medium the next day. From the second day after plating, refresh the medium each day.

3.2.2. Manual Isolation of ICM

This procedure is performed on hatched blastocysts (spontaneously hatched or protease treated), preferably a collapsed one.

1. Transfer the blastocyst to a pre-prepared MEF-coated culture dish containing hESC medium.
2. Fix the embryo (see Note 20) to the dish with the help of a syringe with a 30 G needle. With the other hand, using a pipette with sharp edge prepared as described in Subheading 3.2.1, try to cut away as much as possible of the trophectoderm (see Note 21).
3. Let the piece that contains the ICM on the dish and remove the trophectoderm pieces.

3.2.3. Derivation of hESC from ICM of Blastocyst-Stage Embryos

1. Monitor the plated ICMs daily for their attachment and growth using a phase contrast microscope (see Note 22).
2. After several days, few cells with morphological characteristics of undifferentiated hESC (small cells with few cytoplasm, big nuclei with prominent nucleoli) will appear in the culture dish and eventually expand in the following days forming a small colony (Fig. 3a–c) (see Note 23). When the colony reaches a certain size (Fig. 3d) the cells should be passaged (see Note 24).
3. Using a pulled pipette with sharp edge, slice the undifferentiated part of the colony in small squares (around 400 cells) by parallel cuts, (see Note 25) but not too close to the border of the colony (see Note 26).
4. Aspirate the small clumps and plate them on pre-prepared FL with hESC medium.

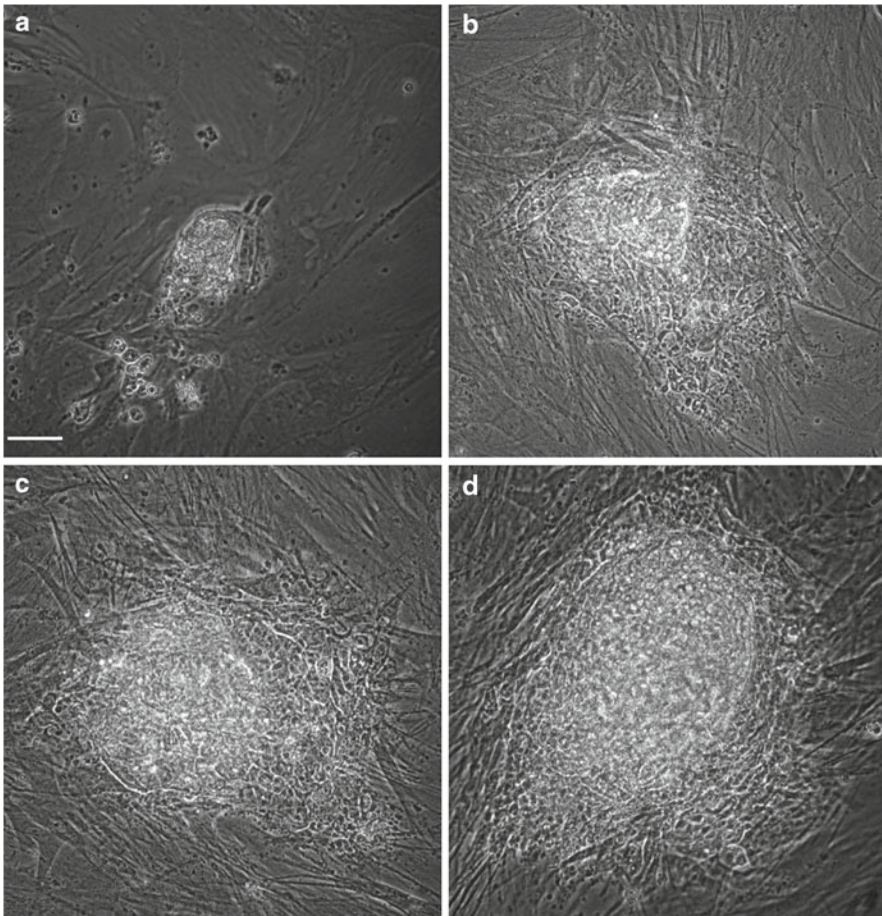

Fig. 3. Derivation of hESC lines. Phase Contrast images of an outgrowth (**a**) 2 days, (**b**) 11 days, (**c**) 12 days and (**d**) 13 days after plating ICM on MEFs. At day 13, a clear central compact group of cells was evident. This was sliced in small pieces with a sharp glass pipette and the resulting pieces plated on freshly prepared feeder layers. Original magnification ×200; Scale bar: 100 μm.

5. Place the dish back into the incubator at 37°C in 5%CO_2.
6. Next day, evaluate the dish and refresh the hESC medium (see Subheading 3.4).

3.3. Derivation of hESC from Single Blastomeres of 4-Cell Stage Embryo

In our attempt to characterize the plasticity/totipotency state of the 4-cell stage human embryos (7), single blastomeres from 4-cellstage embryos were used for the derivation of hESC lines as described below:

1. 4-cell stage embryos on day 2 after oocyte retrieval should be considered for this procedure (Fig. 4a; see Note 27).
2. Perform the biopsy (removal of individual blastomeres from the 4-cell stage embryo) using micromanipulators as described (15) but use a biopsy pipette with an inner diameter of 80 μm (Fig. 4b, c; see Note 28).
3. Place the biopsied blastomere (Fig. 4d) in a 25 μL drop of sequential embryo culture medium (EmbryoAssist) under oil on easy grip culture dishes as used in the IVF programme (see Note 29). The blastomeres are further cultured at 37°C in 6% CO_2, 5% O_2 and 89% N_2 for few days and are evaluated daily using an inverted microscope with Hoffman modulation optics.
4. The following day, transfer the blastomere-derived embryo (Fig. 4e) to another dish with 25-μL droplets of blastocyst culture medium (BlastAssist) under oil.

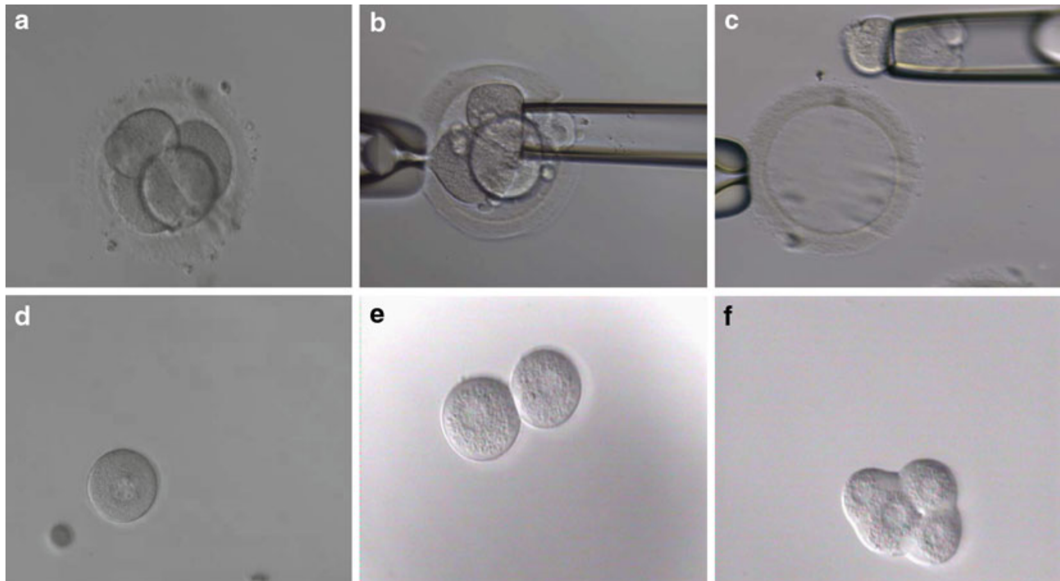

Fig. 4. Isolation and culture of single blastomeres. From a 4-cell stage embryo (**a**) all the blastomeres are removed using a micromanipulator. While the embryo is held in place using a holding pipette (**b**), each blastomere is biopsied (**b**, **c**). The blastomeres (**d**) are further cultured in individual drops for 2 more days until they reach a compaction stage ((**e**): day 1 after biopsy; (**f**): day 2 after biopsy).

5. When the blastomere-derived embryos reach a compaction stage (Fig. 4f; see Note 30), fill the tip of the pipette with hESC medium, and transfer the embryo to a non-coated culture dish containing hESC medium for washing.
6. Take a new pipette, fill the tip with hESC medium, and transfer the embryo to a pre-prepared MEF-coated culture dish containing hESC medium.
7. Place the culture dish in an incubator at 5% CO_2, 37°C.
8. Do not change the culture medium in the dish the first day after plating in order to allow attachment of the cell aggregate to the MEFs (see Note 31).
9. Starting from day 2 after plating, the medium can be refreshed daily (see Note 32).
10. If an outgrowth of cells with undifferentiated hESC morphology appears, passage it manually and culture further as described in Subheading 3.2.3 (see Note 33).

3.4. Maintenance of hESC

Culture of hESC is a time-consuming and labour-intensive process. At present, all the lines in our laboratory are routinely cultured on MEF feeder layers and passaged manually, using the protocols we present here.

Each morning, hESC cultures are evaluated using a phase contrast microscope. The size of the colonies and the presence of spontaneous differentiation are assessed and a decision is reached whether or not the cells need to be passaged. Undifferentiated hESC colonies have clear borders and consist of many tightly packed cells undistinguishable when viewed at small magnification (Fig. 5a). At high magnification the cells show a bright border and visible nucleoli (Fig. 5b, c). Only undifferentiated cells are subject to passaging by manually cutting each colony (Fig. 5d) as described in Subheading 3.2.3. Differentiation may be present in the middle or at the periphery of the colony (Fig. 5e, f).

Generally, in our laboratory the hESC are passaged every 5 days. After the evaluation, we refresh the hESC medium in all the culture dishes with hESC, even the ones considered for passaging. For 60-mm diameter dishes with hESC cultures, 5 mL hESC medium is used.

If during the daily evaluation signs of bacterial infection are observed in the culture dish (FL alone or hESC cultures) the dish is discarded. If the presence of bacterial infection is doubtful, a sample from the medium is sent for analysis to the microbiology department of the hospital and the dish, after the hESC medium was refreshed, is placed in a quarantine incubator.

Preparation of the feeder layer dishes, refreshing the culture media and all the manipulations of the cells are performed in a vertical laminar flow. Passaging of the cells is performed under a stereo microscope located in a horizontal laminar flow.

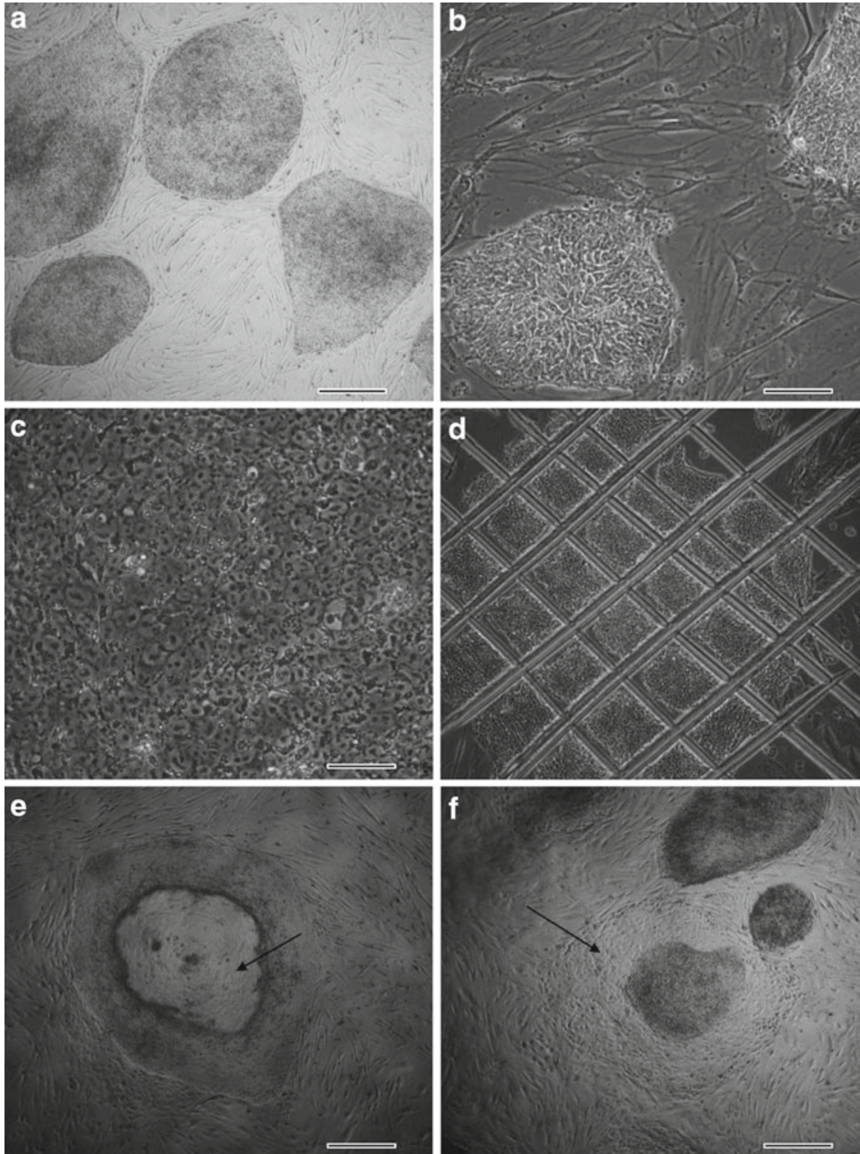

Fig. 5. Maintenance of hESC. Phase contrast image of hESC colonies on MEF at different magnifications (**a**: ×40, **b**: ×200, **c**: ×400). HESC colony passaged by manual cutting using a glass pipette with a sharp edge (**d**). Images of hESC differentiated in the centre (**e**, ×40) and at the periphery (**f**, ×40) of the colonies. Scale bars: **a**, **e**, **f**: (200 μm); **b** (100 μm), **c** (50 μm).

1. For the passage of hESC, FL dishes should be prepared in advance. Take the FL dishes you intend to use from the incubator. They contain MEF medium, so aspirate the MEF medium and add 4 mL hESC medium (4 mL for a 60-mm dish). Place the dishes back in the incubator until the hESC colonies will be manually cut.
2. Take the hESC culture dish intended for passaging out of the incubator and place it on the warm plate under the microscope into the flow.

3. Using a pulled pipette, prepared as described above, cut only the undifferentiated part of the hESC colonies into small squares (not more than 400 cells) leaving the differentiated ones untouched (see Note 34). Most of the small squares will still be attached to the dish but some may already float in the medium.
4. After all the undifferentiated colonies are cut, detach the cells that are still attached by gently blowing hESC medium next to it.
5. Swirl the dish to gather all the sliced pieces (cell clusters) in the middle of the dish.
6. Take the fresh feeder layer dish out of the incubator and bring it into the flow.
7. Collect all cell clusters in a 5-mL pipette and split them into more FL dishes (usually the splitting ratio in our laboratory is 1:3; see Note 35). Add 1 mL of cell cluster suspension per new FL dish so the final volume will be 5 mL hESC medium.
8. Write on the new dish: the cell line ID, passage number, and the date of passaging.
9. Be sure to gently shake the dish before you put it back into the 5 % CO_2 incubator, so all cell clusters are evenly distributed over the feeder layer surface.
10. Evaluate the hESC culture each day as mentioned in the introduction of this subchapter.

3.5. Characterization of hESC

Once hESC are derived and cultured for a few passages, characterization of hESC should be performed. Most commonly, tests are performed to determine: the presence of specific cell surface markers, the expression of a set of pluripotency genes, the ability for in vitro and in vivo pluripotency, and the karyotype.

In addition, each affected line derived from PGD embryos should be tested to confirm the presence of the mutation. The genetic test of the lines depends on the mutation present in the parents. This makes it difficult for us to present a generalized protocol for the genetic testing of the hESC derived from PGD embryos.

In our laboratory, in the case of some lines the same PCR protocols used during PGD procedure were used for the genetic testing of the lines to confirm the presence of the respective mutations. In other cases, direct testing of the mutation was used (9, 10). Detailed protocols for each PCR procedure in case of our hESC lines is not of general interest and therefore not described in this chapter, but they are available upon request.

3.5.1. Cell Surface Markers (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81)

To check the expression of cell surface markers of undifferentiated hESC, cells are cultured on inactivated MEFs in 4-well plates for 3–4 days.

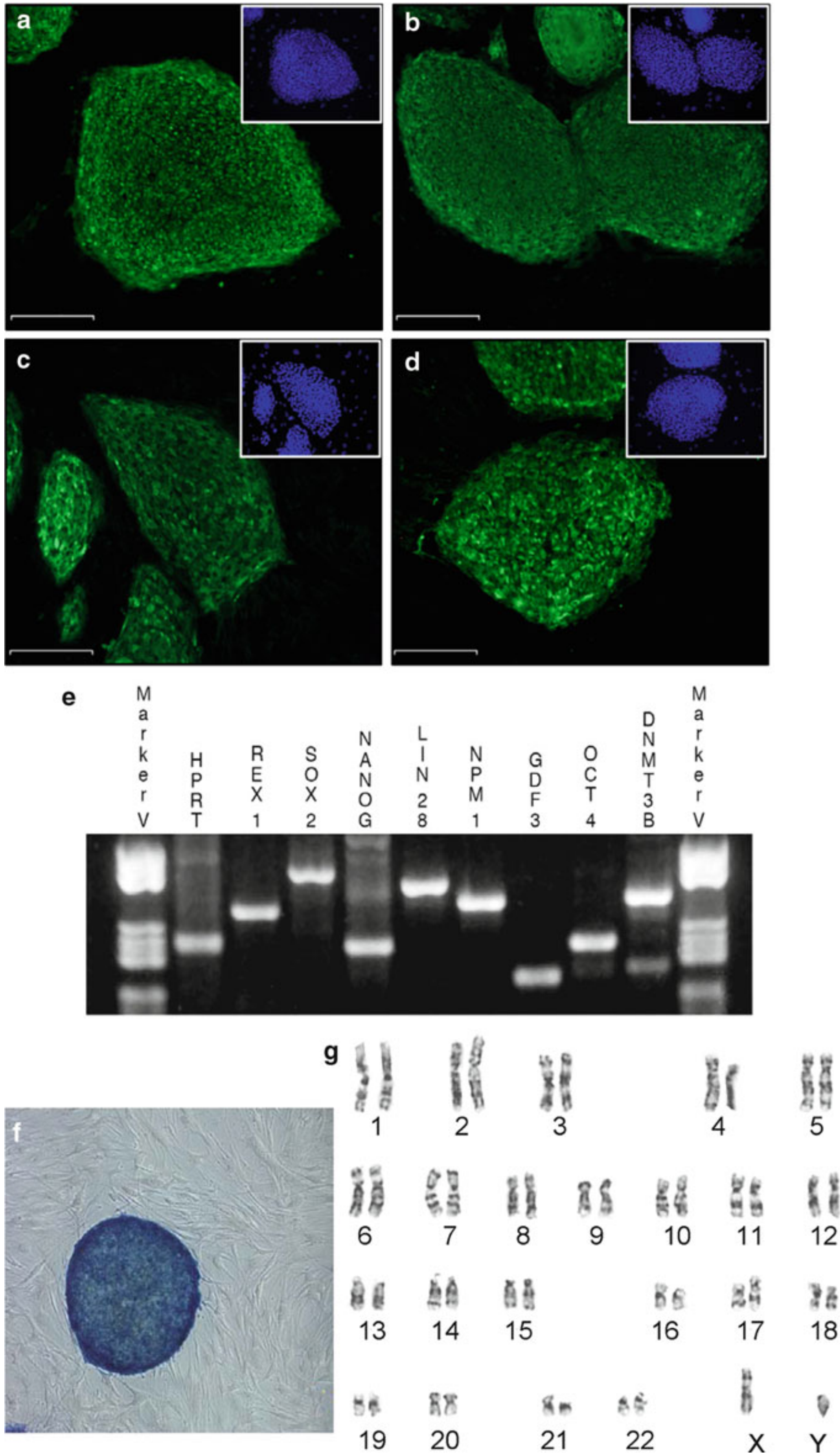

Fig. 6. Characterization of hESC. (**a**–**d**): Immunostaining (*green*) for SSEA3 (**a**), SSEA4 (**b**), TRA 1_60, (**c**) and TRA 1-81 (**d**). Images of DAPI staining (*blue*) in each cassette. (**e**): RT-PCR analysis of *HPRT, REX1, SOX2, NANOG, LIN28, GDF3, OCT4*, and *DNMT3B* in undifferentiated hESC. (**f**): Expression of alkaline phosphatase activity. (**g**): Normal 46XY G-banding karyotype from hESC. Scale bar 100 μm.

1. Aspirate the hESC medium and rinse with PBS.
2. Add 4% neutral phosphate buffered formalin for 10 min at room temperature to fix the cells.
3. Wash three times with PBS.
4. Add PBS/0.1% Triton-X for 20 min at room temperature to permeabilize the cells.
5. Wash three times with PBS (see Note 36).
6. Add primary antibody previously diluted in PBS at the concentration recommended by the manufacturer (see Note 37).
7. Incubate 2 h at room temperature or overnight at 4°C.
8. Wash three times with PBS.
9. Add a labelled secondary antibody diluted at the concentration recommended by the manufacturer (see Note 38).
10. Incubate 1–2 h at room temperature in the dark.
11. Wash three times with PBS.
12. Add one to two drops of mounting medium with DAPI (Vectashield) to identify the nuclei.
13. If not used immediately for the analysis, cover with aluminium foil and store at 4°C.
14. Check staining with the confocal fluorescent microscope (Fig. 6a–d).

3.5.2. Gene Expression

To study the expression of pluripotency markers in our hESC lines, we use Reverse Transcription-PCR (RT-PCR).

1. Extraction of total RNA is performed using a commercially available RNeasy Mini kit.
2. The cDNA synthesis is carried out with *Not*I-d(T)18 primers using the First-Strand cDNA Synthesis Kit, according to the manufacturer's protocol.

For the PCR reactions, the following primer sets are used:

Gene	Forward primer	Reverse primer	Fragment size (bp)
NANOG	CAGAAGGCCTCA-GCACCTAC	CTGTTCCAGG-CCTGATTGTT	216
POU5F1	GACAACAATGAGA-ACCTTCAGGAGA	TTCTGGCGCCG-GTTACAGAACCA	218
HPRT	GCCGGCTCCG-TTATGGCG	AGCCCCCCTT-GAG-CACACAGA	226
REX-1	GCGTACGCAAAT-TAAAGTCCAGA	CAGCATCCTAAA-CAGCTCGCAGAAT	306
SOX-2	CCCCCGGCGG-CAATAGCA	TCGGCGCCGGGG-AGATACAT	448

(continued)

Gene	Forward primer	Reverse primer	Fragment size (bp)
LIN 28	AGTAAGCTGCA-CATGGAAGG	ATTGTGGCTCAA-TTCTGTGC	420
NPM1	TGGTGCAAAG-GATGAGTTGC	GTCATCATCTT-CATCAGCAGC	343
GDF3	AGACTTATGCTAC-GTAAAGGAGCT	CTTTGATGGCA-GACAGGTTAAAGT	150
DNMT3B	TTGTAGCCATG-AAGGTTGGC	TGTGTAGTGC-ACAGGAAAGC	351

1. Prepare the following PCR reaction mix on ice: reaction buffer (as indicated by the supplier), 10 pmol of each primer/reaction, 0.2 mmol dNTP /reaction, 2.5 U Taq polymerase/reaction and H_2O to adjust the final volume to 23 μL/reaction (see Note 39 and 40).
2. Divide 23 μL of PCR reaction mix over the appropriate amount of PCR tubes.
3. Add 2 μL of cDNA to each reaction tube.
4. Place the tubes in a thermocycler and apply the following program: 95°C, 5 min; 35× (94°C, 30 s; Annealing Temperature, 30 s; 72°C, 45 s); 72°C, 5 min; 4°C, ∞.
5. Load the samples onto a 2% agarose gel for visualization and analysis of the results (Fig. 6e; see Note 41).

3.5.3. Alkaline Phosphatase

To detect alkaline phosphatase activity we use the Vector® Blue Alkaline Phosphatase Substrate kit III and the protocol instructed by the manufacturer. In brief:

1. Aspirate hESC medium from the dish (see Note 42).
2. Add 3 mL of freshly prepared substrate working solution provided by the supplier.
3. Place the dish in the dark at room temperature.
4. Monitor the enzymatic reaction each 30 min by visualizing the staining under a bright field optic microscope (Fig. 7f).

3.5.4. Karyotype

Cytogenetic evaluation is an important element in the quality control of hESC lines. In our laboratory we have used karyotyping by G-banding and, more recently, array comparative genomic hybridization (aCGH). G-banding karyotyping is performed on metaphases and is used to study large chromosomal changes such as the gain or loss of a large region of a chromosome at the single-cell level. Array CGH is a more sensitive technique that allows the identification of small chromosomal abnormalities that cannot be

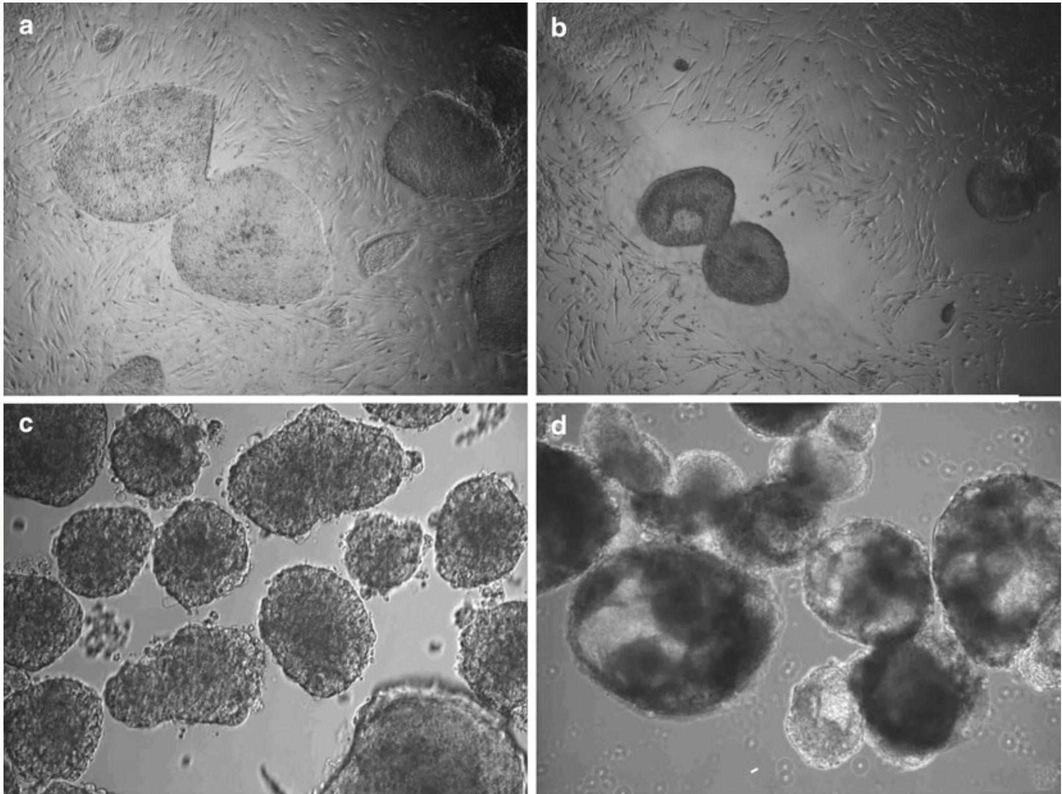

Fig. 7. Formation of EBs: hESC colonies on MEF before (**a**) and after (**b**) collagenase treatment. Two days (**c**) and 12 days old (**d**) EBs.

detected by G-banding. Because, for aCGH, the test is performed on DNA from pooled cells, the method does not allow the detection of low levels of mosaicism in a cell culture.

Array CGH requires know-how, special apparatus and is relatively expensive. Therefore, we will describe the protocol for G-banding karyotype of hESC as this is less technically demanding and is a worldwide approach for the characterization of hESC.

1. One 60-mm diameter culture dish containing a high number of colonies should be used. In order to increase the number of cells in metaphase, the cells should be harvested during active cell division. We are using the cells on day 3–4 after passaging.
2. Aspirate the hESC medium.
3. Add 5 mL hESC medium containing 0.1 μg/mL colcemid in order to block the cell division in the metaphase (mitotic arrest).
4. After 4 h of incubation at 37°C in 5%CO_2 (see Note 43), collect the entire medium in a 15-mL tube and centrifuge at $115\times g$ for 10 min.

5. During centrifugation, add 2 mL of cell dissociation solution to the culture dish with hESC. Regularly monitor the cell detachment under the microscope. After approximately 10 min at 37°C the hESC should detach from the dish and by gentle pipetting a single-cell suspension can be obtained (see Note 44).
6. Add 2 mL of hESC medium and centrifuge for 5 min at 115 × *g*.
7. Discard the supernatant, add 0.5 mL of PBS and add the entire cell suspension to the pellet obtained at step 4 by mixing gently to homogenize the suspension.
8. Add 5 mL of pre-warmed solution of 0.075 M KCl in distillated water to the cell suspension from step 7. The KCl solution should be carefully added; drop wise and with gentle agitation.
9. Incubate the cell suspension for 5 min at 37°C (see Note 45) and centrifuge at 115 × *g* for 8 min.
10. Discard all but 0.5 mL of the supernatant and resuspend the cells.
11. Add 4 mL freshly prepared fixative (3:1 v/v ratio of methanol to acetic acid (see Note 46) drop wise while flicking the tube in between drops to prevent cell clumping). The last 2 mL may be added faster.
12. Centrifuge at 115 × *g* for 8 min.
13. Discard all but 0.5 mL supernatant and resuspend the cells by tapping the bottom of the tube.
14. Repeat steps 11–13 twice.
15. Resuspend the pellet in 4 mL fixative and close it properly with parafilm to avoid evaporation. The tube (on which the name of the line, passage number and date are mentioned) can be kept at −20°C for at least 1 year paying attention not to allow evaporation of the fixative.

In our routine practice the tubes with fixed hESC are sent to the cytogenetic laboratory where trained personnel will prepare high-quality metaphase spreads and will further stain the chromosomes by classical G-banding for karyotype analyses (Fig. 6g) Generally, 20 metaphases are read per sample.

3.5.5. Pluripotency

Pluripotency is one of the defining features of hESC and is currently validated in vitro by formation of three-dimensional multicellular structures formed by non-adherent cultures of differentiating ES cells (embryoid bodies, EBs) and in vivo when a teratoma is obtained after injection of undifferentiated hESC into immunocompromised mice. The validation is accomplished by scoring the presence/absence of ectoderm, endoderm and mesoderm in EBs and in teratomas.

In Vitro Differentiation (EB Formation)

1. Aspirate the hESC medium from the culture dish and wash with PBS.
2. Add 2 mL collagenase (1 mg/mL) and place the dish back in the incubator at 37°C, 5% CO_2 (see Note 47).
3. After 10–15 min check the hESC colonies under the microscope: the border of the colonies should start to detach from the feeder layers while the rest of the colony remains attached to the dish (Fig. 7a, b). If not, return the dish to the incubator and check it again after 5 min (see Note 48).
4. Try to detach the colonies by gently blowing medium next to the colonies.
5. When all the colonies are floating in the culture media, aspirate the cell suspension and transfer it into a 15-mL tube containing 12 mL EB medium.
6. Allow the colonies to sediment for 10 min and aspirate the supernatant, leaving as little medium as possible.
7. Using a 2-mL serological pipette, add 2 mL of EB medium and pipette up and down until the colonies are dissociated in smaller pieces.
8. Add extra 10 mL of EB medium, gently homogenize the suspension, and centrifuge at $115 \times g$ for 3 min.
9. Aspirate the supernatant, resuspend the pellet in 6 mL EB medium and transfer the cell suspension into a 60-mm diameter Petri dish. These dishes do not allow the resulted clumps of cells to attach. As they remain in suspension, they tend to fold and form 3-D structures called EBs that initially have a compact and, days later, more cystic appearance (Fig. 7c, d; see Note 49).
10. Place the dish into the incubator at 37°C, 5% CO_2 overnight.
11. The next day replace the old medium with fresh EB medium.
12. To change the medium, transfer EBs into a 15-mL tube and let them sediment for 5 min.
13. Aspirate the supernatant leaving 0.5 μL and add 6 mL fresh EB medium.
14. Transfer the cell suspension to low attachment bacterial Petri dishes and place the dish into the incubator.
15. Change medium every 2 days (see Note 50).

Often, after approximately 7–10 days, EBs containing spontaneous beating cardiomyocytes can be observed in the culture dish.

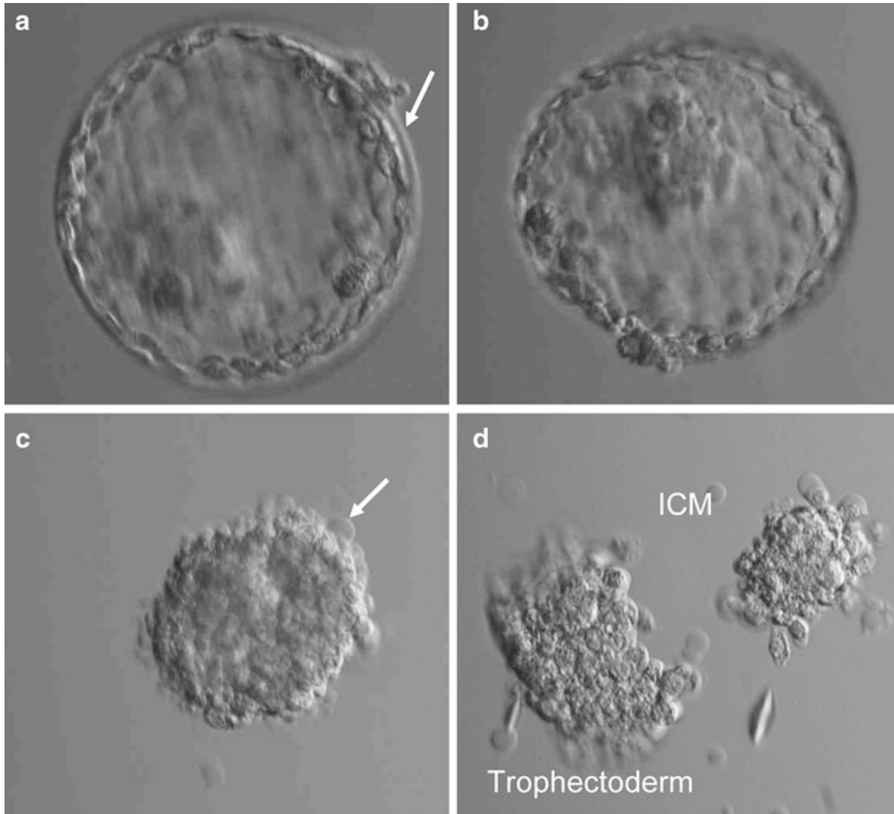

Fig. 8. In vitro and in vivo differentiation of hESC. (**a**–**c**) Immunostaining of EBs performed with markers specific for endoderm (alpha-fetoprotein, **a**), mesoderm (myosin heavy chain, **b**) and ectoderm (tubulin beta III, **c**). Original magnification ×200. (**d**–**f**) Hematoxylin-eosin staining on teratomas showing differentiation into endoderm (**d**), mesoderm (**e**), and ectoderm (**f**).

In our laboratory EBs are analyzed at days 10 and 15: using a 10-mL pipette all the EBs are collected in a 15-mL tube filled with 10 mL EB medium and centrifuged at 115 × *g* for 3 min. The pellet can be fixed, embedded and evaluated histologically and histochemically for the presence of three germ layers: endoderm, ectoderm, and mesoderm (Fig. 8a–c).

The EB medium is used for spontaneous differentiation of hESCs. To enhance differentiation towards a specific cell lineage, the EB media should be adapted by adding required factors (16).

In Vivo Differentiation (Teratoma Formation)

Immunocompromised mice SCID-beige or NOD-SCID can be used for this assay.

1. Two almost confluent 60-mm diameter dishes should be used for the injection of one mouse.

2. Aspirate the hESC medium and wash once with PBS
3. Add 2 mL collagenase (1 mg/mL) per dish and place the dishes back into the incubator at 37°C, 5% CO_2 until the colonies start to detach.
4. Collect the hESC colonies in a 15-mL tube containing 2 mL hESC medium.
5. Gently pipette up and down in order to break the colonies in small pieces (see Note 51).
6. Add an extra 8 mL of hESC medium and centrifuge at $115\times g$ for 3 min at 4°C.
7. Remove the supernatant, add 1 mL hESC medium and transfer the suspension to a 1-mL sterile Eppendorf tube.
8. Centrifuge at $115\times g$ for 5 min at 4°C
9. Remove carefully all the supernatant, resuspend the pellet in 30 μL hESC medium and close the cap.
10. Place the eppendorf tube with hESC on ice and transfer it to the animal facilities (see Note 52).
11. In the animal house, aspirate first 50 μL of sterile hESC medium in an insulin syringe 26Gx1/2″. Next, aspirate the hESC clumps into the syringe (see Note 53).
12. Take the mouse, clean the area for injection and inject all the cells intramuscular into the rear leg (see Note 54).
13. After injection, place the mouse in the pre-prepared fresh cage.
14. Identify the cage: line ID, date of injection, side of injection, and eventual remarks.
15. Place the cage in the sterile room.

Wait for a minimum of 4 weeks. Teratoma forming should be visible after 4–10 weeks.

When the tumour is approximately 1 cm, sacrifice the mouse and very gently, cut loose the tumour with the scissors, starting by removing the skin and fat tissue. Mention the weight and the appearance (cystic) of the tumour.

Collect the pieces in 4% neutral phosphate buffered formalin so that they are ready for embedding and histological analysis (Fig. 8d–f).

3.6. Freezing and Thawing of hESC on MEFs

Cryopreservation of hESC in our laboratory was initially performed using the "vitrification in open-pulled straws" protocol from Reubinoff et al. 2001 (17). The technique requires a relatively skilled person as the timing for different incubation steps during the freezing or/and warming of the cells is crucial for the success of the procedure. In addition, only few pieces of colonies can be loaded per straw during the freezing. This does not represent a problem for the cryopreservation of hESC at the first passages after

derivation when few cells are available for cryopreservation, but represents an inconvenience in case of large amount of hESC available after subsequent passages. Another disadvantage of the procedure is the fact that the straws are open so there is a possible microbiological contamination from one straw to another while stored in the liquid nitrogen. Recently, we successfully applied the mFreSR medium (Stem Cell Technologies) for cryopreservation of our hESC. Our protocol is based on the company's recommendations with a few modifications.

1. Use mFreSR at room temperature.
2. Using a stereo microscope check the quality of the hESC colonies on the culture dish aimed for freezing (see Note 55).
3. If present, cut the differentiated parts of the colonies and remove them from the dish (see Note 56).
4. Using a pulled glass pipette with sharp edges cut all the undifferentiated colonies in small squares, keeping in mind that the size should be bigger than usually applied for passaging (see Note 57).
5. Transfer all the sliced pieces to a 15-mL tube containing 10 mL hESC medium.
6. Centrifuge the cells at $115 \times g$ for 3 min.
7. Remove the supernatant without disturbing the pellet.
8. Using a 2 mL serological pipette add 1 mL mFreSR for a 60-mm diameter dish (see Note 58) and gently resuspend the pellet (see Note 59).
9. Transfer 1 mL of cell suspension to each cryovial.
10. Place the vials into an isopropanol freezing container (room temperature) and transfer the container to –80°C overnight. The next day, place the cryovials into the liquid nitrogen.

For thawing frozen hESC

1. Prepare FL necessary for plating the hESC that will be thawed by removing the MEF medium and replacing it with 4 mL hESC medium (for a 60-mm diameter dish). Place the dish back into the incubator at 37°C, 5% CO_2 until the cells are thawed.
2. Prepare one 15-mL centrifuge tube with 10 mL HESC medium and keep it in the flow.
3. Remove the vial from the liquid nitrogen storage.
4. Keep it in a water bath at 37°C while continuing swirling.
5. When the cell suspension is almost thawed, take the vial out of the water bath and wipe it with alcohol.
6. Transfer the suspension drop by drop into the 15-mL tube with hESC medium (see Note 60).

7. Centrifuge at 115 × *g* for 3 min.
8. Discard the supernatant, add 1 mL hESC and gently resuspend the pellet.
9. Transfer the cell suspension to the previously prepared FL dish.
10. Be sure the cell clumps are equally distributed on the dish before it is put back into the incubator
11. The next day proceed as for hESC cultures 1 day after passage (see Note 61).

3.7. Mycoplasma Detection

Mycoplasma screening is utterly important in any cell culture. There are several methods to detect mycoplasma contamination. We use a highly sensitive PCR-based method and, in parallel, send samples from our culture to the immunology department of the University for alternative testing. An important advantage of the PCR method is that it allows the detection of mycoplasma contamination not only in the medium conditioned by the hESC cultures but also in both DNA and cDNA samples from cultured hESC.

In the routine practice in our laboratory, we are testing the medium after at least 24 h of hESC culture.

In order to detect as many mycoplasma species as possible, we are using two different PCR primer sets (here called MYCO1 and MYCO2). Although there is a significant overlap in the mycoplasma species that can be detected with these two primer sets, there remain a number of species that have only been proven to be detected with one of the sets (18, 19). Moreover, to rule out possible PCR failure, each sample is tested in duplicate.

The following primers are used:

MYCO1 forward: 5′-GGGAGCAAACACGATAGATACCCT-3′

MYCO1 reverse: 5′-TGCACCATCTGTCACTCTGTTAAC-CTC-3′

Positive samples with MYCO1 should result in a 270-bp band.

MYCO2 forward: 5′-GGGAGCAAACAGGATTAGATACCCT-3′

MYCO2 reverse: 5′-TGCACCATCTGTCACTCTGTTAAC-CTC-3′

Positive samples with MYCO2 should result in a 270-bp band.

1. Collect the medium condition overnight by hESC cultures (see Note 62). The cell culture supernatant can be used fresh or, alternatively, it can be incubated for 5 min at 95°C after which it can be stored for 1 week at 4°C.
2. Briefly centrifuge the sample to pellet the cell debris.
3. Prepare the PCR mix on ice with all the components indicated by the manufacturer (see Note 63): reaction buffer (as indicated by the supplier), 10 pmol of each primer/reaction, 0.2 mmol dNTP /reaction, 1.25 U hotstart Taq polymerase/reaction and H_2O to adjust the final volume to 23 μL/reaction.

4. Add 2 μL sample/reaction to the PCR mix according to the manufacturer's instructions (see Note 64).
5. Place the reaction tubes in a thermocycler and apply the following programs:

   For MYCO1: 95°C, 2 min; 35× (95°C, 30 s; 56°C, 30 s; 72°C, 30 s); 72°C, 10 min; 4°C, ∞

   For MYCO2: 95 °C, 5 min; 40× (94°C, 30 s; 55°C, 30 s; 72°C, 1 min); 72°C, 10 min; 4°C, ∞
6. Load the samples of the PCR reactions on a 2% agarose gel.

4. Notes

1. During autoclaving, the foam that appears due to the presence of gelatine may touch the neck of the bottle, raising concerns for sterility. Therefore, we advise to prepare the solution in a bottle with a larger volume. Prepare solution needed for no more than 1 week. Keep the bottle at room temperature.
2. Each time we prepare hESC medium, a freshly prepared 2-Mercaptoethanol working solution is used.
3. hESC culture medium can be kept at 2–8°C for 1 week without bFGF addition; after bFGF is added it cannot be used for more than 2 days.
4. We have used different strains of mice for isolation of MEFs: 129/Sv, C57Bl, and CF1. Due to the lower efficiency of the 129/Sv strain in producing offspring, we focused on C57Bl and CF1. Although no evident difference was observed in the capacity to support hESC derivation and culture, we prefer using CF1 mice as they seem, in our experience, easier to handle.

   We have also cultured hESC on mitomycin-inactivated human foetal fibroblasts. On these feeders, the colonies have a different, more angular shape, as compared to the more round hESC colonies on MEFs. Most of our hESC lines are carrying genetic mutations and therefore cannot be used for clinical purposes. Therefore, we decided to maintain our lines on MEFs for practical reasons as they are easier to access and have the same genetic origin.
5. If the gelatine solution is pre-warmed at 37°C in the water bath, 30 min will be sufficient for the gelatine to properly attach to the dish. When coating the dish with gelatine, attention should be paid to cover the entire bottom of the dish, so gently shake it to assure a homogenous covering of the bottom.
6. It is important to take out the vial from the water bath before the complete thawing of the cell suspension as prolonged contact

of the MEFs with the cryoprotectants in the freezing medium might affect their viability.

7. The volume of the medium depends on the size of the culture dish; e.g., for a 60-mm diameter dish: 3.5 mLof medium; for a 35-mm dish: 1.5 mL of medium.
8. Be sure that the cell suspension is homogenous so that the same number of MEFs will be plated in each gelatine-coated dish. The added volume should be small enough (e.g., 500 μL of cell suspension for a 60-mm dish; 250 μL for a 35-mm dish) to allow gentle shaking of the dish for homogenization without spilling.
9. A homogenous distribution of MEFs in the dishes is very important. If hESC colonies form on areas with low fibroblast concentration, they will have a very flat appearance, while on areas with a high concentration, they will pile up and differentiate more easily. Both situations make the process of passaging more difficult.
10. We do not use antibiotics in the MEF culture medium; therefore, bacterial contamination may be noticed faster.
11. Ideally, feeder layer dishes should be prepared 1 day in advance. In our experience 3 days old feeder layers are still of good quality for hESC culture. However, using older feeder layers will have an impact on the quality of the culture. We advise not to use feeder layers of more than 5 days old.
12. The best dilution for us was 1:1 (v/v) in hESC medium. Batch to batch variation may affect the end result of the procedure. Therefore we advise to test each new batch of antibody or GPC on a bad-quality embryo, if available.
13. For more embryos you can use the same dish by preparing more lines with drops.
14. Glass capillaries have a smaller diameter than Kimble pipettes. We use them in the last step of immunosurgery to separate the ICM from trophectoderm.
15. If no diamond pen is available, slide the two pipettes over each other with a bit of pressure. In this way, the pipettes will break and will have a continuous sharp edge.
16. This step should only be performed with embryos that did not hatch. For hatched embryos, proceed immediately with the antibody treatment. If the embryo is hatching (only a part of the embryo protrudes from the ZP), while still in the IVF culture dish try to aspirate the embryo into a pipette that has a diameter slightly bigger than the part that has already hatched. By going up and down with the embryo in the pipette, the ZP will detach from the embryo.

17. For the transfer steps, in order to avoid dilution of the solutions, fill the tip of the pipette with the solution to which the embryo will be transferred before picking up the embryo and try to aspirate as few of the previous medium or solution as possible.
18. It is useful to gently aspirate the embryo in the last drop of protease up and down several times while monitoring the ZP in the microscope. In this way, the embryo can be removed from the protease solution, as soon as the ZP has disappeared.
19. Try to remove as many trophectoderm cells as possible from the ICM. If more than 10–20% of the trophectoderm cells are left attached to the ICM, this will negatively influence the chance to derive hESC as they might overgrow the ICM cells.
20. If the blastocyst is still expanded, it will be difficult to immobilize it on the dish. Try to collapse the embryo by aspirating it in and out of a pipette with sharp discontinuous edges and a diameter a bit smaller than the embryo.
21. Sometimes it is difficult to distinguish the part of the collapsed embryo where the ICM is located. In this situation, cut the embryo in more pieces and put all of them in the culture dish.
22. Based on our experience, most of the ICM are already attached on the first day after plating.
23. An outgrowth can be evident from day 2 after plating but in many cases will contain mainly cells with trophoblast-like appearance. Cells with hESC morphology usually appear later in culture, but this is not a rule.
24. For some of our hESC lines the first passage was performed 2 weeks after plating ICM.
25. Press the sharp edge of the pipette firmly enough on top of the colony to leave deep marks behind as shown in further in Fig. 5d.
26. The left over cells can expand further after the first passage and could serve as a back-up source of undifferentiated cells at first passages.
27. We used single blastomeres from top quality 4-cell stage embryos with equally sized blastomeres and less than 10% fragmentation (estimated as a proportion of the total embryo volume) for the derivation of hESC. In an attempt to derive multiple lines from the same embryo all blastomeres were put in culture. However, this is not a perquisite for hESC derivation from single blastomeres of 4-cell stage embryos.
28. Blastomeres at day 2 are bigger in size than blastomeres at day 3 when usually biopsy for PGD is performed. The bigger inner diameter is recommended to avoid damage to the blastomeres.
29. For manipulation of the embryos, blastomeres, or cellular aggregates, use denudation pipettes (Swemed) or Eppendorf pipettes with disposable tips (0.1–2.5 μL).

30. In our case, most of the blastomere-derived embryos were compacting 2 days post biopsy (day 4 after oocyte retrieval).
31. In our experience, the aggregates attach within the first 36 h after plating. It is highly unlikely that the aggregates would attach after this time.
32. The same amount of hESC medium as for derivation and culture of hESC derived from blastocyst stage should be used, as described in Subheading 3.4.
33. The timing of appearance and maturation of an outgrowth and its morphology is highly variable. In our experiments, the outgrowths were cut for the first time more than 2 weeks after plating of the embryos. However, it is possible that outgrowths need to be cut earlier, depending on their size and morphology.
34. Cutting the colonies should be done as quickly as possible to avoid rise in pH of the medium.
35. Do not add too many cells into a new culture dish as the quantity of the medium is relatively low. 100 colonies per 60-mm diameter dish should be sufficient.
36. During the complete procedure: for appropriate washing, the cells should be incubated with excess PBS for 5 min at least, preferably on a shaking plate.
37. Always use a negative control for each staining. For this, the primary antibody is replaced by an antibody with the same isotype.
38. To avoid aspecific binding of the secondary antibody, use preferentially Fab2 fragments of secondary antibody.
39. Prepare a reaction mix for two samples more than needed. This will avoid shortage of the mix due to pipetting errors. Include a negative control (H_2O instead of cDNA) control.
40. All PCR reactions are performed with standard Taq polymerase (GE Healthcare), except the one for *DNMT3B* in which Expand High Fidelity Taq (Roche Diagnostics) is used.
41. It is recommended to include an appropriate molecular marker in order to be able to determine the length of the observed DNA fragments.
42. Instead of using a full dish for Alkaline Phosphatase staining, we often leave some colonies attached to the MEFs during passaging of the culture and use these for the evaluation.
43. We have tried different protocols for karyotyping the hESC. The one that gave most of the metaphases is the one using low concentration of colcemid (e.g., 0.1 μg/mL) and long incubation periods (4 h) compared with high concentrations of colcemid (50 μg/mL) and a short incubation time (1 h).

44. Feeder cells are normally inactivated either by Mitomycin treatment or by gamma irradiation, therefore are not able to divide further and should not present metaphases. Nevertheless, some cells may escape the process of inactivation, and interfere during the karyotype interpretation. In case of MEF, the murine chromosomes are easily distinguishable from the human ones due to their specific morphology and banding. In case of human feeder layers, it is advisable to initially manually cut out the hESC colonies, remove them from the human feeders and only then to obtain a single cell suspension of hESC. Afterwards, follow all the subsequent steps presented in this protocol.
45. The KCl solution is hypotonic and will make the cells swell, thereby making easier to break the cells in the next steps and facilitating the exposure of metaphases. It is important not to go over 5-min incubation as the cells become more fragile.
46. The fixative should be kept at all time in –20°C.
47. Alternatively, you can cut all the colonies. This procedure will allow you to obtain more homogenously sized EBs.
48. If more than 30 min are needed for the colonies to start detaching, make sure that the collagenase solution has the correct concentration.
49. By this procedure a heterogeneous population of EBs regarding size and morphology will be obtained. This might affect the reproducibility of the differentiation experiments. A standardized approach to the production of EBs has been reported by using AggreWell (Stem Cell Technologies) that helps to generate EBs consistent in size and morphology.
50. In the first day many cellular debris will be present in the medium. This is normal as many cells will die as a consequence of manipulation.
51. The clumps of cells should be smaller than for passaging, not less than 20–30 cells. If they are too big they can block the needle. Some groups are using single cells for injection.
52. Try to keep the Eppendorf tube always in a vertical position to avoid the cells sticking to the plastic.
53. The cells are on the bottom of the Eppendorf tube, so try to keep the aspirated volume as small as possible. After aspirating the cells in the syringe, keep the syringe in a vertical position with the needle downwards to allow most of the colonies to settle in the needle.
54. Be very careful: the needle should remain in the muscle while injecting; wait a few seconds after emptying the needle before gently removing it.

55. We prefer to do the freezing day 4 post passaging.
56. Proceed as for passaging undifferentiated cells, except that now the target areas are the differentiated cells that usually, in our culture conditions are at the periphery of the colonies.
57. If there are many colonies on the dish or when more dishes are intended for freezing, to reduce the time of the procedure, enzymatic (collagenase) treatment can be used to detach the cells from the FL. Follow the same instructions as for collecting colonies for EBs preparation.
58. The company advises to apply 1 mL of mFreSR™ for every well of a 6-well plate being frozen. However, if the wells are at low density (less than 50% confluent) 1 mL of mFreSR™ may be used for every 2 wells.
59. As many cells are dying during the freezing procedure, the clumps should be large enough to assure the survival of sufficient cells after thawing. On the other hand, too large colonies form large compact clumps after thawing and replating on MEFs that do not further expand in culture.
60. The cells should be very carefully manipulated as they are fragile after thawing. Use 1 mL tips or 2 mL serological pipettes to avoid cell damaging.
61. In our experience, there is often more differentiation than usually present at the periphery of the colonies in the first days after thawing. Some colonies can be totally differentiated and should be removed from the culture dish on day 1 or 2 after thawing.
62. To optimize the detection of mycoplasma in cultures with only few colonies, leave the dishes from which colonies were cut for passaging for 4–7 days without changing medium before performing the test.
63. Always prepare a reaction mix for two samples more than needed. This will avoid shortage of the mix due to pipetting errors.
64. Always include positive and negative controls. These can be provided together with the detection.

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Chapter 7

Analysis of LINE-1 Expression in Human Pluripotent Cells

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Abstract

Half of the human genome is composed of repeated DNA, and some types are mobile within our genome (transposons and retrotransposons). Despite their abundance, only a small fraction of them are currently active in our genome (Long Interspersed Element-1 (LINE-1), Alu, and SVA elements). LINE-1 or L1 elements are a family of active non-LTR retrotransposons, the ongoing mobilization of which still impacts our genome. As selfish DNA elements, L1 activity is more prominent in early human development, where new insertions would be transmitted to the progeny. Here, we describe the conventional methods aimed to determine the expression level of LINE-1 elements in pluripotent human cells.

Key words: LINE-1, Retrotransposon, Retrotransposition, Expression, DNA methylation, Real-time PCR, Immunoblots, Human embryonic stem cells, Induced pluripotent stem cells

1. Introduction

Long Interspersed Element-1 (LINE-1 or L1) is a non-long terminal repeat (non-LTR) retrotransposon that makes up about 17% of the human genome (1). Although these elements are typically inactive because they are 5′ truncated and harbor different mutations, an average genome contains ~100 copies of Retrotransposition-Competent LINE-1 elements (RC-L1s) (2, 3). RC-L1s are ~6 kb in length and contain two open reading frames (ORF1 and ORF2) flanked by a 5′ UTR and a 3′ UTR that ends in a poly (A) tail (Fig. 1a (4)). ORF1 encodes a 40-kDa protein (ORF1p) that possesses nucleic acid chaperone activity (5, 6), and ORF2 encodes a 150-kDa protein (ORF2p) with endonuclease and reverse transcriptase activity (7, 8). Both proteins show a strong preference to bind their encoding RNA (*cis*-preference) forming a ribonucleoprotein particle (RNP), which is a proposed retrotransposition

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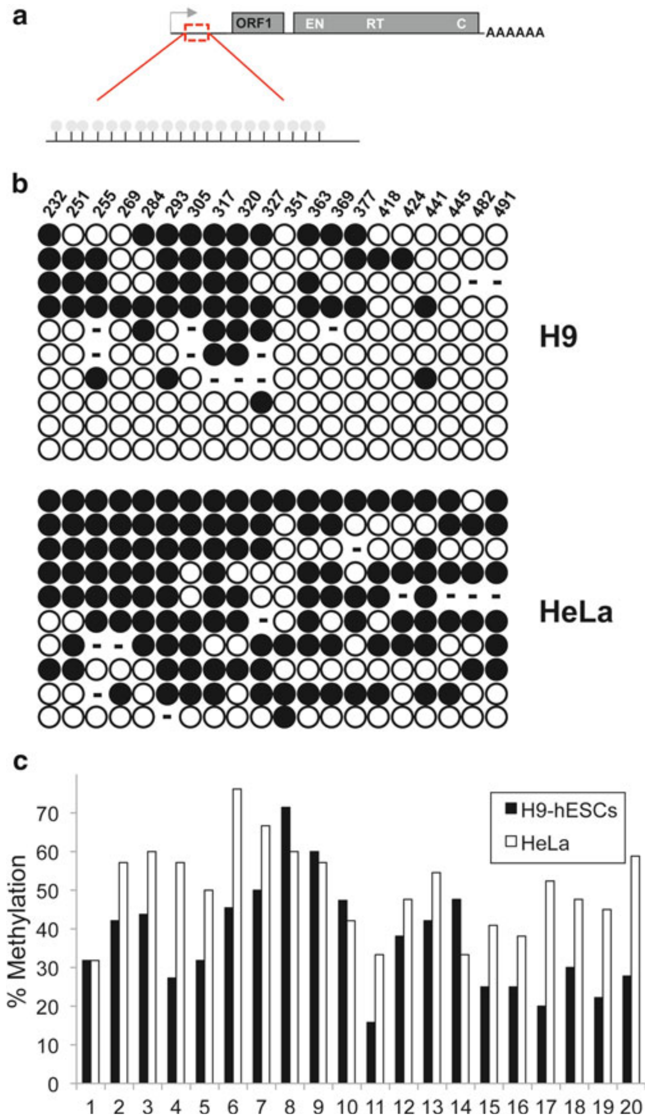

Fig. 1. Methylation analyses of the LINE-1 5′ UTR CpG island. (**a**) The cartoon shows a schematic representation of a human LINE-1 element and the relative position of the CpG island contained within the 5′ UTR (4, 19). The promoter of an L1PA1 element contains 20 CpG residues (*black and grey lollipops*). ORF1 and ORF2 are depicted as *grey boxes*, and UTR regions as *black lines*. Within ORF2, the relative position of the ENdonuclease (EN), Reverse Transcriptase (RT), and Cysteine-rich (C) domains is also indicated. (**b**) Below is shown the individual methylation status of ten sequences with the highest sequence homology to a consensus L1Hs element analyzed in H9 hESCs and differentiated HeLa cells as a control. The positioning of each CpG residue has been numbered using the sequence of L1.3 (34). *Open and closed circles* indicate unmethylated and methylated CpG nucleotides, respectively. *Black small squares* denote mutated CpG positions. (**c**) The *graph* displays the percentage of methylation in the 20 CpG dinucleotides present in a consensus L1Hs element (numbering using L1.3 as a reference sequence, accession number L19088.1 (34)) analyzed in H9 hESCs (*black bars*) and HeLa cells (*white bars*).

intermediate (9–11). L1 retrotransposition takes place by a mechanism known as Target-site Primed Reverse Transcription (TPRT) (7, 11–13) that requires both encoded proteins to succeed (5–8).

The movement of LINE-1 impacts human genome evolution and represents the origin of different diseases, such as muscular dystrophy, hemophilia A, and cancer (reviewed in refs. 14–17). In addition, recent studies have revealed that L1 mobilization processes are a source of genomic variation among humans (18), including our somatic genome (2, 19–21). It has been reported that LINE-1 retrotransposition occurs in the germ line, during early development, and in selected somatic tissues (22–25). More recently, in a mouse model of human L1 retrotransposition, it has been found that most heritable LINE-1 insertions occur during early embryogenesis (26).

In human embryonic stem cells (hESCs), not only the expression of endogenous LINE-1 elements has been demonstrated, but also that their expression is higher than in differentiated cells (27, 28). In vitro LINE-1 retrotransposition assays in hESCs have shown that L1 insertions can take place into genes and lead to small deletions of genomic DNA at the target site (27). In addition, the expression of LINE-1 elements has also been reported in other pluripotent cells (28), hESC-derived neuronal progenitor cells (NPCs) (19), and recently in induced pluripotent stem cells (iPSCs) upon reprogramming (29). Regarding their expression, DNA methylation is considered a host defense mechanism against transposable elements (30, 31). Indeed, it has been demonstrated that deletion of the de novo methyltransferase 3-like protein in mice leads to overexpression of LINE-1 mRNA and meiotic failure in the germ line (32). In addition, L1 mRNA levels in pluripotent cells seem to correlate with the methylation status of their promoters, specifically within a CpG island contained within its 5′ UTR region (19, 29, 32). In sum, pluripotent cells offer a unique model to study the accumulation and regulation of LINE-1 elements in humans.

In the present chapter, we provide several protocols aimed to dissect the expression of L1s in pluripotent cells, from the study of their promoter methylation status to the quantification of their expression at the RNA and protein levels.

2. Materials

2.1. Components and Recipes for Culture Cell Media

1. For HeLa or human embryonic fibroblasts (HEFs): DMEM–high glucose (Invitrogen)—supplemented with 10% FBS (Invitrogen), 1 mM L-glutamine (Invitrogen), and 1× penicillin/streptomycin (Invitrogen). Cells are passaged using 0.05% Trypsin–EDTA (Invitrogen).

2. For hESCs or iPSCs: KO-DMEM (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 1% non-essential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 8 ng/ml of b-FGF (Invitrogen). Cells are grown on Matrigel (BD), and passaged manually as described (27, 28).

All cell lines are grown in a humidified incubator with 7% CO_2. We routinely analyze the absence of *Mycoplasma* spp. using a PCR-based kit (Mycoplasma-PCR-Detection Kit VenorGeM, Minerva Biolabs).

2.2. Methylation Analyses

1. Genomic DNA extraction: Blood & Tissue DNeasy Mini kit (Qiagen) or Blood & Cell Culture DNA Midi Kit (Qiagen). Follow manufacturer's instructions.
2. Bisulfite conversion: Epitect Kit (Qiagen). Follow manufacturer's instructions.
3. Primers: For: 5′-AAGGGGTTAGGGAGTTTTTTT and Rev: 5′-TATCTATACCCTACCCCCAAAA (both from Sigma, see ref. (19)).
4. Cloning and sequencing: QIAquick gel extraction kit (Qiagen) is used to purify amplified products, which are subsequently cloned in pGEM-T Easy-II (Promega). Follow manufacturer's instructions. Sanger-based DNA sequencing is performed following standard procedures.

2.3. LINE-1 Expression Analyses

1. RNA isolation: Use either TRIZol (Invitrogen) or an RNeasy Mini Kit (Qiagen). Follow manufacturer's instructions.
2. DNase step: RNase-free DNase I (Invitrogen). Follow manufacturer's instructions.
3. Reverse transcription step: High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Follow manufacturer's instructions.
4. Conventional RT-PCR: High fidelity expand taq (Roche). Follow manufacturer's instructions.
5. Real-Time RT-PCR: Brilliant SYBR Green QPCRMix (Stratagene) and a MX3005P Real-Time PCR machine (Stratagene). Follow manufacturer's instructions.
6. Western blot: To isolate LINE-1 RNPs, use a previously described protocol (10, 11, 27, 28). 10% SDS-PAGE gel preparation, gel running, and transfer procedures are conducted following standard procedures (10, 11, 27, 28).
7. Antibodies: Mouse monoclonal anti β-Actin (1/20,000, Sigma) and custom anti-ORF1p (purified from rabbits inoculated with a C-terminal peptide of L1.3-ORF1p (CERNNRYQPLQNHAKM), 1/1,000, gently provided by

Dr. Gael Cristofari, CNRS, France). Goat anti-rabbit or anti-mouse HRP conjugate secondary antibodies (1/20,000, Jackson Immunoresearch).

8. Real Time PCR oligonucleotides (Sigma): 5′UTRfwd 5′-GAATGATTTTGACGAGCTGAGAGAA, 5′UTRrev 5′-GTCCTCCCGTAGCTCAGAGTAATT, ORF2fwd 5′-CAAACACCGCATATTCTCACTCA, ORF2rev 5′-CTTCCTGTGTCCATGTGATCTCA; see ref. (19).
9. Conventional RT-PCR oligonucleotides (Sigma): ORF1fwd 5′-AGGAAATACAGAGAACGCCACAA and ORF1rev 5′-GCTGGATATGAAATTCTGGGTTGA (see ref. 27).
10. Cloning and sequencing: pGEM-T Easy-II (Promega). Follow manufacturer's instructions. DNA sequencing is performed following standard procedures.

3. Methods

3.1. LINE-1 Promoter Methylation Analyses

1. Isolate total genomic DNA (gDNA) from samples. Although desired, obtaining high-weight molecular DNA is not crucial for the analysis.
2. Next, conduct bisulfite conversion of gDNAs (2 μg) using the Epitect kit from Qiagen. Follow the protocol provided by the manufacturer. Due to its high density in the genome and to ensure complete conversion on all LINEs, repeat the conversion step twice (see Note 1).
3. Purify your converted DNA using the buffers and columns provided by the Epitect kit (Qiagen).
4. Set up a conventional PCR reaction to amplify a 363-bp region that contains the CpG island of the LINE-1 5′ UTR using converted gDNAs and primers For and Rev (see Subheading 2). Set up PCR as follows: 2 min at 95°C, 35 cycles of 30 s at 94°C followed by 30 s at 54°C and 60 s at 72°C, and a final extension of 5 min at 72°C. PCR reactions are carried out in 50-μl volumes using 10 Us of Taq polymerase, 0.2 mM dNTPs (Invitrogen), and 200 ng of each primer. Include a negative control (RNA/DNA-free water (Invitrogen), see also Note 6). We routinely use 300–500 ng of converted gDNA per reaction. After the PCR, one-fifth of the reaction is loaded on a 1% agarose gel (with either SYBR green or ethidium bromide) to ensure amplification. Next, the rest of the PCR is loaded on a new gel, the ~350-bp amplification band excised, purified using a QIAquick gel extraction kit (Qiagen), and DNA concentration is quantified using a Nanodrop (Thermo).

5. The PCR products are cloned into the pGEM-T Easy Vector (Promega), and X-gal/IPTG is used to screen clones with or without insert following manufacturer's instructions. Next 50–100 bacterial colonies are cultured, plasmid DNA prepared using a Promega plasmid DNA isolation kit (Promega), and inserts sequenced using conventional Sanger DNA sequencing and universal primers as described (19) (see Note 2).
6. Next, the unique sequence in each clone is extracted, and the LINE-1 promoter contained is identified using Repeatmasker at http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker (33). In general, most of the sequences should correspond to elements belonging to the subfamily L1HS or L1P1. Data is next analyzed in two ways.
7. First, the fraction of unmethylated CpG sites is calculated by comparing sequenced amplicons with a consensus L1 sequence in each of the 20 positions (see Fig. 1b). Furthermore, each amplicon is compared to a consensus active L1Hs element (L1.3, accession number L19088.1 (34)), and the ten sequences with the highest homology to this sequence are used to graphically represent the overall level of LINE-1 promoter methylation as shown in Fig. 1b. The percentage of methylation for each CpG position is also plotted (Fig. 1c). In addition, collective results from the 20 CpG sites are collapsed into one data point for each analyzed cell line. Next, the proportion of CpG converted to TpG by bisulfite treatment is compared between samples using the Chi-square test ($df = 1$; $\alpha = 0.05$).

Representative data of the methylation status of the L1PA1 promoter in hESCs and HeLa cells is shown in Fig. 1a–c. As shown, hESCs are characterized for containing L1PA1 hypomethylated promoters when compared to differentiated cells, like HeLa (Fig. 1b, c) or HEFs (29).

3.2. LINE-1 Expression Analyses by Real-Time PCR

1. Isolate total RNA from cultured cells using either TRIzol or an RNeasy mini kit. Quantify your RNA concentration using a Nanodrop (Thermo) (see Note 3).
2. Treat total RNA with RNase-free DNase I. To do that, 2 μg of total RNA is treated with 4 Us of RNase-free DNase I (Invitrogen) for 30 min at room temperature. Stop the reaction by adding 1 μl of EDTA solution (Invitrogen) and incubate samples for 5 min at 65°C using a Thermoblock (Eppendorf). To prevent gDNA contamination, this step is repeated twice (see Note 4). Store RNAs in ice.
3. Set up a reverse transcription reaction. To do that, use 1 μg of DNase I-treated total RNA and a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) following manufacturer's instructions (see Note 5). Store cDNAs on ice.

4. Dilute cDNAs 1/5 and 1/10 using RNA/DNA-free water (Invitrogen). Keep diluted cDNAs on ice.
5. Set up a real-time PCR reaction (see Note 6). PCR reactions are carried out in 20-μl volumes. In addition, reactions are conducted in triplicate using both sets of diluted cDNAs (1/5 and 1/10) and LINE-1 and GAPDH (35) primer sets. For each PCR, use 10 μl of Brilliant SYBR Green QPCRMix (Stratagene), 1 μl of diluted primers (50 μM each), 2 μl of diluted cDNAs, and 7 μl of RNA/DNA-free water (Invitrogen). To prevent variability, we prepare a master mix containing 3.2× volumes of each component in a DNA-free tube, which is subsequently aliquoted in three PCR tubes. Next, PCR is run on a MX3005P Real-Time PCR machine (Stratagene) or a similar platform, and a melting curve from 50 to 95°C with reads every 0.2°C is included to confirm the identity of the amplified products.

   To determine L1 mRNA levels, we use two sets of oligonucleotide pairs (19) to amplify 67- and 64-bp amplicons from the 5′-UTR and ORF2 regions of an L1Hs element, respectively (Fig. 2a, and Note 7). Notably, both sets of primers preferentially amplify the youngest subfamily of LINE-1 elements (19). Although a single set of primers could be enough, it is more appropriate to include both sets of LINE-1 primers, as the 5′ UTR set determines the expression level of full-length transcripts, while the ORF2 set provides an estimation of full-length transcripts plus other non-L1 transcripts present in a cell due to the high density of truncated LINEs in the human genome. It is, thus, anticipated that the level of L1 mRNA

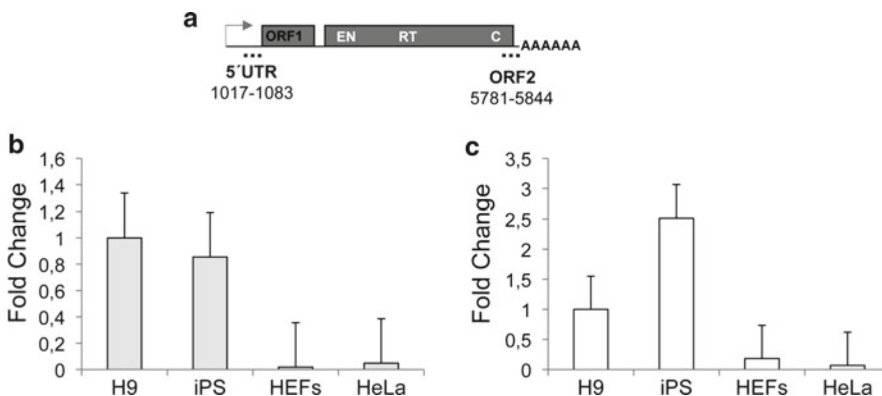

Fig. 2. Quantification of LINE-1 mRNA expression levels. (**a**) The cartoon shows a scheme of a human LINE-1 element (see Fig. 1). The relative position and amplification lengths (*dashed lines*) of the two sets of primers (5′ UTR and ORF2 (19)) used in the quantitative-RT-PCR reaction are indicated below the scheme. The position of the amplified region is based on the L1.3 sequence (L19088.1 (34)). (**b**, **c**) Quantitative RT-PCR results for L1 RNA expression using the 5′UTR (**b**) or ORF2 (**c**) primer set analyzed in H9-hESCs, an iPSC line (iAND-4, *see* ref. (29) and its parental human embryonic fibroblast (HEFs), and in HeLa cells. The *graphs* show the fold change in L1 expression with respect to H9-hESCs. To normalize results, we determined the amplification Ct for GAPDH (35) and compared to the rest of the samples.

obtained using the ORF2 set is higher than the level obtained with the 5′UTR set (19).

6. In the real-time RT-PCR, GAPDH (or other housekeeping gene) is amplified as an internal normalization control. To calculate expression differences, we determine the cycle threshold (C(t)) for LINE-1 and GAPDH, and the C(t) obtained from the GAPDH PCR is used to normalize the mRNA content in the samples. We routinely use the ΔΔC(t) method to represent results (36). In doing that, a sample is designed as a reference, and the rest are compared to this sample (Fig. 2b, c). In general, pluripotent cells (hESCs and iPSCs) express 10–25 times more L1 mRNA than differentiated cells like HeLa or HEFs (Fig. 2b, c).

3.3. LINE-1 Expression Analyses by Conventional PCR

1. Prepare RNA and cDNAs as described above (Subheading 3.2, steps 1–3; see also Notes 3–7).
2. Use cDNAs to set up a conventional PCR reaction using a set of primers that amplify a 236-bp region of LINE-1-ORF1 (see Fig. 3a and Note 6). PCR reactions are carried out in 50-μl volumes using 10 Us of Taq polymerase, 0.2 mM dNTPs

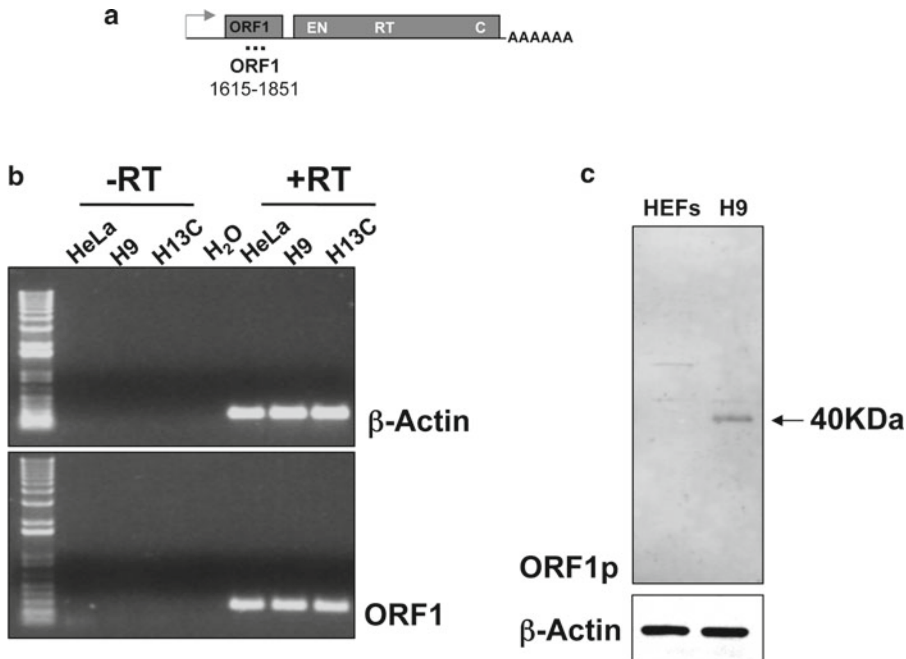

Fig. 3. LINE-1 expression in pluripotent cells. (**a**) A cartoon shows a scheme of a human LINE-1 element (see Fig. 1). The relative position and amplification length (*dashed line*) of the set of primers used in the RT-PCR reaction (ORF1 (27)) are indicated below the scheme. The position of the amplified region is based on the L1.3 sequence (L19088.1 (34)). (**b**) RT-PCR analysis of LINE-1 RNA in HeLa, H9-hESCs, and H13C-hESCs cell lines. H_2O is an RT-PCR reaction lacking input RNA. –RT = control reaction lacking reverse transcriptase. MW denotes 1 kb size standard (Invitrogen). (**c**) Western-blot analysis using a polyclonal antibody against ORF1p in RNPs isolated from H9-hESCs and human embryonic fibroblast (HEFs). Below is shown a western-blot control using a monoclonal antibody against β-actin as a loading control.

(Invitrogen), and 200 ng of each primer. We use 0.5–1 μl of cDNAs per reaction. Set up PCR as follows: 2 min at 95°C, 35 cycles of 30 s at 94°C followed by 30 s at 56°C and 60 s at 72°C, and a final extension of 10 min at 72°C. The set of primers for ORF1 were designed to amplify a region of ORF1 that allows a precise classification of the LINE-1 mRNA expressed (Fig. 3a (27, 37–39)). Include the amplification of a housekeeping gene (GAPDH, β-actin, etc.) as a control for RNA integrity (27) and a negative control (use RNA/DNA-free water (Invitrogen), Notes 4 and 6).

3. Usually, one-fifth of the reaction is loaded on a 1% agarose gel (with either SYBR green or ethidium bromide) to ensure amplification (see Fig. 3b). Using conventional RT-PCR, it is expected to detect L1 mRNA in any human cell line or tissue (see Fig. 3b, Note 8, and refs. 27, 40).
4. As described above, the rest of the PCR is loaded on a gel, the ~250-bp amplification band excised, purified using a QIAquick gel extraction kit (Qiagen), and DNA concentration is quantified using a Nanodrop (Thermo).
5. Also as described above, purified PCR products are cloned into pGEM-T Easy Vector (Promega), and X-gal/IPTG is used to screen clones with or without insert following manufacturer's instructions. To infer the subfamily of LINE-1 expressed in a sample, we routinely sequence 20–30 bacterial colonies per sample. Bacterial clones are cultured, plasmid DNA prepared using a Promega plasmid DNA isolation kit, and inserts sequenced using conventional Sanger DNA sequencing and universal primers as described (27).
6. Next, the unique sequence in each clone is extracted, and the LINE-1 contained is identified using Repeatmasker at http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker (33). In several human cell lines (hESCs, HeLa, hECs, etc.), it is common to detect the expression of a wide constellation of LINE-1 subfamilies, including both inactive and active subfamilies (19, 27, 28, 41).

3.4. LINE-1 Expression Analyses by Western Blot

The LINE-1 RNP is a proposed L1 retrotransposition intermediate (10, 11, 42, 43). Thus, LINE-1 expression can also be inspected by western blot in RNP fractions using antibodies directed against ORF1p (19, 27, 28, 44–46). To do that, RNPs are isolated from cultured cells using ultracentrifugation and a sucrose cushion (see Notes 9 and 10), resolved on an SDS-PAGE, and transferred to a membrane, and the use of an L1-ORF1p antibody allows to determine the level of LINE-1 RNP expression (see Fig. 3c). In general, pluripotent cells are characterized for expressing detectable levels of LINE-1 RNPs when compared to differentiated cell lines as HeLa, 293T, or primary human fibroblasts (see Fig. 3c and refs. 19, 27, 28, 47–49).

4. Notes

1. When conducting the bisulfite conversion on gDNAs, use a PCR machine, but make sure that all liquid is equally heated. If needed, use two PCR tubes per sample.
2. For the methylation analysis, 50 clones per sample should be enough to obtain significant data. However, 100 clones will likely ensure a more robust comparison between samples.
3. Although L1 mRNA levels are routinely calculated on total RNA, the protocol can be applied to either nuclear or cytoplasmic RNA. Indeed, recent reports have demonstrated that in certain circumstances there are significant changes in L1 mRNA levels in fractions that cannot be observed in total RNA (50).
4. To prevent gDNA contamination in RT-PCRs, we routinely repeat the DNase I step twice. In addition and prior to its use in any RT-PCR, we analyze the presence of contaminant gDNA using LINE-1 ORF1 primer sets (see Fig. 3b).
5. To prime the reverse transcription reaction, we previously used either random hexamers or OligodT-based methods, obtaining similar results. However, to detect only the sense strand of LINE-1 mRNA, a specific primer must be used in the RT reaction.
6. Any PCR reaction for LINE-1 is extremely sensitive to external contaminations due to its high abundance in human gDNA. Thus, a PCR hood is required to obtain reliable data.
7. In addition to SYBR green, the same real-time PCR can be adapted to TaqMan probes as described (19).
8. Although less amplification cycles can be used in the RT-PCR, due to its density in the human genome, it is very likely that L1 mRNA can be found in most human cell lines even with low cycling conditions.
9. For RNP expression analyses, use fresh nonfrozen cell pellets.
10. A protocol to isolate RNPs is out of the scope of this chapter. However, excellent protocols have been described previously (10, 11, 19, 27, 28, 47–49).

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Chapter 8

Characterization and Gene Expression Profiling of Five Human Embryonic Stem Cell Lines Derived in Taiwan

Steven Shoei-Lung Li

Abstract

Human embryonic stem cell (hESC) lines have been derived from the inner cell mass of blastocysts. Five hESC lines have been derived from 32 discarded blastocysts in Taiwan, and these lines have since been continuously cultured on mitotically inactivated mouse embryonic fibroblasts as feeder in the hESC medium for more than 44 passages and underwent freezing/thawing processes. All of five hESC lines expressed characteristic undifferentiated hESC markers such as SSEA-4, TRA-1-81, alkaline phosphatase, TERT, transcription factors POU5F1 (OCT4), and NANOG. The hESC lines T1 and T3 possess normal female karyotypes, whereas lines T4 and T5 are normal male, but line T2 is male trisomy 12 (47XY,+12). The hESC lines T1, T2, T3, and T5 were able to produce teratomas in SCID mice, and line T4 could only form embryoid bodies in vitro. Global gene expression profiles of single colonies of these five hESC lines were analyzed using Affymetrix human genome U133 plus 2.0 GeneChip. The results showed that 4,145 transcripts, including 19% of unknown functions, were detected in all five hESC lines. Comparison of the 4,145 genes commonly expressed in the five hESC lines with those genes expressed in teratoma produced by hESC line T1 and placenta revealed 40 genes exclusively expressed in all five hESC lines. These 40 genes include the previously reported stemness genes such as POU5F1 (OCT4), NANOG, TDGF1 (CRIPTO), SALL4, LECT1, and BUB1 responsible for self-renewal and pluripotent differentiation. The global gene expression analysis also indicated that the TGFβ/activin branch components inhibin BC, ACVR2A, ACVR1 (ALK2), TGFBR1 (ALK5), and SMAD2 were found to be highly expressed in undifferentiated states of these five hESC lines and decreased upon differentiation. The epigenetic states and expression of 32 known imprinted genes in these five hESC lines and/or differentiated derivatives were also investigated. In short, the hESC nature of these five hESC lines is supported by the undifferentiated state, extensive renewal capacity, and pluripotency, including the ability to form teratomas and/or embryoid bodies; and these cell lines will be useful for research on human embryonic stem cell biology and drug development/toxicity testing. The epigenetic states and expression of imprinted genes in hESC lines should be thoroughly studied after extended culture and upon differentiation in order to understand epigenetic stability in hESC lines before their clinical applications.

Key words: Human embryonic stem cells, hESC, DNA microarray, Stemness genes, Self-renewal, Pluripotency, Imprinting

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1. Introduction

In 1998 Thomson and his collaborators reported the first successful derivation of human embryonic stem cell (hESC) lines from inner cell mass of blastocysts produced by in vitro fertilization using mitotically inactivated mouse embryonic fibroblasts (MEF) as feeder layer (1). Since then, many hESC lines have been derived and characterized (2–4). The hESC lines can proliferate indefinitely in undifferentiated states and were capable of differentiating in vivo and in vitro into various cell type derivatives of all three embryonic germ layers, ectoderm, mesoderm, and endoderm. Because of the dual abilities to proliferate indefinitely and differentiate into multiple cell types, the hESC lines could potentially provide an unlimited supply of different cell types for transplantation therapy to treat a variety of degenerative diseases such as Parkinson's disease, spinal cord injury, diabetes, and heart failure (5).

The hESC lines are also useful for research to understand the mechanisms of cell differentiation during early human embryo development, and the molecular bases that govern self-renewal and pluripotency of hESCs remain to be fully understood. Several groups have attempted to identify the "stemness" genes responsible for self-renewal and pluripotency of mammalian embryonic stem cells by comparing the highly expressed genes between hESC lines and their differentiated derivatives (6–9). However, the stemness genes may not be highly expressed ones. Further, analysis of pooled population of a given hESC line may not reveal different events of initial differentiation among heterogeneous colonies. The recent advancements on linear amplification of very small amount of RNA samples for successful microarray detection have allowed one to analyze global gene expression profiles among single colonies of hESC lines (10–12).

The in vitro fertilization has been reported to increase human diseases caused by aberrant genomic imprinting (13), and abnormal imprinting has also been reported in human embryonic stem cells (14–16). Therefore, it is important to monitor and maintain epigenetic stabilities in hESC lines for transplantation purpose. However, little is known about the epigenetic states and expression of 32 known imprinted genes in hESC lines following extended culture and upon differentiation (17). The epigenetic states and expression of imprinted genes in hESC lines should be thoroughly studied after extended culture and upon differentiation in order to understand epigenetic stability in hESC lines before their clinical applications.

In my laboratory in Taiwan, five hESC lines have been derived from 32 discarded blastocysts, and their gene expression profiling of single colonies determined (18). The gene expression of potential

stemness genes, as well as TGFβ/activin branch components, was compared among these five hESC lines, T1 teratoma, T4 embryoid bodies, and human placenta. The epigenetic states and expression levels of 32 imprinted genes in these five hESC lines and/or differentiated derivatives were also investigated (19).

2. Materials

2.1. Derivation and Culture of hESC Lines

1. Human embryos: 32 discarded blastocysts (six Day 5 embryos, 19 Day 6 embryos, and seven Day 7 embryos) (18), which usually would have been discarded because of inferior quality, were donated with informed consent by couples undergoing in vitro fertilization treatment and Institutional Review Board (IRB) approval in Taiwan (see Note 1).
2. The hESC culture medium: DMEM/F12 (1:1, 11330) supplemented with 20% KSR, 1% non-essential amino acids, 2 mM L-glutamine, 0.1 mM beta-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (bFGF).
3. Dulbecco's modified Eagle's medium (DMEM, GIBCO, http://www.invitrogen.com).
4. Knockout serum replacement (KSR, GIBCO).
5. Basic fibroblast growth factor (bFGF, Invitrogen, http://www.invitrogen.com).
6. Blastocyst medium (MediCult, http://www.medicult.com).
7. Rabbit anti-BeWo cell antibodies (Sigma-Aldrich, http://www.sigmaaldrich.com).
8. Guinea pig complement (Rockland Immunochemicals, http://www.rockland-inc.com).
9. Pronase (Sigma-Aldrich).
10. Collagenase (type IV, Invitrogen).
11. DMSO (Sigma-Aldrich).
12. FBS (GIBCO).
13. D-PBS (GIBCO).
14. Four-well culture plate (Nunc).
15. CO_2 incubator (NU-5510, NUAIRE, http://www.nuaire.com).

2.2. Preparation of MEF Feeder

1. C57/BL6 inbred strain mice (National Laboratory Animal Center, Taiwan).
2. MEF medium: DMEM supplemented with 10% FBS, 1% non-essential amino acids, and 2 mM glutamine.

3. Mitomycin C (Sigma-Aldrich).
4. Six-well culture plate (Nunc).

2.3. Karyotyping

1. Ethidium bromide (Amresco, http://www.amresco.com).
2. Colcemid (Invitrogen).
3. Trypsin-EDTA (GIBCO).
4. Carnoy's fixative (methanol:acetic acid, 3:1).
5. Modified Wright's staining (Sigma-Aldrich).

2.4. Staining of Cell-Surface Markers

1. Antibodies for SSEA-1 (Sc-21702), SSEA-4 (Sc-21704), TRA-1-81 (Sc-21706), and alkaline phosphatase (AP, Sc-21707) (Santa Cruz Biotechnology, http://www.scbt.com).
2. Vector ABC detection kits (Vector Laboratories, http://www.vectorlabs.com).

2.5. Formation of Teratoma and Embryoid Bodies

1. Severe combined immunodeficient (SCID) 4-week-old beige male mice (National Taiwan University Animal Center, Taipei, Taiwan).
2. "RNA later solution" (Ambion, http://www.ambion.com).

2.6. DNA Microarray Analysis

1. Single colonies of hESC lines T1 at Passage 32, T2 at Passage 26, T3 at Passage 16, T4 at Passage 10, and T5 at Passage 6 (18).
2. Lysis buffer: 0.5% NP-40 (Vysis, http://www.vysis.com), 20 mM DTT (dithiothreitol, Sigma-Aldrich), and 1 μl of 0.5 μg/μl oligo$(dT)_{12-18}$ primer (Invitrogen).
3. SUPERSCRIPT™ one-step RT-PCR kit (Invitrogen).
4. 2× Reaction Mix (Invitrogen).
5. RT/PLANTINUM Taq Mix (Invitrogen).
6. Microarray Target Purification Kit (Roche Applied Science, http://www.roche-applied-science.com).
7. Microarray Target Amplification Kit (Roche Applied Science).
8. Microarray RNA Target Systhesis Kit (Roche Applied Science).
9. Human placenta (Stratagene/Agilent Technologies, http://www.genomics.agilent.com).
10. Affymetrix Human Genome U133 plus 2.0 GeneChip (see Note 2, http://www.affymetrix.com).
11. Affymetrix GeneChip scanner 3000.
12. Affymetrix GeneChip Operating Software MAS 5.0.
13. GeneSpring software version 7.2 (Silicon Genetics, http://www.sigenetics.com).
14. GC-RMA.

Table 1
PCR primer sequences and polymorphisms

Gene	Primer sequence (5′→3′)	Size (bp)	SNP	Acc. no Location
IGF2	F CTTGGACTTTGAGTCAAATTGG R CCTCCTTTGGTCTTACTGGG	235	G→A	X07868 Pos. 820
IPW	F GGGAACTCTTCTGGGAGTGAAT GTTATCA R GGGAGGTTCATTGCACAGAAATTTGG Seq. TGGATAGATGCACACAAACAC	1,550 868	C→T	U12897 Pos. 1670
NESP55 gDNA[a] NESP55 cDNA	F GGCTCCTTGTGCTGTCTGTCTTGTAG R CCACACAAGTCGGGGTGTAGCTTA F TCGGAATCTGACCACGCGCA R CACGAAGATGATGGCAGTCAC Seq. CAACCTGAAAGAGGCGATTGAA	233 1,141	T→C	M21741 Pos. 299
PEG10	F TCATTTTCCTGCCTGGTTGC R GGAGCCTCTCATTCACAGC	405	C→T	XM_496907 Pos. 4404
KCNQ1 gDNA[a] KCNQ1 cDNA	F CACTGCCTGCACTTTGAGCC R GTGAGGAGAAGGGGGTGGTT F GGACCTGGAAGGGGAGACT R GCGATCCTTGCTCTTTTCT	282 282	G→A	AJ006345 Pos. 331010
ATP10A	F AAAGACACCACCGACAGGAA R ATGCTCATGTCCACTGTGCT	318	G→C	BC052251 Pos. 4006
TCEB3C	F CCAGAGCTGAGAGAAAGTGC R TTTCCTGGCGAGACGATTTG	249	C→G	NM_145653 Pos. 772

This table is adapted with permission from Li et al. (19)
[a]gDNA indicates genomic DNA

2.7. Analyses of Epigenetic States and Expression of Imprinted Genes

1. Wizard SV Genomic DNA Purification System (Promega, http://www.promega.com).
2. Absolutely RNA Nanoprep Kit (Stratagene/Agilent Technologies, http://www.genomics.agilent.com).
3. Microarray Target Amplification Kit and Microarray Target Purification Kit (Roche Applied Science).
4. Go Tag Flexi DNA polymerase (Promega).
5. PCR primer sequences (Table 1).
6. Wizard SV Gel and PCR Clean-up System (Promega).
7. BigDye terminator cycle Sequencing Kit (3.1 version) and ABI 3730 DNA sequencer (Applied Biosystems, http://www.appliedbiosystems.com).
8. Affymetrix human genome U133 plus 2.0 GeneChip.

3. Methods

3.1. Derivation and Culture of New hESC Lines

The procedures used for derivation of the five hESC lines in Taiwan are those described previously (1–3, 18) and briefly summarized here.

1. The donated embryos were cultured in blastocyst medium under 5% CO_2 at 37°C, and the embryos that grew to blastocyst stage on day 5, day 6, or day 7 after fertilization were collected in a four-well culture plate.
2. The zona Pelucida of the embryos were removed by pronase digestion (250 iu/ml) for 1–3 min, and the zona-free blastocysts were then rinsed in blastocyst medium for 5 min to remove the remaining pronase activity.
3. The blastocysts were treated with rabbit anti-BeWo cell antibodies (1:100 dilution) in high glucose DMEM supplemented with 20% KSR for 15 min, rinsed in blastocyst medium for 3 min, and then subjected to guinea pig complement (1:10 dilution) in DMEM for 15 min.
4. The trophectoderm of blastocysts were attacked by the antibody-complement immunoreaction, gradually swelled and ruptured, and the remaining trophectoderms were removed by glass-micropipette.
5. The inner cell masses were gently transferred to a Nunc 4-well dishes pre-plated with mitotically inactivated MEF feeder layer, and cultured in hESC medium.
6. Once the inner cell mass adhered to the bottom of feeder layer and expanded in cell numbers, it formed a distinct colony after 10-day culture.
7. The primary colonies were propagated to new well with mitotically inactivated MEF feeder (see Note 3) by mechanical slicing with a glass micropipette.
8. Daily change of hESC medium is necessary (see Note 4).
9. Routine passages of hESC lines every 7 days were done with collagenase (type IV, 1 mg/ml) treatment and mechanical scrape.
10. All hESC lines were cryopreserved by a slow freezing method (–1°C/min) with a cryoprotectant of 10% DMSO, 40% FBS, and 50% hES medium.

3.2. Preparation of MEF Feeder

Mouse embryonic fibroblasts (MEF) feeder was prepared as previously reported (20) and briefly described here.

1. The pups (C57/BL6) at gestation 13–14 days were removed from uteri, and the head and abdominal organ were resected.

2. After rinsed in D-PBS, the trunks were minced with scissors and grinded with glass slide in 1× trypsin-EDTA in DMEM until no chunk remained, and they became glutinous.
3. These embryonic cells were washed, diluted, and plated at concentration of 60,000 cells/cm^2 in MEF medium in tissue culture T_{75} flask.
4. The MEF were propagated until passage 5 or 6, and inactivated with mitomycin C (10 μg/ml) and replated in a gelatin-treated Nunc 6-well plate as feeder.

3.3. Karyotyping

The karyotyping of hESC lines is briefly described here (see Note 5).

1. The hESC lines were cultured in the hESC medium in 6-well plate and treated with 0.1 μg/ml ethidium bromide at 37°C for 30 min and then 0.1 μg/ml colcemid for 1 h.
2. The cells were subsequently dislodged with trypsin-EDTA, fixed in Carnoy's fixative (methanol:acetic acid, 3:1).
3. The cell suspension was dropped on precleaned glass slide to make chromosome spread.
4. The chromosomes were visualized by using modified Wright's staining.

3.4. Staining of Cell-Surface Markers

1. The hESC colonies were fixed in the culture dishes by 100% ethanol for immunostaining of cell-surface markers SSEA-1 and TRA-1-81, whereas 90% acetone in H_2O fixation was used for SSEA-4.
2. The antibodies for SSEA-1 (Sc-21702), SSEA-4 (Sc-21704), TRA-1-81 (Sc-21706), and alkaline phosphatase (AP, Sc-21707) were used using Vector ABC detection kits.

3.5. Formation of Teratomas and Embryoid Bodies

1. SCID mice were used as animal hosts for the xenografted hESC lines (see Note 6).
2. Approximately one million cells of hESC lines were injected into the rear leg muscles.
3. The resulting teratomas were excised after 7–8 weeks.
4. Teratomas were fixed in neutral buffered 10% formalin, embedded in paraffin, and examined histologically after hematoxylin and eosin staining (see Note 7).
5. One teratoma produced by hESC line T1 was cut into small pieces and immediately stored in the "RNA later solution" for RNA extraction and Affymetrix GeneChip analysis.
6. Formation of embryoid bodies (EB) was induced by mechanically dissecting undifferentiated hESC line T4 colonies into pieces which were transferred and grown in hESC medium without MEF feeder at 37°C, 5% CO_2.

3.6. DNA Microarray Analysis

The gene expression profiling of single colonies (see Note 8) from five undifferentiated hESC lines, as well as T1 teratoma, T4 embryoid bodies and placenta, were analyzed using the following procedure (12, 18).

1. Total RNAs were extracted from single colonies of hESC lines, as well as one T1 teratoma and T4 embryoid bodies.
2. Approximately 200–300 hESCs from each colony were washed with PBS twice and then transferred to Eppendorf in 2 μl lysis buffer.
3. Samples were incubated for 5 min at 65°C to lyse the cells, to release RNAs, and to anneal oligo$(dT)_{12-18}$ primer with RNAs.
4. The cDNA synthesis was carried out by using SUPERSCRIPT™ one-step RT-PCR kit.
5. For the reverse transcription step, the whole 5 μl of the resuspended RNAs were incubated for 60 min at 42°C, then 15 min at 72°C in 50 μl of reaction mixture containing 25 μl of 2× Reaction Mix, 1 μl of RT/PLANTINUM Taq Mix.
6. 28.5 μl of the cDNAs present in the 50 μl RT reaction mixture purified by Microarray Target Purification Kit were used as templates to amply cDNA by Microarray Target Amplification Kit.
7. The amplified cDNA were purified by Microarray Target Purification Kit.
8. The complementary RNAs (cRNAs) were synthesized from 200 ng cDNA with Microarray RNA Target Synthesis Kit.
9. 500 ng RNAs each from the teratoma produced by hESC line T1 and embryoid bodies formed by hESC line T4, as well as 5 ng and 500 ng RNAs of human placenta, were used for cDNA amplification and cRNA synthesis.
10. The purified 5 μg cRNAs were analyzed using Affymetrix Human Genome U133 plus 2.0 GeneChip according to the manufacturer's protocols (see Note 9).
11. GeneChips from the hybridization experiments were read by the Affymetrix GeneChip scanner 3000.
12. The raw data were processed using Affymetrix GeneChip Operating Software MAS 5.0 and its default analysis parameters. A gene probe with either the detection call of "present" or "marginal" was considered present for that array.
13. The raw data were also analyzed by GeneSpring software version 7.2.
14. The correlation coefficients of gene probes expressed between any two colonies of five hES cell lines, as well as two placenta samples, were calculated from the normalized values by using GC-RMA.

3.7. Analyses of Epigenetic States and Expression of Imprinted Genes

The allele-specific expressions of seven imprinted genes in these five hESC lines were investigated using single nucleotide polymorphism (SNP) markers. Expression levels of 32 known imprinted genes from undifferentiated hESC lines, embryoid bodies, and teratoma were analyzed using Affymetrix human genome U133 plus 2.0 GeneChip (19).

1. Genomic DNAs (gDNA) were isolated using the Wizard SV Genomic DNA Purification System.
2. Total RNAs were extracted using the Absolutely RNA Nanoprep Kit from undifferentiated cells, embryoid bodies, and/or teratomas of hESC lines.
3. The cDNAs were synthesized using the Microarray Target Amplification Kit and purified with Microarray Target Purification Kit.
4. PCR amplification of genomic DNA and cDNA was carried out in a 25-μl reaction volume, with two units of the Go Tag Flexi DNA polymerase, 1× supplied reaction buffer, 0.12 μmol/L of each primer, 0.75 mM $MgCl_2$, 0.2 mM of dNTPs, and 10–200 ng DNA template.
5. Cycle conditions are as follows: initial denaturation at 95°C for 2 min, then 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 3 min, followed by a final extension at 72°C for 5 min.
6. Amplified DNA was purified using the Wizard SV Gel and PCR Clean-up System and sequenced with the BigDye terminator cycle Sequencing Kit (3.1 version) and ABI 3730 DNA sequencer.
7. Expression levels of imprinted genes from undifferentiated hESC lines, embryoid bodies, and teratoma were analyzed using Affymetrix human genome U133 plus 2.0 GeneChip according to the manufacturer's protocols.

3.8. Results

3.8.1. Derivation and Characterization of Five hESC Lines

Five hESC lines T1, T2, T3, T4, and T5 were successfully derived from 32 discarded blastocysts (two from six Day 5 embryos, three from 19 Day 6 embryos, and none from seven Day 7 embryos) in Taiwan (see Note 10), and they have since been continuously cultured on mitotically inactivated MEF feeder in the hESC medium for more than 44 passages and underwent freezing/thawing processes (18). Some characteristics of these five hESC lines are summarized in Table 2. The karyotype analyses of these hESC lines revealed that lines T1 and T3 are normal female, whereas lines T4 and T5 are normal male, but line T2 is male trisomy 12 (47XY,+12) (see Note 11). All of these hES cell lines were stained positively for a number of undifferentiated hESC markers, including SSEA-4, TRA-1-81, and alkaline phosphatase (AP), but negatively for SSEA-1 (a positive marker for mouse embryonic stem cells).

Table 2
Characterization of five hESC lines derived in Taiwan

hESC lines	T1	T2	T3	T4	T5
Karyotype	XX	XY	XX	XY	XY
SSEA-1	–	–	–	–	–
SSEA-4	+	+	+	+	+
TRA-1-81	+	+	+	+	+
AP	+	+	+	+	+
Oct4	+	+	+	+	+
Nanog	+	+	+	+	+
Teratoma	+	+	+	*	+
Freezing/thawing	+	+	+	+	+
Passages (5-5-05)	P56	P57	P47	P39	P35
Stock vials	154	86	109	38	121

This table is adapted with permission from Li et al. (18)
"+" indicates the positive results, whereas "–" means the non-expression
"*" denotes embryoid bodies formation

The hESC lines T1, T2, T3, and T5 were able to produce teratomas in SCID mice, and histological examinations revealed many cell type derivatives of ectoderm, mesoderm, and endoderm. The hESC line T4 failed to produce teratoma in SCID mice (see Note 12), but was able to form in vitro embryoid bodies. The microarray analysis of T4 embryoid bodies indicated the presence of all three germ-layer markers. These data indicate that after many passages these five hESC lines maintained the pluripotent capacity to differentiate into cell type derivatives of all three embryonic germ layers.

3.8.2. Gene Expression Profiles of Single Colonies of Five hESC Lines

The gene expression profiles of single colonies of five hESC lines were analyzed using Affymetrix HG-U133 plus 2.0 GeneChip (18). The single colonies of five hESC lines expressed 16–33% (average 25%) of the 54,675 gene probes. The common expression of gene probes between any two colonies of five hESC lines was found to range from 36% to 67%, with average of 47%, while their correlation coefficients were from 0.79 to 0.98, with average of 0.88. However, only 4,145 genes (15%) out of the total 27,571 gene probes detected on the array were found to express in all single colonies of five hESC lines (see Note 13). The molecular functions of these 4,145 genes were analyzed according to the Gene Ontology database (Fig. 1). It may be noted that the molecular functions for 19% of the 4,145 genes are unknown (see Note 14). On the other hand, those genes exhibiting differential

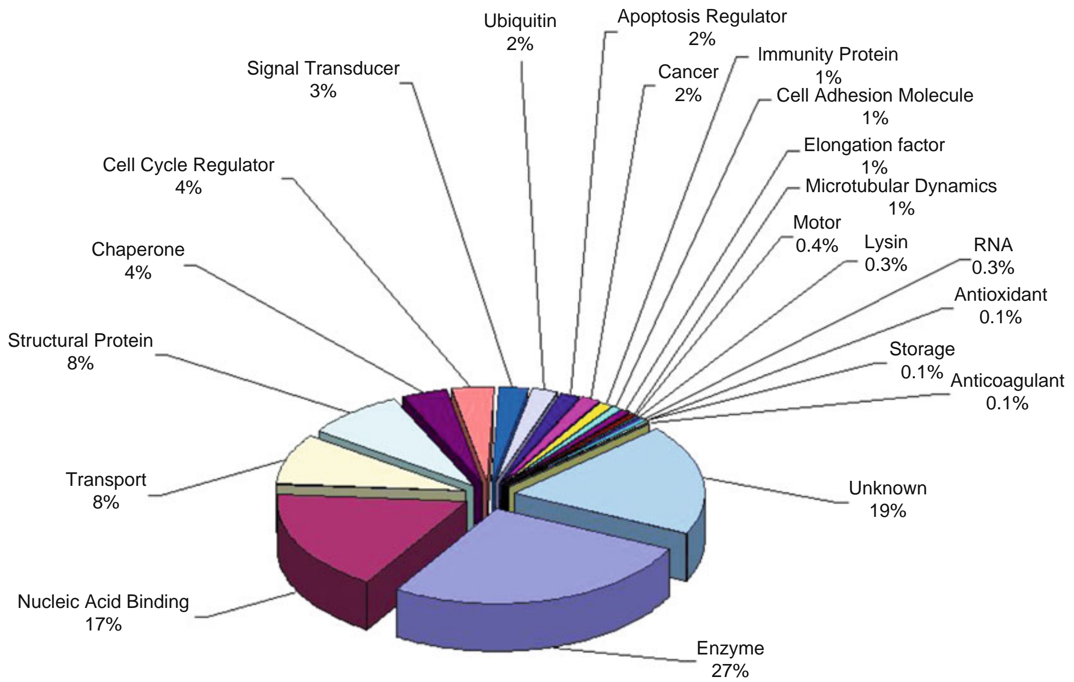

Fig. 1. Molecular functions of the 4,145 genes expressed in all five hES cell lines. The 4,145 genes were functionally annotated according to the GeneOntology database. Some of these genes were assigned to more than one molecular function. This figure is adapted with permission from Li et al. (18).

expression among five hESC lines do provide unique identity (signature) for each hESC line. For example, the chromosome Y-linked genes such as RSP4Y1, EIF1AY, DDX3Y (H-2 minor antigen), and JARID1D (H-Y antigen) (21) were expressed only in male hESC lines T2, T4, and T5, but not in female hESC lines T1 and T3 (see Note 15).

3.8.3. Gene Expression Profiles of Placenta, Teratoma, and Embryoid Bodies

To confirm a linear amplification using small amount of RNAs, 5 ng and 500 ng RNAs of human placenta were used for cDNA amplification and cRNA synthesis for microarray detection using Affymetrix HU-U133 plus 2.0 GeneChip. A scatter plot of gene probes detected between two placenta samples showed similar patterns of gene expression with a correlation coefficient of 0.94, indicating no significant bias during cDNA amplification (18). One of four teratomas produced by hESC line T1 was used for gene expression profiling, and other three teratomas were used for histological examinations, indicating different cell type derivatives of three germ layers. A total of 25,989 gene probes (48%) on the array were detected in T1 teratoma. The GeneChip analysis of T4 embryoid bodies detected the expression of 17,541 gene probes (32%) out of 54,675 gene probes on the array. 88% of these gene probes expressed in T4 embryoid bodies were also detected in the

T1 teratoma, and these commonly expressed gene probes include many markers for all three germ layers, ectoderm, mesoderm, and endoderm.

3.8.4. Comparison of Gene Expression Between hES Cell Lines, Teratoma, and Placenta

When the 4,145 genes commonly expressed in all single colonies of five hESC lines were compared with the genes expressed in T1 teratoma and human placenta (18), only 40 genes were found to be exclusively expressed in all hESC lines, but not in T1 teratoma and placenta (Fig. 2). Thus, these 40 genes are potential embryonic stemness genes responsible for self-renewal and pluripotency (Table 3). Among these 40 genes, 25 genes have known functions, which include the previously reported stemness genes POU5F1 (OCT4), NANOG, SALL4, TDGF1 (CRIPTO), LECT1, and BUB1 (6–11), telomerase reverse transcriptase, SOX 15, DHX9, NMU, and three ubiquitin specific proteases. The remaining 15 genes of unknown functions are: three POU domain containing genes POU5F1P1, POU5F1L, and ASH1L, two developmental pluripotency associated DPPA2 (see Note 16) and DPPA4, two hypothetical zinc finger proteins ZNF208 and ZNF670, two chromosome open reading frames C1orf31 and C14orf115, one LOC390411, two IMAGE clones 2344436, and 3448785, three EST sequences of germ cells.

As indicated in Fig. 2, the 97 genes, including SOX2, FGF2, VEGF, and CDC25A, were commonly expressed in all hESC lines

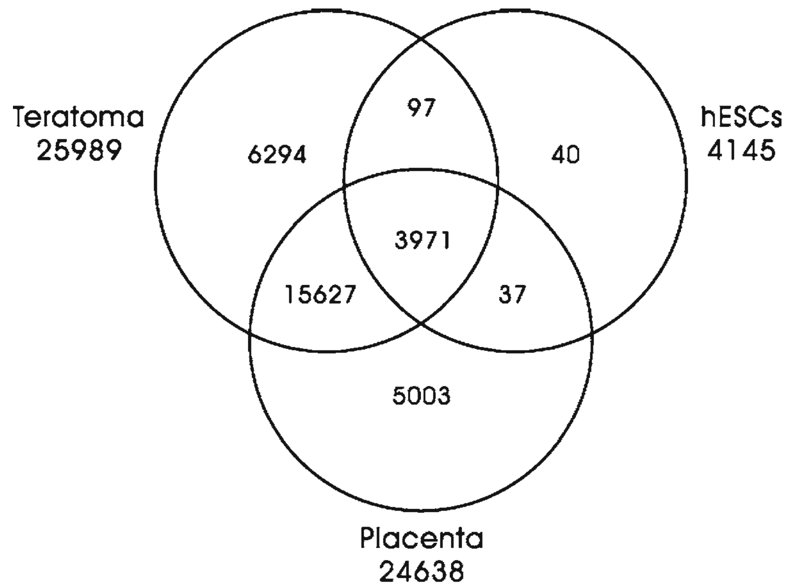

Fig. 2. Venn diagram of the 4,145 genes commonly expressed in all single colonies of five hESC lines, the 25,989 gene probes expressed in teratoma produced by hESC line T1, and the 24,638 gene probes expressed in two placenta samples. This figure is adapted with permission from Li et al. (18).

Table 3
List of 40 potential embryonic stemness genes

(a) 25 genes of known functions

Symbol	Gene description	Probe ID
POU5F1	POU domain Class 5 Transcription Factor 1,Oct4, 360aa	208286_x_at
NANOG	Nanog homeobox	220184_at
SALL4	Sal-like 4 (Drosophila), zinc finger protein	229661_at
TDGF1	Teratocarcinoma-derived growth factor 1, CRIPTO	206286_s_at
LECT1	Leukocyte cell-derived chemotaxin 1	206309_at
BUB1	BUB1 budding (yeast), human spindle check point kinase	215509_s_at
TERT	Telomerase reverse transcriptase	207199_at
SOX15	SRY-related box 15 (Oct4 associated)	217040_x_at
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9, ATP-dependent RNA helicase	212105_s_at
NMU	Neuromedin U	206023_at
USP10	Ubiquitin specific protease 10	209136_s_at
USP28	Ubiquitin specific protease 28	230623_x_at
USP44	Ubiquitin specific protease 44	224048_at
COBL	Cordon-bleu homolog (mouse)	213050_at
MAP7	Microtubule-associated protein 7	202889_x_at
KPNB1	Karyopherin (importin) beta 1	217027_x_at
MNAT1	Menage a trois 1 (CAK assembly factor, ubiquitin ligase activity)	203565_s_at
PSAT1	Phosphoserine aminotransferase 1	220892_s_at
AASS	Aminoadipate-semialdehyde synthase	214829_at
SMPDL3B	Acid sphingomyelinase-like phosphodiesterase	205309_at
UGP2	UDP-glucose pyrophosphorylase 2	231698_at
ALDH1B1	Aldehyde dehydrogenase 1 family, member B1	209646_x_at
HIST1H1B	Histone 1, H1b	214534_at
GRID1	Glutamate receptor, ionotropic, delta 1	1555267_at
MST1R	Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	205455_at

(continued)

Table 3 (continued)

(b) 15 genes of unknown functions

Symbol	Gene description	Probe ID
POU5F1P1	POU domain, OCT3A-related intron-less gene, hypothetical protein, 359aa	214532_x_at
POU5F1L	Similar to POU domain Class 5 transcription factor 1	210265_x_at
ASH1L	ASH1L (Drosophila), hypothetical protein, 2969aa	210905_x_at
DPPA2	Developmental pluripotency associated 2, hypothetical protein, 221aa	240301_at
DPPA4	Developmental pluripotency associated 4, hypothetical protein, 294aa	219651_at
ZNF208	Zinc finger protein 208	208542_x_at
ZNF670	Hypothetical zinc finger protein, 389aa	223898_at
C1orf31	cDNA clone IMAGE:6150603, CIN_DROME Molybdenum cofactor synthesis protein cinnamon (Drosophila)	225638_at
C14orf115	Hypothetical protein, 702aa, transposase, DNA binding	220536_at
LOC390411	Similar to nucleophosmin 1; nucleolar phosphoprotein B23; numatrin; nucleophosmin/nucleoplasmin family, member 1	216387_x_at
	EUROIMAGE cDNA clone 2344436	237192_at
		237193_s_at
	IMAGE clone 3448785, hypothetical protein, 122aa	237563_s_at
	Testis 3′ mRNA	240681_at
	Germ cell 3′ mRNA	230356_at
	Germ cell 3′ mRNA	231079_at

This table is adapted with permission from Li et al. (18)

and T1 teratoma produced by hESC line T1 (see Note 17). The 37 genes, including DDX3X and BOMB, were also detected in both all hESC lines and placenta. The 3,971 genes were found to be expressed in all five hESC lines, T1 teratoma, and placenta. These 3,971 genes, as well as the 4,145 genes, include many house-keeping genes and 19% of genes without known molecular functions (18).

As to the differentiation marker genes expressed in T1 teratoma and placenta, 84 gene probes were detected as ectoderm (31 gene probes), mesoderm (23 gene probes), and endoderm (30 gene probes) markers (18). Among 84 gene probes, two of four gene probes for trophoblast markers were detected among 5,003 gene probes of placenta, while the other two gene probes were found among 15,627 gene probes of both placenta and T1 teratoma. It may be noted that T4 embryoid bodies were also found to express many of these 84 gene probes for all three germ layers and trophoblast markers. However, none of these 88 gene probes for three germ

layers and trophoblast markers was found among the 4,145 genes commonly expressed in all colonies of five hESC lines. These results indicate that T1 teratoma and T4 embryoid bodies did consist of many cell type derivatives of all three germ layers, and that the 4,145 genes were expressed by undifferentiated hESC colonies.

The expression values and the relative indices (fold changes) of TGFβ superfamily components, including ligands, receptors, signaling SMADs, and regulatory proteins, as well as their target genes, in undifferentiated conditions (average of five hESC lines) and differentiated T4 embryoid bodies and T1 teratoma, as well as placenta, were analyzed (18). Several genes, including IHBC, LEFTY1 (LEFTY B), LEFTY 2 (LEFTY A), BMP2, ACVR2A, TGFBR1, ACVR1, TDGF1 (CRIPTO), BMPR1A, SMAD2, SMAD4, SMAD5, LTBP1, SMURF2, and FST, showed significantly higher expression in undifferentiated hESC lines, and decreased drastically in differentiated T4 embryoid bodies, T1 teratoma, and placenta. On the other hand, many genes such as TGFB2, INHBA, INHBE, BMP7, GDF3, GDF15, NODAL, TGFBR2, BMPR2, ACVRL1, BMPR1B, SMAD6, NOG, ENG, RUNX1, and SMURF1 were up-regulated significantly upon differentiation (see Note 18).

3.8.5. Allele-Specific Expression of Seven Imprinted Genes

In order to distinguish mRNA transcripts from each parental allele, the potential SNPs of the 32 known imprinted genes (17) were searched from the literature (14, 15) and SNP database of NCBI (19). The heterozygous alleles of SNPs at seven genes IPW, PEG10, NESP55, KCNQ1, ATP10A, TCEB3C, and IGF2 genes were found by sequencing genomic DNAs of hESC lines (Table 4). The genomic DNAs of hESC lines T1 and T2 exhibited T and C alleles of IPW gene, whereas the cDNA sequencing of undifferentiated cells and teratomas from hESC lines T1 and T2 showed only T allele of IPW gene (Fig. 3). The genomic DNA from hESC lines T2 and T3 exhibited C and T alleles of PEG10 gene, whereas the cDNA sequencing of undifferentiated hESC lines T2 and T3 cells, as well as hESC line T2 teratoma, showed only C allele of PEG10 gene. The T and C alleles of NESP55 gene were identified in the genomic DNA of hESC line T1, whereas the cDNA from hESC line T1 teratoma showed only T allele of NESP55 gene. The G and A alleles of KCNQ1 gene were identified in genomic DNA of hESC line T3, whereas only G allele of KCNQ1 was found in the sequencing cDNA from undifferentiated hESC line T3 cells. The C and G alleles of ATP10A gene were found in the genomic DNA of hESC line T2, whereas the sequencing cDNA products from undifferentiated cells and teratoma of hESC line T2 showed only C allele of ATP10A gene. The G and C alleles of TCEB3C gene were found in the genomic DNAs of hESC lines T1, whereas only G allele of TCEB3C gene was identified in the cDNAs of hESC line T1 teratoma. These results clearly demonstrated the monoallelic

Table 4
Allele-specific expression of imprinted genes in hESC lines

Genes	hESC lines	Genomic DNA	cDNA		
			Undiff. cells	Tetratoma	EB[a]
IPW	T1	T/C	T	T	
	T2	T/C	T	T	
PEG10	T2	C/T	C	C	
	T3	C/T	C		
NESP55	T1	T/C		T	
KCNQ1	T3	G/A	G		
ATP10A	T2	C/G	C	C	
TCEB3C	T1	G/C		G	
IGF2	T2	A/G	A	A	
	T3	G/A	G>A[b]		
	T4				A/G[c]

This table is adapted with permission from Li et al. (19)
[a]EB means embryoid bodies
[b]Partially expressed A allele
[c]Bi-allelic expression

expression of six imprinted genes IPW, PEG10, NESP55, KCNQ1, ATP10A, and TCEB3C in undifferentiated hESC lines and/or differentiated derivatives (see Note 19). As to IGF2 gene, the A and G alleles were identified by sequencing genomic DNA of hESC lines T2 and T3, whereas the cDNA sequencing of undifferentiated cells and teratoma from hESC line T2 showed only A allele. However, the cDNA of undifferentiated cells from hESC line T3 detected the full expression of G allele and partial expression of A allele, indicating the partially relaxed imprinting of IGF2 gene. Further, the embryoid bodies of hESC line T4 (EB4) showed equal expression of both A and G alleles, indicating no imprinting of IGF2 gene (see Note 20).

Expression levels of the 32 known imprinted genes from five undifferentiated hESC lines, T4 embryoid bodies (EB4) and T1 teratoma (TT1) were analyzed using Affymetrix human genome U133 plus 2.0 GeneChip (19). Ten imprinted genes, namely, GRB10, PEG10, SGCE, MEST, SDHD, SNRPN, SNURF, NDN, IPW, and NESP55, were found to be highly expressed in the undifferentiated hESC lines and down-regulated in differentiated derivatives (T4 embryoid bodies and T1 teratoma). The UBE3A gene abundantly expressed in undifferentiated hESC lines, and further up-regulated in differentiated tissues (T4 embryoid bodies and T1 teratoma).

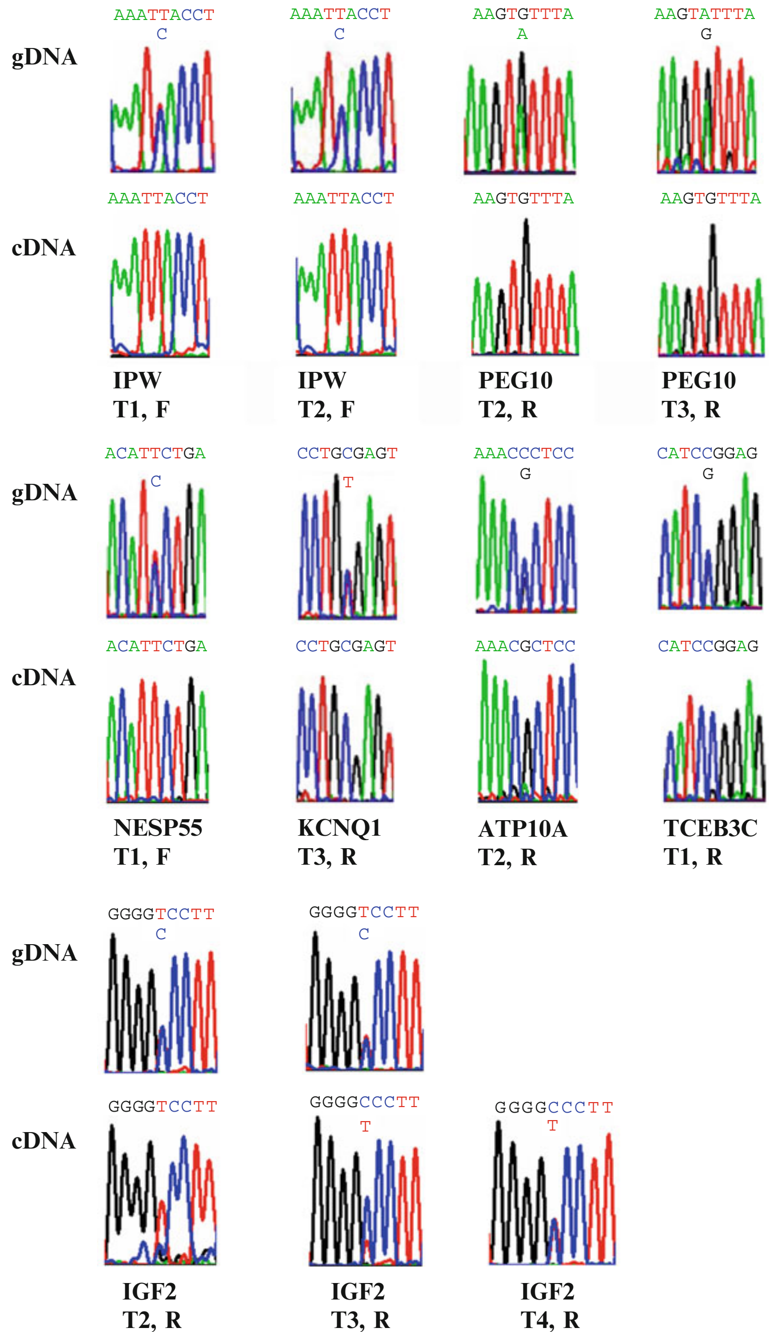

Fig. 3. Allele-specific expression of seven imprinted genes. The heterozygous alleles of SNPs at seven genes IPW, PEG10, NESP55, KCNQ1, ATP10A, TCEB3C, and IGF2 genes were found by sequencing genomic DNAs (gDNA) of hESC lines T1, T2, T3, and T4 (Table 4). The cDNA sequences of undifferentiated cells and teratomas from hESC lines T1, T2, and/or T3, as well as embryoid bodies of hESC line T4, were determined using either forward (F) or reverse (R) primers (Table 1). It may be noted that the peak height of heterozygous alleles (nucleotides) was lower than that of homozygous allele (nucleotide) in addition to two different colors instead of single color. When the noninformative homozygotes at SNPs were detected in genomic DNAs, no cDNA sequencing was carried out. This figure is adapted with permission from Li et al. (19).

The expression levels of other 21 imprinted genes were relatively low in undifferentiated hESC lines, and six of them (TP73, COPG2, OSBPL5, IGF2, ATP10A, and PEG) were found to be up-regulated in differentiated tissues (T4 embryoid bodies and T1 teratoma) (see Note 21).

4. Notes

1. The experimental protocols using human embryos were approved by the IRB of Kaohsiung Medical University Hospital.
2. The Affymetrix GeneChip contains 54,675 probe sets to analyze the expression level of 47,400 transcripts and variants, including 38,500 well-characterized human genes.
3. The established hESC lines may also be grown on either feeder-free Matrigel-coated dish in MEF-conditioned medium with additional 4 ng/ml bFGF or hESC-differentiated fibroblast-like cells as feeder.

   The feeder-free culture dish was coated with Matrigel diluted with DMEM/F12 (1:30) overnight at 4°C. The BD Martigel™ (Matrix 354234) is the manufacturer's Trademark for extracellular matrix extracted from the Engelbreth-Holm-Swarm tumor. The MEF-conditioned medium is prepared as described previously (22). The MEF cells were cultured in MEF medium overnight, and the mitotically inactivated MEF cells were maintained in hES medium containing 4 ng/ml bFGF. After 24 h, the MEF-conditioned medium was collected and filtered through 0.2 um membrane (PN4612, Pall Life Sciences). The culture dish was coated with Matrigel diluted with DMEM/F12 (1:30) overnight at 4°C.

   The hESC-differentiated fibroblast-like cells with capacity to support the growth of undifferentiated hESCs were established according to the previously published procedure (23). The hESC-T3 of female normal karyotype (passage 19) cells were transferred into feeder-free and noncoated plate (10 cm) in DMEM supplemented with 10% FBS (GIBCO) under 5% CO_2 at 37°C. After 10 days, cells appeared as fibroblast-like morphology, that is, flat cells with elongated nucleus and branching pseudopodia. These *h*ESC-*T3* *d*ifferentiated *f*ibroblast-like cells are designated as T3HDF. The expression of transcription factors OCT4, SOX2, and NANOG, which were highly expressed in hESC-T3 cells, was shown to be down-regulated in differentiated T3HDF cells. The expression profiles of mRNAs, microRNAs, and proteins between hESC-T3 and T3HDF cells were also found to be very different (S.S.-L. Li, unpublished). The T3HDF cells were passaged

using trypsin (0.05%, GIBCO) every 4 days or cryopreserved. After inactivation using mitomycin C (10 μg/ml), T3DF cells (passage 8) as feeder have been shown to support the undifferentiated growth of hES-T3 cells (34 passages on MEF) for more than 14 passages (24).

4. The hESC medium may be changed every two days, but 4 ng/ml bFGF should be added when hESC medium is not changed daily.

5. The karyotypes of hESC lines were analyzed by the Cytogenetics Laboratory of Kaohsiung Medical University Hospital.

6. The experimental protocol was approved by the Laboratory Animal Management Board of Kaohsiung Medical University.

7. The histological sections of teratoma were examined after hematoxylin and eosin staining by the Pathology Laboratory of Kaohsiung Medical University Hospital.

8. The gene expression profiling of single human oocyte, 4-cell and 8-cell embryos were successfully analyzed using the same amplification procedure (12).

9. The Affymetrix GeneChip expression analyses were done by the DNA Microarray Core Facility of National Taiwan University College of Medicine for Genomic Medicine Program of National Science Council in Taiwan. It may be noted that Affymetrix GeneChip expression analysis can be used as a stand-alone quantitative comparison, since the correlation between Affymetrix GeneChip results and TagMan RT-qPCR results was shown in a good linearity of $R^2 = 0.95$ by the MicroArray Quality Control Study, a collaborative effort of 137 scientists led by the US-FDA (25, 26).

10. Li et al. (18) reported that two of these five hESC lines were derived from six day 5 embryos (2/6 = 33%), and the other three hESC lines were from 19 day 6 embryos (3/19 = 16%). No hESC line was obtained from seven day 7 embryos (0/7 = 0%). Mitalipova et al. (27) reported that four hESC lines were derived from 19 discarded blastocysts in which three hESC lines were from nine day 6 blastocysts (3/9 = 33%) and one hESC line was from ten day 7 blastocysts (1/10 = 10%). Although the sample sizes in these two studies were relatively small, these results appear to indicate a decreasing ability of the slowly developing blastocysts to give rise hESC lines.

11. The hESC line T2 at passage 37 was found to possess very high frequency of trisomy 12 (47XY,+12). The trisomy 12 was previously reported to increase in long-term cultured hESC lines (3, 28). The existence of chromosomal abnormalities in hESC lines is detrimental for the future use of transplantation therapy because of potential oncogenesis.

12. The hESC line T4 failed to produce teratoma in 4 SCID mice, while teratomas were induced using the same procedure at the same time in the same SCID mice by other hESC lines T1, T2, T3, and T5. The hESC line T4 failed again to form teratoma in another 4 SCID mice in the repeated experiment.
13. 4,145 genes were found to be expressed in all single colonies of five hESC lines derived in Taiwan (18); 3,031 genes of them (73%) were also identified among the 7,385 genes commonly expressed in all three hESC lines HSF-1, HSF-6, and H9 previously reported using Affymetrix HG-U133A and B GeneChips containing 44,794 gene probes (29). Some of the non-overlapped 1,114 genes are due to the absence of gene probes on Affymetrix HG-U133A and B GeneChips. These two studies used similar Affymetrix GeneChips, but different cDNA amplification procedures to analyze gene expression profiles of different hESC lines grown on different culture conditions. The high frequency of common genes exclusively expressed in our five hESC lines (18) and those three hESC lines reported previously (29) indicates that these genes are important for maintaining hESC phenotype, i.e., unlimited self-renewal and pluripotent differentiation. These results also confirm that the linear amplification procedure described previously (12, 18) can indeed be used for the gene expression profiling among single colonies of hESC lines.
14. The gene expression profiles of hESC lines and/or their in vitro differentiated embryoid bodies have been analyzed using cDNA microarray (8, 30, 31), oligonucleotide microarrary (32–39), EST (expressed sequence tags) (40), SAGE (serial analysis of gene expression) (41), and MPSS (massively parallel signature sequencing) (42). The results of these approaches show that approximately 25% of genes remain uncharacterized in the hESC lines representative of early embryo development. The transcriptional profiles of human oocytes and preimplantation embryos were reported to have more than 40% of their transcripts without known functions (11). In our report (18), 19% of 4,145 genes commonly expressed in all five hES cell lines (Fig. 1) and 15 genes (37.5%) of the 40 potential embryonic stemness genes do not have known functions (Table 2). The roles of these genes without known functions during early embryo development remain to be elucidated in the future.
15. The expression of H-2 minor antigen (DDX3Y) and H-Y antigen (JARID1D) in differentiated cells will have significant implications in the future use of hESC lines for cell-based regenerative or reparative therapy. On the other hand, the 11 testis-specific genes (i.e., TSPY, testis-specific protein Y; DAZ, deleted in azoospermia) of ampliconic region of chromosome Y (21) were not detected in the male hESC lines T2, T4, and T5.

16. DPPA2 gene was recently shown to play a role in maintenance of the undifferentiated state and proliferation of mouse ES cells (43).
17. The 97 genes exclusively expressed by both five hESC lines and T1 teratoma are also likely to contain some stemness genes such as SOX2 and FGF2, since teratoma cells also contain undifferentiated stem cells.
18. The TGFβ superfamily members control many aspects of early embryonic development and signal through two main branches, TGFβ/activin and BMP/GDF. The TGFβ/activin branch components ligand inhibin BC, type II receptor ACVR2A, type I receptor ACVR1 (ALK2) and TGFBR1 (ALK5), and SMAD 2, as well as SMAD4, were found to be highly expressed in undifferentiated hESC lines and decreased upon differentiation. Conversely, significant increase of BMP7, GDF3, GDF15, and NODAL in BMP/GDF branch signaling via BMPR2 and BMPR1B was observed in T4 embryoid bodies, T1 teratoma, and placenta. These results were consistent with the report that TGFbeta/activin signaling is necessary for the maintenance of pluripotency in human embryonic stem cells (44, 45).
19. The monoallelic expression of PEG10, NESP55, and KCNQ1 genes was also reported previously in hESC lines (14, 15).
20. The IGF2, as well as H19 in the same chromosomal region 11P15.5, was also reported to be more variable and thus could potentially provide a sensitive indication of epigenetic status of hESC lines (16). The IGF2 gene was also shown to be only partially imprinted in human germ cell-derived lines (46).
21. The expression of imprinted genes plays important roles during early embryo development. The hESC lines and their differentiated derivatives offer an opportunity for studying the expression of different imprinted genes shortly before and after the embryonic implantation. The expression levels of the 32 known imprinted genes were relatively consistent among five hESC lines. It may be noted that five (SNRPN, SNURF, NDN, IPW, and UBE3A) of 11 highly expressed imprinted genes in undifferentiated hESC lines are located on chromosomal region 15q11-q13, and that abnormal expression of SNRPN and NDN genes results in the neurogenetic disorder known as Prader-Willi Syndrome (47).

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Chapter 9

Derivation of Human Embryonic Stem Cell Lines from Poor Quality Embryos

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Abstract

A serious shortcoming in the derivation of human embryonic stem cell (hESC) lines has been the availability of human embryos. About 60% of human embryos generated by in vitro fertilization (IVF) fail to develop normally and are unusable for fertility treatment. Such embryos often retain sufficient pluripotent cells that can generate genetically normal, pluripotent hESC lines with stable phenotype. We describe here a simple protocol for isolating pluripotent stem cells from abnormally developed grade III human embryos that are an unutilized byproduct of in vitro fertility treatment. Embryos that progress to the blastocyst stage are subjected to immunosurgery or mechanical surgery to isolate the inner cell mass (ICM). Isolated cells are plated on to fibroblast feeders in hESC derivation media. Pluripotent cells that grow from the ICM are isolated mechanically and cultured to obtain a stable hESC line. In this way, we derived two sibling hESC lines BJNhem19 and BJNhem20 that represent the Indian ethnic background and show stable phenotype upon long-term continuous culture of over 225 passages.

Key words: Pluripotent, Human ES cell, Poor quality embryo, hES cell derivation, Blastocyst, BJNhem19, BJNhem20, Sibling hES cell lines

1. Introduction

The utility of hESC lines in understanding mechanisms of human development and disease progression is well established. In just over a decade since the first derivation of hESCs was reported (1), their cell products are already in use in clinical trials (Geron, http://www.geron.com), reaffirming their tremendous application to regenerative medicine. While recently described reprogramming methods allow the generation of induced pluripotent stem cells (iPSCs) from somatic cells without the use of human embryos (2), hESCs still remain the accepted standard for pluripotency. They are a genetically unmanipulated and hence a safer cell type.

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Additionally, efficient generation of bonafide iPSCs requires a good understanding of the nuances of hESC derivation and culture. Supernumerary human embryos of good quality are difficult to obtain due to their primary requirement in fertility treatment. In vitro fertilization (IVF) also generates abnormally developed embryos that are not usable for treatment and discarded. Such abnormal embryos are easier to obtain with informed consent and appropriate ethical clearance for use in hESC research. Several hESC lines have been derived from poor quality embryos (3–6). Thus, a more accessible resource is available for researchers to train in derivation and culture of human pluripotent cells.

In this chapter, we detail the approach and technique required to efficiently obtain and propagate hESC lines from grade III human blastocysts. Detailed characterization of derived hESC lines is described in several research papers (7–12) and protocols and is beyond the scope of this chapter. We believe that the information given here will help even the novice to successfully practice and derive pluripotent cells.

2. Materials

2.1. Derivation of hESC Lines

2.1.1. Cell Culture Media

Dulbecco's phosphate-buffered saline (DPBS), Ca^{2+}- and Mg^{2+}-free (Gibco; Cat. No. 14190144).

Mineral oil embryo grade (Sigma; Cat. No. M5904).

Sterile ultrapure water, embryo grade (Sigma; Cat. No. W1503).

Acid Tyrode's solution (AT) (Sigma; Cat. No. T1788) is aliquoted and stored at –80 °C.

Rabbit antihuman antiserum and guinea pig serum complement (Sigma Chemical Co., USA; Cat. No. S1639) are aliquoted and stored at –80°C.

Mouse embryonic fibroblast (MEF) media: Dulbecco's modified Eagle's medium (DMEM) (GIBCO; Cat. No. 10313021) supplemented with 10% fetal bovine serum (FBS) (Hyclone. South Logan, UT, USA; Cat. No. SH3007003).

Mitotically inactivated MEFs (ATCC SCRC-1040).

To prepare basic fibroblast growth factor (bFGF) (Sigma; Cat. No. F0291) working solution, 10 μg of bFGF is reconstituted in 1 ml of 0.1% fraction V bovine serum albumin (BSA, Sigma; Cat. No. A8806 prepared in sterile DPBS). Aliquot 0.1 ml into sterile tubes and store at –80°C. Use 32μl of the reconstituted bFGF in 100 ml of hESC medium.

hESC derivation media: Knockout DMEM (Invitrogen; Cat. No. 10829018) supplemented with 5% Knockout Serum Replacement (Invitrogen; Cat. No. 10828028), 5% FBS (Hyclone, South Logan, UT, USA; Cat. No. SH3007003), 2 mM L-glutamine

(Invitrogen; Cat. No. 25030-081), 0.1 mM β-mercaptoethanol (Invitrogen; Cat. No. 2185-023), 1% nonessential amino acids (Invitrogen; Cat. No. 11140050), 8 ng/ml bFGF, 20 ng/ml human leukemia inhibitory factor (LIF) (Sigma; Cat. No. L5283), 1× antibiotic–antimycotic (Invitrogen; Cat. No. 15240062) (see Note 1).

DMSO (Sigma Chemical Co; Cat. No. D2650).

2.1.2. Cell Culture Supplies

Organ culture dish (BD Falcon; Cat. No. 353037).

Dissection microscope/stereozoom microscope with warm stage.

Glass Pasteur pipettes (Sigma; Cat. No. S6286).

35 mm tissue cultures dishes (BD Falcon; Cat. No. 353001).

Disposable serological pipettes: 10 ml (Greiner; Cat. No. 607180); 5 ml (Greiner; Cat. No. 606180) and 1 ml (Nunc; Cat. No. 159609).

Glass Pasteur pipettes (Sigma; S6268).

Spirit lamp and capillary cutter.

Flexible autoclavable tubing with mouthpiece and capillary holder.

0.22 μm syringe filter (Sartorius stedim, Cat. No.16634).

Gelatin (Sigma; Cat. No. G1393): 0.1% (w/v) in water. Autoclave to sterilize.

2.2. Pluripotency Marker Analysis

Fixative: 2% paraformaldehyde in DPBS.

Staining media: 3% FBS in PBS.

Antibodies are diluted in 1% FBS

Antibodies specific to pluripotency antigens, such as OCT4 (BD Pharmingen; Cat. No. 611202); Stage-specific embryonic antigen-4 (SSEA-4) (Chemicon; Cat. No. MAB4304); TRA-1–60 (Chemicon; Cat. No. MAB4360); TRA-1–81 (Chemicon; Cat. No. MAB4381). Alexa Fluor-conjugated secondary antibodies (all from Molecular Probes, USA): goat anti-mouse IgG 568-Cat. No. A11004; goat anti-mouse IgG 488-Cat. No. A11001.

2.3. Routine Culture and Maintenance of hESC Lines

2.3.1. Cell Culture Media

hESC culture media: Knockout DMEM (Invitrogen; Cat. No. 10829018) supplemented with 20% Knockout Serum Replacement (Invitrogen; Cat. No. 10828028), 2 mM L-glutamine (Invitrogen; Cat. No. 25030081), 0.1 mM β-mercaptoethanol (Invitrogen; Cat No. 2185-023), 1% nonessential amino acids (Invitrogen; Cat. No. 11140050), 8 ng/ml bFGF.

2.3.2. Cell Culture Supplies

Pasteur pipettes (Sigma; Cat. No. S6268).

35 mm dish (BD Falcon; Cat. No. 353001).

To prepare 1× Trypsin-EDTA working solution, 10× Trypsin-EDTA (Invitrogen; Cat. No. 15400054) is diluted to 1× with DPBS just before use.

DPBS.

DMEM.

2.4. Freezing Human Embryonic Stem Cells at Early Passage by Vitrification

2.4.1. Vitrification Media

Holding Media: DMEM containing 1 ml of 1 M HEPES buffer (Invitrogen; Cat. No. 15630106), 10 ml FBS and 0.5 ml 100× NEAA (Invitrogen; Cat. No. 11140050) made up to 50 ml.

Vitrification solution (VS1): To 8 ml Holding media add 1 ml DMSO (Sigma; Cat. No. D2650) and 1 ml Ethylene glycol (Sigma; Cat. No. E9129).

Vitrification solution (VS2): To Holding media add 2 ml DMSO, 2 ml Ethylene glycol, and 1.71 g Sucrose. Make up the volume to 10 ml with Holding media.

Thawing media: 0.684 g Sucrose dissolved in Holding media. Make up the volume to 10 ml.

2.4.2. Vitrification Supplies

Cryocan and cryocane.

Glass pipette or capillaries pulled to about 300 μm in diameter.

Dissection microscope/stereozoom microscope with warm stage.

Organ culture dish (BD Falcon; Cat. No. 353037).

90 mm Petri dish.

5 ml Cryovials (Nunc; Cat. No. 366656), punctured with a needle.

Embryo freezing straws (sterile).

Organ culture dish.

3. Methods

3.1. Generation of Tools for Embryo Transfer and hESC Manipulation (see Note 2)

1. Embryo transfer pipette: Heat a capillary or Pasteur pipette over a low blue flame till the glass softens. Remove it from the flame and pull slowly to generate a narrow tube of 150–200 μm. Cut the tube midway with a glass cutter to generate two capillaries. Fire–polish the tip by holding it at the base of the flame for 1–2 s. Make several transfer pipettes ahead of time.
2. Microneedles: Heat a capillary or Pasteur pipette over a low blue flame till the glass softens. Pull the capillary ends apart quickly to generate a fine tip of 15–20 μm for removing the trophectoderm or 5–10 μm for manipulating the ICM. As Grade III embryos and their ICMs vary greatly in size, keep microneedles in a range of sizes handy.
3. Colony cutting capillaries: Make microneedles as above. Aim for a thickness of about 100 μm. The speed of pulling will determine the thickness of the capillary. Pass the tip quickly over the top part of the flame to create a J shaped tip.

3.2. Preparation of MEF Feeder Dishes

1. Gelatin coat culture dishes for 2 h before use, then remove excess gelatin.

2. Thaw a vial of mitotically inactivated MEFs into 10 ml MEF media, spin at 1,000 rpm (about $100 \times g$) for 2 min and resuspend in MEF media to get desired cell concentration.
3. Plate 50,000 cells/cm^2 onto gelatin-coated dishes and allow cells to attach for at least 8 h (see Note 3).
4. Replace MEF media with hESC derivation media and incubate for at least 24 h to precondition the medium. The dishes may be used up to 5 days later for plating hESCs (see Note 4).

3.3. Derivation of Human Embryonic Stem Cells (10)

All embryo manipulations are done by viewing through a stereo-zoom microscope equipped with a warm stage. Embryos, cultures, and reagents must be kept at 37°C at all times unless mentioned otherwise.

1. Transport embryos from IVF clinic to laboratory in drops of human embryo medium used by the clinic, under a layer of mineral oil in an embryo culture dish. Place the dish containing embryos in a 10 cm Petri dish, seal it with Parafilm and place in a warm light-proof box. Embryos must be returned to a CO_2 incubator as soon as possible, within 1 h (see Note 5).
2. Examine and photograph embryos and assign a number to each embryo for future reference.
3. Let embryos recover for at least 2 h at 37°C, 5% CO_2. Leave early stage embryos until they become expanded blastocyst or show no further development.
4. Prepare microscope and warm stage for use in the biosafety cabinet. Attach embryo transfer pipette to the end of sterile tubing fitted with a capillary holder and 0.2 μm filter (see Fig. 1).

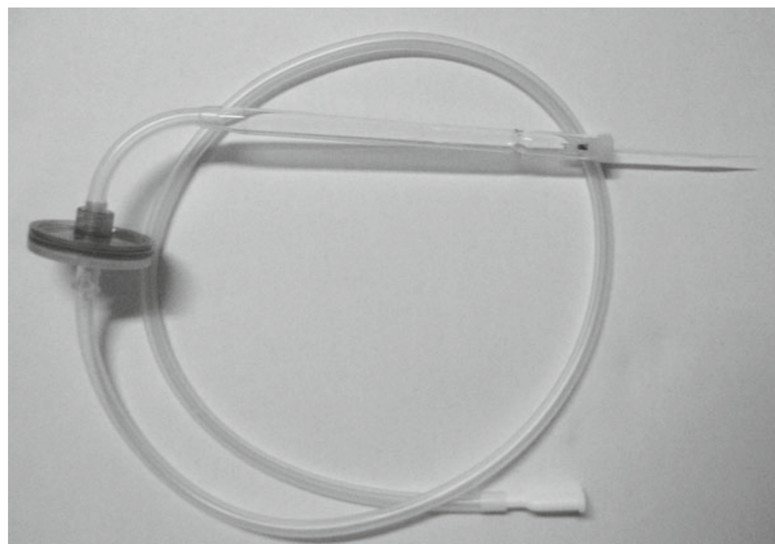

Fig. 1. Embryo transfer pipette fitted to the end of sterile tubing with a capillary holder and 0.2 μm filter.

5. Prepare a 10 cm Petri dish with two 50 μl microdrops each of DPBS, acid Tyrode's solution (AT) and MEF medium.
6. Transfer embryo to be used for derivation to a fresh drop of DPBS. Move embryo to drop 2 of DPBS and ensure that any mineral oil is not carried over. Examine carefully and observe the zona pellucida and ICM.
7. Transfer embryo to drop 1 of AT, rinse by pipetting once and transfer to drop 2 of AT. Observe embryo and as soon as zona pellucida starts dissolving (about 20 s), pipette it into drop 1 of MEF media (see Note 6).
8. Rinse zona-free embryo in MEF media and transfer it to drop 2 of MEF media and return it to the incubator in a covered dish, ensuring that it will not dry out (see Notes 7 and 8).
9. Set up two 50 μl microdrops each of DBPS and guinea pig serum complement in a 35 mm Petri dish.
10. Remove the zona-free embryo from incubator and pipette it into drop 1 of DPBS. Rinse and transfer to drop 2 of DPBS (see Note 9).
11. Transfer embryo to drop 1 of guinea pig serum complement, rinse and transfer to drop 2 for 30 min at 37°C, 5% CO_2 (see Note 10).
12. Meanwhile set up two microdrops each of DPBS, anti-human antiserum and MEF media in a fresh 35 mm dish and equilibrate at 37°C, 5% CO_2.
13. After complement incubation, remove dishes containing embryo and fresh microdrops from incubator.
14. Transfer embryo to drop 1 of PBS, rinse and transfer to drop 2 DBPS for 5 min.
15. Transfer embryo to drop 1 of antiserum, rinse and transfer to drop 2 at 37°C, 5% CO_2. Observe embryo every 5 min and monitor for lysed trophectoderm (TE).
16. When most of the TE cells are lysed or after a maximum of 20 min wash embryo through two drops of DPBS for 2 min each and transfer to MEF media.
17. After immunosurgery, transfer embryo to an embryo culture dish containing a mitotically inactivated MEF feeder layer.
18. Using two fine microneedles of about 20 μm, position the embryo with the ICM region to the right. If the ICM is discernable, cut the embryo into two parts as close to the ICM as possible and discard the TE part. If the ICM is not apparent cut the TE to open up the blastocyst.
19. Using a 20 μm microneedle to hold the TE in place, dissect out as much of the ICM as possible using a 10 μm microneedle.

20. Remove any cells that are clearly TE from the dish and leave the remaining cells in the dish. Add 2 ml of sterile embryo grade water to the edge of the dish around the center well to maintain humidity (see Note 11).
21. Return the dish containing the ICM to the incubator and leave undisturbed for at least 4 days.
22. After 4 days give a 50% medium change every alternate day while viewing the dish through the microscope to avoid disturbing any cells that may have attached.
23. After 6–7 days a small ICM outgrowth should be visible surrounded by TE cells (see Note 12).
24. Allow ICM to grow for 8–14 days since plating but before it starts showing signs of overt differentiation. Cells should be of uniform size, with a high nuclear:cytoplasmic ratio and smooth edge to the colony.
25. Cut ICM into two to four pieces using a cutting capillary and transfer to a fresh feeder dish in hESC derivation media. This is passage number 1. Leave behind a bit of the original outgrowth and allow it to regrow (see Note 13).
26. Leave the dishes undisturbed for 2 days and then feed every alternate day. A pluripotent colony should be apparent in 3–4 days and should be ready to cut again in 5–7 days.
27. After the third passage, omit FBS from the growth medium and replace with hESC culture media. After 25 passages or when sufficient stocks have been made and tested, cultures can be grown without antibiotics.
28. After passage number 2, vitrify one to two colonies and test the stocks by thawing to ensure that a usable early passage stock is generated.
29. After passage number 5, cells may be plated on to 35 mm feeder dishes if the cell number is sufficient. By this time, the colonies should be ready to cut in 3–4 days.

3.4. Cryopreservation of Early Passage hESCs by Vitrification (13) (see Note 14)

1. Feed the dish containing good undifferentiated hESC colonies that are ready to be passaged, with fresh hESC culture media 2–4 h before vitrification.
2. Label cryovials, open, fix to a cryocane and partially immerse in liquid nitrogen.
3. Label straws at the broad end with cell line name, passage number, and straw number.
4. On the lid of a 90-mm Petri dish, place a 100 μl drop of holding medium, one 20 μl drop of VS1, and two 20 μl drops of VS2.
5. Cut the colony in pieces and transfer to an organ culture dish containing holding medium.

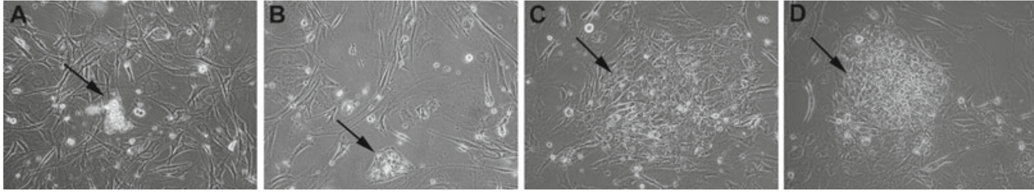

Fig. 2. Appearance of colonies after vitrified hESCs are thawed onto MEFs. *Arrow points* to colony at (**A**) day 0, (**B**) day 2, (**C**) day 4, and (**D**) day 5 after thawing. Note the decrease in feeder density over time.

6. Use an embryo transfer pipette for all subsequent manipulations.
7. Place four or five colony pieces into the 100 μl drop of holding medium.
8. Collect the pieces in a minimal volume and transfer them to the VS1 drop. Leave for 1 min.
9. Then, transfer the pieces in a minimal volume to drop 1 and then drop 2 of VS2 for 20 s each.
10. Collect pieces in 3 μL of VS2 and make a small drop.
11. Touch the narrow end of a straw to the drop and collect all pieces into the straw by capillary action.
12. Very quickly plunge the straw into the vial of liquid nitrogen and cap the vial.
13. Transfer to a liquid nitrogen storage tank without letting the straws thaw.
14. To thaw vitrified cells for culture, remove the straw from liquid nitrogen, plunge quickly into a dish containing holding medium and let the pieces fall out into the medium. Transfer pieces to an appropriate feeder dish containing hESC culture media and leave undisturbed for 3–4 days.
15. Each thawed piece should attach within 2 days and should start forming an undifferentiated colony (see Fig. 2).
16. Monitor the dish and feed every other day till the colonies are ready to passage (7–10 days).

3.5. Preliminary Characterization of ICM-Derived Cultures

Once the ICM-derived culture reaches passage no. 2 or later, preliminary characterization for pluripotency markers can be done.

1. Set up MEFs in hES derivation medium on a 4-well dish as described before. Place two to four colony pieces in each well and culture until colonies are ready to passage.
2. Wash wells in PBS and fix in paraformaldehyde for 20 min.
3. Wash off fixative with PBS and add staining media. Incubate at 37°C for 1 h.

4. Replace staining media with appropriate primary antibody diluted in 1% FBS. Incubate at 37°C for 1 h.
5. Wash off antibody with PBS and then add appropriate secondary antibody diluted in staining media. Incubate at 37°C for 1 h (in dark).
6. Wash off antibody with PBS. Observe staining for pluripotency marker using a fluorescence microscope.

   This preliminary analysis can be done as soon as spare colonies are available and even on regrown dishes. Once a growing ICM culture is stabilized and sufficient cells are available, detailed pluripotency analysis can be done as described elsewhere (8–12).

4. Notes

1. Cells should be grown in antibiotic-free media after the initial stocks are made as the use of antibiotics could affect hES characteristics over long-term culture.
2. Use of quick, practiced steps and tools that the user can adapt to their individual needs is a key to efficient hESC derivation. A primary requirement in handling human embryos is the skill required to manipulate them in the desired manner without damaging or losing the required embryonic cells. Hence, it is worth investing sufficient time in practicing how to view, transfer and manipulate embryos even before starting on hESC derivation. Depending on availability, spare human embryos or mouse embryos or microbeads can be used for practice. Mouse embryos are an excellent alternative to train in all manipulations required for hESC derivation.
3. Feeder density greatly affects how pluripotent cells will grow. High density feeders promote the growth of thick colonies that are prone to differentiation. Medium and low-density feeders promote uniform growth and typical hESC morphology (see Fig. 3).
4. The feeder density decreases further in hES media as the cells detach due to the absence of serum. Interestingly, as the feeder density decreases, colonies grow better, probably due to matrix deposited by the displaced fibroblasts.
5. Portable CO_2 incubator may be used where available.
6. Poor quality embryos often have very thick zona pellucida and may require longer incubation in AT. If the zona is not dissolved in 30 s, transfer the embryo to PBS and try to dissect out the ICM straight away without immunosurgery.

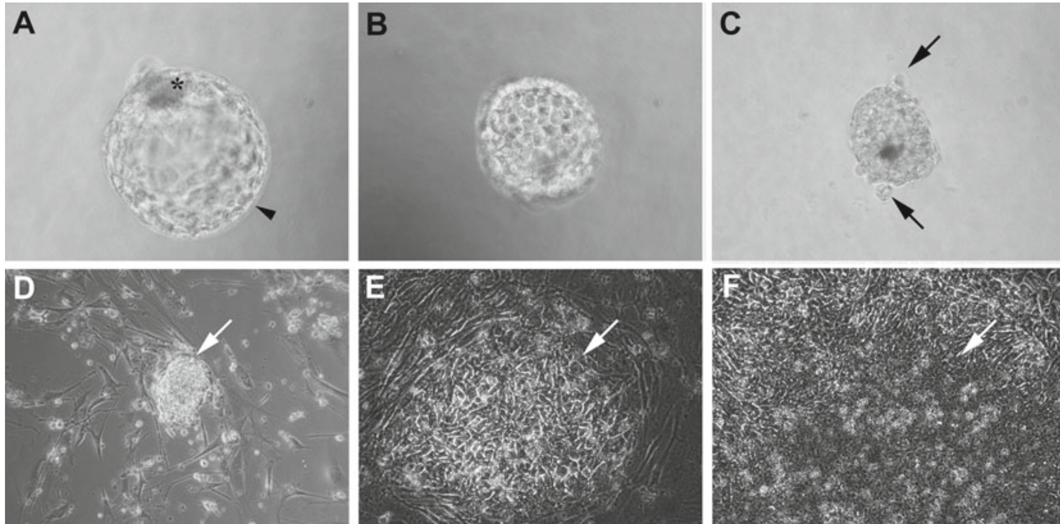

Fig. 3. (**A**–**C**) Poor quality human blastocyst at various stages of derivation. (**A**) The ICM has some dead cells and appears dark (*asterisk*). The translucent zona pellucida is marked by an *arrowhead*. (**B**) Blastocyst after acid Tyrode's treatment. (**C**) Blastocyst after immunosurgery. *Arrows point* to lysed TE cells. (**D**–**F**) ICM (*white arrow*) cultured on MEF feeders seen at day 5 (**D**), day 9 (**E**), and day 12 (**F**) of culture. Note the cells of uniform size and with a high nuclear:cytoplasmic ratio in (**F**).

7. If embryo is to be cultured whole, transfer it to a MEF feeder dish with hESC derivation media.
8. With some practice the ICM can be dissected out directly after zona removal without immunosurgery. This can be done directly on a feeder dish and requires minimum manipulation of the embryo which helps maintain cell health. Care should be taken to remove most of the TE cells as they could cause the ICM to differentiate.
9. Before starting complement treatment make sure the embryo has an intact TE. A ruptured TE will allow complement to bind ICM cells, which also get lysed on serum treatment. If the TE is damaged, isolate the ICM mechanically, without immunosurgery.
10. Complement activity may vary with each batch and upon storage. It is advisable to test various dilutions of each new batch.
11. It is worth using good quality water as in case it accidentally spills into the culture well, embryo/ICM health will not be affected. However, sterile cell culture grade water may also be used.
12. If the TE is overgrowing the ICM, the TE can be separated away using a microcapillary. This helps prevent ICM differentiation.
13. Do not discard any dishes in the initial stage. They can be healthy for at least a month.

14. Pluripotent hESCs do not survive bulk freezing in the initial stages of derivation as they are dissociated as colony pieces/clumps and not single cells. Vitrification is a quick and efficient way to make a large batch of stocks especially when colonies available are limited. Vitrification can be done as early as p2. One large pluripotent colony of 0.5 mm diameter can be vitrified to generate at least five straws.

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Chapter 10

Derivation, Expansion, and Characterization of Human Embryonic Stem Cell Lines from Aneuploid Embryos

Juan-Carlos Biancotti and Neta Lavon

Abstract

Human embryonic stem cells (hESCs) are an invaluable cell source to study human embryogenesis and development and for exploring the nature of human diseases. Moreover, hESCs can serve as an unlimited source of cells for cell therapy. The first hESC lines were derived from frozen blastocyst-stage embryos. In the past 12 years, the field evolved and the hESC lines are derived from pre-embryos in various developmental stages using several techniques. In parallel, the wide use of hESCs triggered the development of materials and methods for expansion of the cell lines derived. Here, we describe our method for derivation, expansion, and characterization of hESC lines from pre-embryos that were diagnosed to carry aneuploid cells and were destined to be discarded.

Key words: Blastocyst, Human embryonic stem cells, hESC, Derivation, Expansion, Characterization, Aneuploidy, Trisomy, Preimplantation genetic screening

1. Introduction

Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of preimplantation embryos (1). hESCs have the unique properties of unlimited proliferation and capacity to differentiate into the cells comprising the entire human body (2, 3). These cells are used for studying human embryogenesis and disease modeling and as a source for cell therapy. hESCs are commonly isolated from frozen surplus embryos obtained from in vitro fertilization (IVF) treatments (1, 4). Most pre-embryos are donated for research by couples that have finished their IVF program. Other sources of embryos for research are poor-quality embryos, morula-stage embryos, and single blastomeres (5–7).

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We have used another source of preimplantation embryos for derivation of hESCs. These embryos were studied by preimplantation genetic screening (PGS) and were found to be aneuploid, having abnormal number of chromosomes. They were destined to be discarded unless donated for research. The embryos were biopsied at the eight-cell stage, corresponding to 3-day old embryos. One or two blastomeres were removed and analyzed using single-cell fluorescent in situ hybridization. By the time the embryo reached its blastocyst stage (5-day old embryo), the healthy euploid embryos were identified and separated, and the abnormal aneuploid embryos were donated for research. We have shown that we can derive hESC lines from blastocyst-stage embryos that were diagnosed by PGS as aneuploid (8–10). Some of the cell lines derived resulted in euploid hESCs carrying a normal karyotype (9, 10). We have shown that the euploidy was not achieved through chromosome duplication. Alternatively, we suggested that the euploid hESC lines originated from mosaic embryos consisting of aneuploid and euploid cells, and in vitro selection occurred to favor euploid cells (9). We have further shown that these embryos are also source for aneuploid hESC lines with either extra or missing chromosomes (8). The resulting hESC lines carried either trisomy 13 (Patau Syndrome), 16, 17, 21 (Down Syndrome), X (Triple X Syndrome), or monosomy X (Turner Syndrome) (8). These aneuploid hESC lines are an invaluable source of cells for studying syndromes caused by these abnormalities. All the hESC lines exhibited morphology and markers typical of hESCs and the capacity for long-term proliferation. The derived hESC lines manifested pluripotent differentiation potential both in vivo and in vitro (8–10).

In this chapter, we describe the methods used in the derivation of 25 hESC lines from embryos that were prebiopsied for PGS. We developed an alternative method to separate the blastocyst from the zona pellucida by spontaneous hatching through the orifice created during the biopsy. We also detail our method of enzymatic passage for expansion of the cell culture allowing their large-scale production and frozen storage. And lastly, we describe our methods for characterization of hESCs using in vitro and in vivo methodologies.

2. Materials

2.1. Derivation and Cell Culture Components

1. Blastocyst media supplemented with HSA (G-2V5 plus; Vitrolife, Englewood, CO, USA).
2. Anti-human whole serum (developed in rabbit; catalog number H3383; Sigma, Saint Louis, MO, USA).

3. Light mineral oil for embryo culture (catalog number 9305; Irvine Scientific, Santa Ana, CA, USA).
4. Stripper, device for the manipulation of embryos with minimal amount of fluid transfer (catalog number MXL3-STR; Mid Atlanta Diagnostics, Mount Laurel, NJ, USA).
5. Barrier filter mouth pipette: Insert a 0.22 μm syringe filter close to the distal end of an aspirator tube assembly (catalog number A5177; Sigma, Saint Louis, MO, USA). Using a burner, heat the thin part of a glass Pasteur pipette to fold it approximately at 45° angle. Repeat the heating more distally just before the glass melt and pull both sides apart to make the glass tube very thin. Cut the glass around 1 in. from the fold, and flame the tip on the burner to round the edges of the glass tube, paying attention not to block the path. Autoclave the glass pipettes. When needed for use, connect the thick end of the glass pipette to the distal end of the latex tube.
6. Guinea pig complement serum (catalog number S1639; Sigma, Saint Louis, MO, USA).
7. Gelatin (from porcine skin, type A, catalog number G1890; Sigma, Saint Louis, MO, USA), 0.1% solution. Add 0.3 g of gelatin to 300 ml of double distilled water (DDW) in a 500 ml bottle. Close the cap, although not tight, and sterilize in autoclave. It can be kept at room temperature for several weeks (see Note 1).
8. Mitomycin-treated mouse embryonic fibroblasts (M-MEF).
9. MEF media: Add 50 ml of fetal bovine serum (Omega Scientific, Tarzana, CA, USA) and 2.5 ml penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (Invitrogen, Carlsbad, CA, USA) to 500 ml of DMEM high glucose (4.5 g/l) with L-glutamine (Sigma, Saint Louis, MO, USA). The media can be stored at +4°C up to 2 weeks.
10. Basic Fibroblast Growth Factor (bFGF) (Invitrogen, Carlsbad, CA, USA), 10 μg. Prepare 0.1% BSA/PBS: dissolve 0.1gr BSA in 100 ml PBS + Ca/+Mg, sterilize by filtration and store at +4°C. Add 1 ml of the 0.1% BSA/PBS solution into the ampoule containing 10 μg of lyophilized bFGF. Transfer the solution to a sterile 15 ml tube and wash the bFGF ampoule four times with 1 ml of 0.1% BSA/PBS each time, combining the 5 ml in the same tube. Mix and aliquot 1.2 ml of the stock solution in 1.7 ml microcentrifuge tubes (2 μg/ml stock solution). Store aliquots at –20°C (see Note 2).
11. Embryoid body (EB) media: Add to 400 ml of Knock Out DMEM (KO-DMEM), 70 ml of Knock Out Serum Replacement (KOSR; Invitrogen, Carlsbad, CA, USA) (see Note 3), 5 ml of 200 mM glutamine, 5 ml of 100× non-essential amino acids (NEAA), 2 ml of ITS (Insulin, Transferrin, Selenium), 40 μl of

1 M β-mercaptoethanol (β-ME), and 2.5 ml of penicillin (10,000 U/ml)/streptomycin (10 mg/ml) (Pen/Strep). Store EB media at +4°C up to 2 weeks.

12. hESC media: Add 1.2 ml of 2 μg/ml stock bFGF to a bottle (480 ml) of EB media and mix well (5 ng bFGF/ml). To prepare 80 ml of ES media with high bFGF concentration (30 ng/ml), add 1.2 ml of stock bFGF to 78.8 ml of EB media and mix. Store the ES media at +4°C for up to 1 week.
13. MEF-conditioned medium (MEF-CM): the day before preparing the CM, plate M-MEF on gelatin-coated plates and leave it in the incubator overnight. The following day, remove the MEF media, rinse once with PBS + Ca/+Mg, and add EB media. Keep the plate for 2 days in the incubator. Collect the MEF-CM and filter through 0.22 μm membrane. Preferably use the same day, otherwise, store at +4°C for up to 2 days. Before using to grow hESCs add bFGF accordingly.
14. Trypsin/EDTA 0.25% (Invitrogen, Carlsbad, CA, USA).
15. Collagenase type IV (catalog number 17104-019, Invitrogen, Carlsbad, CA, USA). Dissolve 3 mg of collagenase type IV per ml of DMEM media without any other additive. Filter the solution through a 0.22 μm membrane and store at 4°C protected from light for up to 1 week.
16. Dispase (catalog number 17105-041, Invitrogen, Carlsbad, CA, USA). Dissolve 10 mg dispase per ml PBS Ca/Mg-free. Filter the solution through a 0.22 μm membrane. Aliquot in small volumes and store at −20°C. Working solution is a 1:20 dilution of the stock in PBS Ca/Mg-free or DMEM with no additives.
17. Four wells multidish Nunclon surface (catalog number 176740 (Nunc); VWR Scientific Products, West Chester, PA, USA).
18. 60 mm low cell binding culture dishes (catalog number 145389 (Nunc); VWR Scientific Products, West Chester, PA, USA).
19. Inverted microscope placed inside a biological hood (see Note 4).

2.2. Immune Detection Components

1. FACS-PBS: Dissolve 3 g of BSA and 0.1 g of sodium azide in PBS Ca/Mg-free (see Note 5).
2. Antibodies: mouse IgG anti-human SSEA4, mouse IgM anti-human TRA1-60, mouse IgG anti-human OCT4, goat anti-mouse IgG-FITC, goat anti-mouse IgM-FITC, goat anti-mouse IgM-PE (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).
3. 12 mm diameter glass cover slips.
4. Paraformaldehyde: 4% solution in PBS Ca/Mg-free. Prepare 1:4 dilution from 16% stock electron microscopy grade (Electron Microscopy Sciences, Hatfield, PA, USA).

5. Hoechst 33258: 1 μg/ml solution in PBS from 10 mg/ml stock.
6. Anti-fade mounting media (Gel/Mount; Biomeda, Foster City, CA, USA).
7. Fluorescent-activated cell sorting analysis (FACS) tubes: 5 ml polypropylene round bottom tubes (Falcon catalog number 352063, BD, Bedford, MA, USA).

2.3. Surgical Tools and Drugs

1. Sterile PBS.
2. Sterile saline (0.9% NaCl).
3. 70% ethanol.
4. Povidone iodine scrub solution.
5. Ketamine and Xylazine (10 mg/ml each).
6. Carprofen (1 mg/ml) and Buprenorphine (0.01 mg/ml).
7. Eye ointment.
8. Sterile gauze.
9. Lacrimal probe (catalog number 15020; Surgical Tools, Bedford, VA, USA).
10. Lacrimal cannula (catalog number 15151; Surgical Tools, Bedford, VA, USA).
11. Scissors and forceps.
12. 1 ml syringes, 27 G needles, and 21 G needles.
13. Sutures or clips.
14. Electric heat pad.

3. Methods

3.1. Derivation of hESC from Biopsied Embryos and Expansion in Culture

3.1.1. Derivation

1. Upon obtaining day 5–6 blastocyst, transfer it to a well of 4-wells culture plate containing 0.5 ml of pre-equilibrated blastocyst media using the stripper, and cover the well with 0.35 ml of light mineral oil (see Note 6). Keep in a low O_2 incubator (+37°C, 6% CO_2/5% O_2) to allow for spontaneous hatching through the orifice created in the zona pellucida during the biopsy. This may take few hours to overnight (see Note 7), see Fig. 1.
2. The naked blastocyst is treated by immunosurgery to remove the trophectoderm from the inner cell mass (ICM). From this point onwards, all incubations are carried out at +37°C and 5% CO_2. Prepare a four-wells plate containing 50 μl of a 1:5 dilution of anti-human serum in blastocyst media in one well, and 0.5 ml of blastocyst media in each of the remnant three wells.

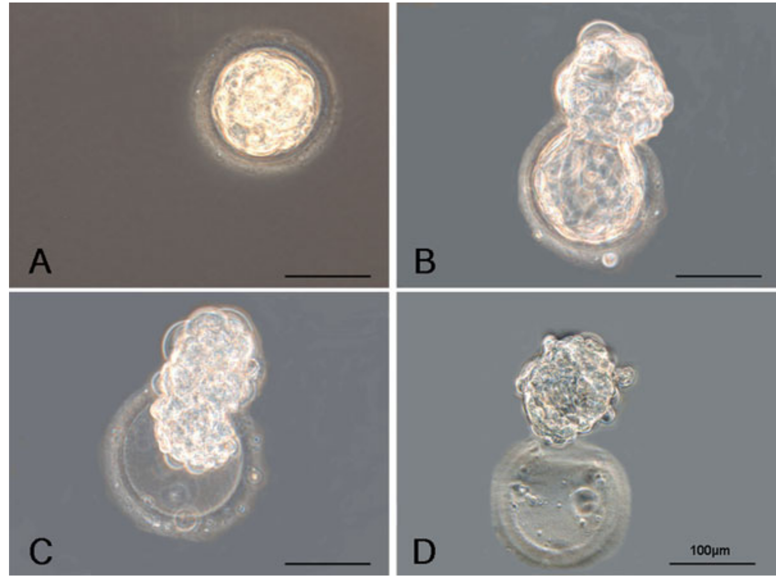

Fig. 1. Separation of blastocyst from the zona pellucida by spontaneous hatching. (**a**) 5 days old blastocyst contained inside the zona pellucida; (**b**) initial stages of hatching through the orifice performed during biopsy; (**c**) most of the blastocyst has been extruded from inside the zona pellucida; (**d**) fully hatched naked blastocyst.

Cover the well containing the anti-human serum with 0.35 ml of light mineral oil and keep the plate in the incubator for up to 15 min.

3. Using the Stripper, transfer the naked blastocyst to the well filled with anti-human serum and place the plate in the incubator for 30 min (see Note 8).
4. In the meantime, prepare a second four-well plate by adding 50 μl of a 1:5 dilution of guinea pig complement in blastocyst media in one well, and 0.5 ml of blastocyst media in each of the remnant three wells. Add 0.35 ml of light mineral oil to the well containing the guinea pig complement and keep the plate in the incubator for up to 15 min.
5. Using the Stripper, transfer the blastocyst from the well containing anti-human serum to the first well filled with blastocyst media, and aspirate up and down five times in different places of the well for a complete wash. Repeat the washes in the other 2 wells.
6. Transfer the blastocyst to the well filled with guinea pig complement in the second plate and leave in the incubator for 30 min.
7. Repeat the washes as in step 5. Check under microscope for blabbing within the trophectoderm (TE).

8. Transfer the ICM (see Note 9) from the complement plate to a well of 12 well-plate seeded with M-MEF and containing ES medium supplemented with 30 ng/ml bFGF (see Note 10). Place the 12-well plate in the incubator without disturbing for 48 h.
9. Change the media every other day. Starting at day 6, use media composed of 50% MEF-CM and 50% ES medium (final concentration 30 ng/ml bFGF).
10. Around day 8–12, when the colony (Passage 0—P0) is approximately 300 μm in diameter, cut it into three to four pieces using a barrier-filter mouth pipette with a rounded tip, and transfer the pieces to a new plate (Passage 1—P1) leaving untouched a piece of the colony in the original well (see Note 11). Repeat the procedure with P1 colonies to generate more P2 starts.
11. Continue transferring hESCs in the same fashion until P4–P8; when the cell line is more established, freeze manually picked colonies of hESCs.
12. At this point, transfer in parallel, one well manually with mouth pipette and one well enzymatically with either collagenase type IV or dispase at a 1:2 ratio.
13. When the cell line is well established and allows expansion by enzymatic treatment, reduce the concentration of bFGF in the media to 5 ng/ml (see Note 12).

3.1.2. Expansion

1. The day before cell passage, prepare 3 wells of a 6-well plate with M-MEF ($1.7–1.9 \times 10^5$ cells/well) on gelatin-coated plates and keep those in MEF media overnight in the incubator. The volume of media and amount of M-MEF plated are calculated for the splitting of hESC from one well of a 6-well plate (9.6 cm^2) at a split ratio of 1:3. If using culture plates of a different size, adjust the values accordingly.
2. Next day, aspirate the medium from the well containing hESCs to transfer and wash once with PBS + Ca/+Mg.
3. Add enough dispase solution to cover the surface of the well and incubate for 15–20 min in the incubator, or until colonies detach from the surface of the plate.
4. Collect the cells and rinse the well with 1–1.5 ml of EB media to recover colonies left behind. Combine collections in a 15 ml conical tube and centrifuge for 5 min at $600 \times g$.
5. Discard the supernatant by aspiration and resuspend the pellet in 7.5 ml of ES media. Aspirate up and down several times to break the big colonies into small clumps.
6. Aspirate the MEF media from the 3 wells containing M-MEF and wash once with PBS + Ca/+Mg.

7. Remove the PBS, distribute 2.5 ml of the cell suspension into each of the 3 wells and move the plate from side to side and front to back to distribute cells evenly.
8. Let cells sit without disturbing until the next day. Change the media on the second day after plating and every other day henceforth.

3.1.3. Freeze Storage

1. Remove the media from the wells containing the cells to freeze and wash with PBS + Ca/+Mg.
2. Detach cells by treatment with trypsin/EDTA, dispase, or collagenase type IV. Incubation time and condition will vary according to the enzyme used: 2–3 min at room temperature for trypsin, 15–20 min in incubator for dispase, and 30–40 min in incubator for collagenase type IV.
3. If using trypsin/EDTA, inactivate by addition of MEF media and collect detached cells into a 15 ml conical tube.
4. Centrifuge for 5 min at 600 × *g*, discard the supernatant, and resuspend in 0.9 ml times the number of vials to freeze of ES media.
5. Aliquot the cell suspension in 0.9 ml per vial and add 100 µl of DMSO.
6. Mix well by inversion, place the vial immediately in a cryobox and store overnight at −80°C. Next day, transfer to liquid nitrogen.

3.2. Characterization of hESC Lines

3.2.1. Alkaline Phosphatase Activity of hESCs

Use the Alkaline Phosphatase kit (catalog number 86R; Sigma, Saint Louis, MO, USA) according to manufacturer's instructions. See Fig. 2a.

3.2.2. Immunostaining for the Typical hESC Markers OCT4, NANOG, and Tra-1-60

1. Grow hESC on 12 mm diameter gelatin-coated glass cover slips containing M-MEF in 24-well plates (see Note 13).
2. Remove the media by aspiration and wash once with PBS.
3. Add 200 µl of 4% paraformaldehyde and incubate 10 min at room temperature to fix the cells.
4. Wash three times with PBS + Ca/+Mg, 5 min each.
5. Add 200 µl per well of 0.1% Triton X-100 in PBS and incubate for 10 min at room temperature to permeabilize the cells.
6. Wash with PBS + Ca/+Mg for 5 min once.
7. Block nonspecific binding sites with 10% normal goat serum in PBS for 30 min at room temperature.
8. Aspirate the solution and replace with 200 µl of blocking solution containing both primary antibodies mouse IgG

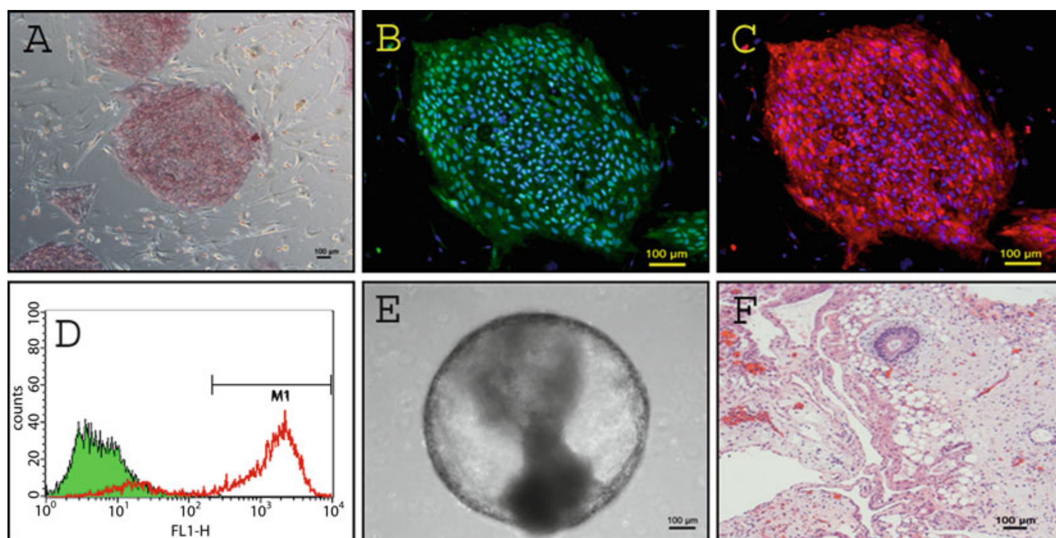

Fig. 2. Characterization of hESC lines. (**a**) Alkaline phosphatase staining showing a positive hESC colony grown on M-MEF. Immunostaining for the transcription factor Oct4 (**b**) localized in the cell nucleus, or the cell surface keratan sulfate TRA-1-60 (**c**), on cells grown on gelatin-coated glass cover slips. (**d**) shows FACS analysis for SSEA4 glycolipid. (**e**) A 20 days cystic embryoid body in suspension formed by in vitro differentiation of hESC. (**f**) shows a section of a paraffin-embedded teratoma stained with hematoxilin and eosin. The teratoma was generated by in vivo differentiation of hESC injected under the kidney capsule of immunocompromised mice.

anti-human OCT4 1:20 and mouse IgM anti-human-TRA1-60 1:100. Incubate for 1 h at room temperature.

9. Wash three times with 0.05% Tween 20 in PBS, 5 min each.
10. Incubate each cover slip for 1 h at room temperature in the dark with 200 μl of the following combination of secondary antibodies in blocking solution: goat anti-mouse IgG-FITC 1:300 and goat anti-mouse IgM-PE 1:200.
11. Wash three times with 0.05% Tween 20 in PBS, 5 min each in the dark.
12. Nuclear staining is performed by incubation with 200 μl of 1 μg/ml bis-benzimide (Hoechst 33258) for 10 min in the dark.
13. Wash once with PBS.
14. On a clean glass slide, put a drop of anti-fade mounting solution and using thin tip forceps, place the cover slip with the cells facing the mounting media (upside down). Remove air bubbles by gently pressing the cover slip against the slide; let it dry overnight at room temperature in the dark and seal the edges with nail polish.
15. Examine the slides under a fluorescent microscope using the corresponding filters, see Fig. 2b, c.

3.2.3. Fluorescent-Activated Cell Sorting Analysis for the Typical hESC Surface Markers, SSEA4 and TRA-1-60

1. Detach hESC from 4 wells of a 12-well plate by incubation with 0.25% trypsin/EDTA for 3–4 min at room temperature.
2. Inactivate trypsin by addition of MEF media, collect the cells in a 15 ml tube and spin down for 5 min at 600 × *g*.
3. Resuspend the cells in 800 μl of FACS-PBS and transfer 200 μl aliquots into four 1.7 ml microcentrifuge tubes.
4. Centrifuge for 5 min at 600 × *g* and discard the supernatant by aspiration.
5. Resuspend the cell pellet in 100 μl of FACS-PBS containing either mouse IgG anti-human SSEA4 or mouse IgM anti-human TRA1-60 antibodies, and incubate on ice for 20 min. Leave two tubes with FACS-PBS and no primary antibodies as negative controls.
6. Centrifuge for 5 min at 600 × *g* and remove the supernatant by aspiration.
7. Resuspend the cell pellets from the two tubes previously incubated with the primary antibodies, in 100 μl of FACS-PBS containing either goat anti-mouse IgG-FITC 1:200 or goat anti-mouse IgM-FITC 1:200, respectively, and incubate for 20 min on ice in the dark. Incubate likewise both control tubes, each one with a secondary antibody solution.
8. Centrifuge for 5 min at 600 × *g* and discard the supernatant.
9. Resuspend in 400 μl of FACS-PBS and transfer into a FACS tube (see Note 14).
10. Examine the samples in a FACS analyzer same day and calculate the percentage of positive cells for each marker against the correspondent control, see Fig. 2d.

3.2.4. In Vitro Differentiation into Embryoid Bodies

1. Grow hESC on M-MEF in 2 wells of a 6-well plate.
2. Remove the media and wash once with PBS + Ca/+Mg.
3. Collect the cells by gentle trypsinization (1 min or less at room temperature), just enough to detach the cells from the plate, leave them as clumps, do not allow single cell suspension.
4. Spin the cells down in a 15 ml conical tube and aspirate the supernatant.
5. Resuspend the pellet in 3 ml of EB media, dissociate big cell clumps into homogenous smaller-clump suspension by gently pipeting up and down.
6. Transfer the cell suspension (3 ml) into a 60 mm low attachment IVF dish prefilled with 4 ml EB media.
7. Rock the dish from side to side and from back to front to distribute the cells, and incubate at +37°C, 5% CO_2 for 3 days without moving the dish. On the third day, remove 3–3.5 ml

of media by gently tilting the dish and aspirating the media from the surface with a 5 ml pipette avoiding the EBs.

8. Replace 3.5–4 ml of fresh EB media, and repeat the process every other day (see Note 15).
9. Take images of the EBs every 7 days to follow its differentiation, see Fig. 2e.

3.2.5. In Vivo Differentiation into Teratomas

To generate teratomas, 5×10^6 hESC should be injected under the kidney capsule of a 6–8 weeks old SCID mouse. The teratoma should develop for at least 30 days before collecting the tissue.

1. Collect by trypsinization (trypsin/EDTA) hESC from a full 6-well plate or a 10 cm culture dish (approximately 5×10^6 cells) and spin them down in a 15 ml conical tube. Remove the supernatant and wash once with PBS + Ca/+Mg.
2. Resuspend the pellet in no more than 100 μl of PBS + Ca/+Mg and load the cell suspension into a 1 ml syringe using a 21 G needle.
3. In parallel, take the weight and anesthetize the mouse by intraperitoneal injection of 0.1 ml mixture of ketamine:xylazine (1:1) (10 mg/ml each) per 20 g body weight. Administer 0.1 ml of 1 mg/ml carprofen by subcutaneous (sc) injection, and apply ophthalmic ointment on both eyes to prevent dryness.
4. Position the mouse on its right side, facing the left-hand of the operator. Disinfect the dorso-lateral flank with povidone–iodine scrub solution and then with 70% alcohol.
5. Localize the kidney by full flexion of the ipsilateral inferior limb, and make a small oblique incision with scissors over the kidney, just below the ribs.
6. Pull the kidney from its distal pole out of the abdominal cavity and fix it outside the body inserting a 21 G needle through the connective tissue under the organ, taking care not to damage the renal artery. Prevent the kidney surface to dry, dropping periodically warm sterile PBS on top of the organ (see Note 16).
7. Using a lacrimal probe, open very gently the space between the kidney and the capsule moving the probe in a windshield wipe manner parallel to the surface of the kidney.
8. Remove the probe, introduce the lacrimal cannula in the subcapsular space and inject the cells very slowly to avoid reflux and loss of cells.
9. Replace very carefully the kidney in its original position inside the abdominal cavity and suture the muscular wall and skin.
10. Administer by sc injection 0.3 ml of saline per mouse, and, when recovering from anesthesia, inject the mouse with 0.1 ml of 0.01 mg/ml buprenorphine solution subcutaneous.

11. Allow the mouse to fully recover from anesthesia in a cage warmed by an electric heat pad.
12. Thirty days after the injection, euthanize the mouse and remove the injected kidney with the teratomas (see Note 17) see Fig. 2f.

3.3. Summary of the Derived hESC Lines and Its Characterization

In Table 1, we detail for each cell line the PGS results of the pre-embryo used for derivation, the full karyotype of the cell line and the profile used for characterization.

4. Notes

1. After removing the gelatin from the autoclave while still hot, mix well by swirling to ensure complete solubility and avoid localized deposition of solid gelatin when cools down.
2. Store bFGF as single-use aliquots. Once thawed, do not refreeze, use the entire volume.
3. There is variation in the ability of different batches of KOSR to support the growth of undifferentiated hESCs. It is highly recommended to test few batches and save the one that matches your cells best.
4. Due to its small size, handling of the blastocyst needs to be done under microscope in a sterile environment.
5. Sodium azide is a toxic chemical that inhibit enzymatic activity. Use always gloves when handling. Its addition to the cell suspension prevents the internalization of surface markers, improving the sensitivity of detection.
6. Blastocysts are usually obtained in tubes containing blastocyst media at the bottom covered by light mineral oil on top. To help find the embryo, transfer the bottom phase (aqueous media) to a 60 mm dish and look for the embryo under microscope. If not found in the aqueous phase, transfer the oily phase to another 60 mm dish and look under microscope again. Sometimes, it can be found at the edges of the liquid phase. Blastocysts are very sticky and have the tendency to attach to the plastic walls. When transferring the blastocyst using the stripper at any step of the derivation, aspirate up and down two to three times blastocyst media before picking the blastocyst to prevent its loss.
7. If the complete hatching of the blastocyst does not occur by next day, it may require cutting the zona pellucida to remove the embryo. This can be performed under microscope using a surgical blade.

Table 1
Summary of the 25 hESC lines derived following the methodology described above, which includes for each of them PGS analysis of the source blastocyst, karyotype of the derived hESC line, and panel of in vitro and in vivo characterization

Cell line	PGS	Karyotype	AP	ICC (Oct4/Nanog/ TRA-1–60)	FACS (SSEA4/TRA-1–60)	EB	Teratoma
CSES1	Mon 21	46,XX (20)	✓	✓	85%/78%	✓	✓
CSES2	Haploid	46,XX (20)	✓	✓	83%/78%	✓	✓
CSES3	Mon 14, 16, 18	46,XX (20)	✓	✓	86%/84%	✓	✓
CSES4	Mon 8, 21	46,XY (20)	✓	✓	79%/82%	✓	✓
CSES5	Tri 18	46,XX (25), 46,XX,+11 (2), 46,XX,+11, t(11;17) (1), 46,XX,+11,-15 (1)	✓	✓	87%/91%	✓	✓
CSES6	Tri 18	46,XX (19), 47,XXX,t(12;17) (1), 47,XX, +17 (1), 46,XX,-8,+11 brokenX (1)	✓	✓	84%/89%	✓	✓
CSES7	Mon 21	46,XX (20)	✓	✓	84%/87%	✓	✓
CSES8	Tri 17, 20	47,XX,+17 (19), 46,XX,-14,+17 (1), 45,XX, -3,-8,+17 (1)	✓	✓	76%/80%	✓	✓
CSES9	Not analyzed	46,XY (20)	✓	✓	84%/87%	✓	✓
CSES10	Not analyzed	46,XX (19), 45,XX, -19 (1)	✓	✓	78%/84%	✓	✓
CSES11	Not analyzed	46,XX (17), 44,XX,-10,-21 (1), 45,XX,-19 (1), 45,XX,-3 (1), 47,XX,+18 (1)	✓	✓	92%/91%	✓	✓
CSES12	Tri 22, XYY	46,XY (19), 45,XY,-11 (1)	✓	✓	93%/92%	✓	✓
CSES13	Mon 14, Tri 21	47,XY,+21 (18), 46,XY,-10,+21 (1), 46,XY,-6,+21 (1)	✓	✓	85%/87%	✓	✓
CSES14	Mon 13, Tri X	46,XX,t(7;17) (15), 46,XX (6)	✓	✓	96%/94%	✓	✓

(continued)

Table 1 (continued)

Cell line	PGS	Karyotype	AP	ICC (Oct4/Nanog/ TRA-1–60)	FACS (SSEA4/TRA-1–60)	EB	Teratoma
CSES15	Tri 15	46,XY (20)	✓	✓	89%/88%	✓	✓
CSES16	Tri 21	47,XY,+13 (20)	✓	✓	91%/85%	✓	✓
CSES17	Mon 8, 13, 14, 16, 17, 22, Null 20	46,XX (20)	✓	✓	94%/91%	✓	✓
CSES18	Mon 20	46,XY (20)	✓	✓	91%/85%	✓	✓
CSES19	Tri 16,17,22, Tet 13,15, 21, 2X,2Y, NR 18	46,XY (20)	✓	✓	85%/86%	✓	✓
CSES20	Mon 14, Tri 21	47,XY,+21 (20)	✓	✓	79%/79%	✓	✓
CSES21	Mon 16, Tri 15, 21, 22	47,XX,+21 (20)	✓	✓	92%/86%	✓	✓
CSES22	Tri 16, 22	47,XX,+16 (15), 47,XX,+15 (2), 46,XX, t(16;22) (1), 47,XX,+5, t(16;22) (1), 46,XX (1)	✓	✓	86%/87%	✓	✓
CSES23	Mon 15, 16, 22	47.XXX (19), 46,XX (1)	✓	✓	95%/93%	✓	✓
CSES24	Mon 22, Tri 14	46,XX (13), 45,X (7)	✓	✓	88%/87%	✓	✓
CSES25	Tri 14	46,XX (22)	✓	✓	97%/96%	✓	✓

8. Most of the TE cells are lost during the washes following incubation with guinea pig complement. There may be however, remnant TE attached to the ICM, which neither significantly affect attachment to the plate, nor growth of hESC.
9. When transferring the blastocyst to the wells containing either human antiserum or guinea pig complement, confirm that the tip of the Stripper is immersed in the aqueous solution and not in the covering oil phase before releasing the blastocyst to ensure the success of the immunosurgery and prevent the loss of the embryo.
10. All media and solutions should be prewarmed before use at room temperature instead of 37°C to preserve composition, unless aliquoted for single use.
11. In some cases, the first manual transfer of fragments of the P0 colony may fail. It is recommended to leave one third of the colony intact as a backup in case of transfer failure, and to recut to generate more P1 colonies after it re-grows.
12. For continuous passage and expansion, it is recommended to use dispase or collagenase type IV instead of trypsin. Different from trypsin, both dispase and collagenase type IV detach the entire hESC colony leaving the feeder layer intact. The collected cell aggregates can be disrupted into smaller cell clumps by pipetting up and down before plating.
13. Glass cover slips usually contain an oily film on the surface that needs to be removed in order to improve cell attachment. One easy way to clean this film is by washing the cover slips inside a bicker with 100% ethanol twice with agitation for 4–5 h, followed by rinse four to five times in distilled water. Finally, they can be autoclaved.
14. It is important to have no cell clumps in the cell suspension that may block the FACS analyzer. If this is the case, extend the incubation time with trypsin to 5 min, the temperature of incubation to 37°C, and/or improve the mechanic cell dissociation and analyze immediately.
15. Following 6–10 days of differentiation, some EBs become cystic and can be found floating in suspension instead of sank at the bottom of the dish. This represents a problem at the moment of changing the media. If there are floating EBs, tilt the dish and aspirate in different parts from the surface of the media, trying to avoid picking them up.
16. If the kidney capsule dries up, it loses elasticity making it more difficult to separate from the kidney surface and increases the risk of perforation by the probe or the cannula. Keeping the kidney wet with warm sterile saline prevents this to happen.
17. Teratomas are not an invasive type of tumor. It grows within the subcapsular space and can thus be easily separated from mouse kidney.

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Chapter 11

Mutated Human Embryonic Stem Cells for the Study of Human Genetic Disorders

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Abstract

Human embryonic stem cells (HESCs) are of great interest in biology and medicine due to their ability to grow indefinitely in culture while maintaining their ability to differentiate into all different cell types in the human body. In addition, HESCs can be used for better understanding the key developmental processes and can, therefore, serve for studying genetic disorders for which no good research model exists. Preimplantation genetic diagnosis of in vitro derived embryos results in affected-spare blastocysts with specific known inherited mutations .These affected blastocysts can be used for the derivation of disease-bearing HESCs, which would serve for studying the molecular and pathophysiological mechanisms underlying the genetic disease for which they were diagnosed. This chapter describes the methods to derive HESCs carrying mutations for inherited disorders.

Key words: Preimplantation genetic diagnosis, Human embryonic stem cells, Embryos, Genetic diseases, PCR, Fluorescent in situ hybridization

1. Introduction

Human embryonic stem cells (HESCs) can be grown in culture for extended periods of time while maintaining a normal karyotype and pluripotency, i.e., the ability to differentiate into cells that represent tissues from the three germ layers (1). As such, HESCs have been proposed as a useful tool for cell replacement in regenerative medicine for the treatment of different pathologies, such as Juvenile Diabetes, Parkinsons, Alzheimer's, or spinal cord injury (1–3). In addition, due to their ability to differentiate, HESCs have been proposed as a valuable tool for studying the early stages of human embryogensis (2). This attribute is particularly important in pathologies that are developmentally regulated and/or their pathogenesis is not yet

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fully understood. For this, HESC lines carrying specific mutations can be used as a powerful tool in the study of human genetics and the exploration of new therapeutic protocols, including gene therapy-based treatments and disease-oriented drug screening and discovery. This chapter describes protocols for the derivation and culture of mutated HESC lines from IVF embryos following PGD, and their application in studying the pathophysiology of specific disorders.

HESCs are derived from the inner cell mass (ICM) cells of blastocyst-stage embryos. These cells are considered to have the greatest developmental potential, differentiating into the widest range of cell types both in vivo (as teratomas) and in vitro (as embryoid bodies) that consist of cells from all three germ layers (4–8). Both the in vivo as well as the in vitro differentiation protocols mimic, to some extent, early human embryonic development (9), and therefore are used to study developmentally regulated events, such as X inactivation in females (reviewed by ref. 10) and globin gene switching (11). Moreover, since HESCs can now be differentiated into specific cell types (neurons, cardiac cells, pancreatic beta cells, as well as many others), key molecules of the differentiation process can be studied for better understanding of the regulatory mechanisms of these processes.

1.1. Study of Inherited Disorders Using Current Available Models

Different models have been developed to study genetic disorders in humans. Perhaps the easiest to establish is primary cell cultures biopsied from affected patients. Such primary disease cell models have been developed in the study of many diseases, including Galactosemia (12), Hurler's syndrome (13), and even cancer (14) or aging (15), in which tissues such as the skin are obtained. However, this approach is restricted to cell types that can be cultured and proliferated in vitro. Therefore, syndromes, such as Fragile X, manifested mainly in nerve cells, or the storage pathology ML4, are difficult to biopsy and culture and, hence, are practically impossible to be studied by primary cultures (16). Moreover, due to the limitation of short life span of the biopsied tissue, some cell cultures must be transformed. The resulting cells do not fully resemble the primary culture and usually carry chromosomal abnormalities in addition to the specific mutation that characterizes the syndrome. Thus, for most diseases, these cellular models do not serve as an optimal model for research.

To overcome these limitations, genetically engineered animals that carry specific mutations associated with the pathologies have been generated, such as transgenic mice for human inherited diseases (reviewed by refs. 17, 18). Many of these animal models, however, do not faithfully represent the abnormal phenotype as manifested in humans (19). They often diverge considerably from the human phenotype due to intraspecific variations. Moreover, there are several naturally occurring mutations in humans which have no counterparts in rodents. For example, the unstable triplet

repeat expansion disorders have no complement in mice (i.e., Fragile X, Myotonic Dystrophy, Androgen Receptor, Spinobulbar Muscular Atrophy, and Huntington's disease, HD). Finally, fundamental differences in DNA and gene expression levels limit the power of knockout (KO) mice in accurately mimicking human disorders, such as in the case of neurodegenerative disorders (20).

1.2. Diseased Embryonic Stem Cells as a Tool for Studying Genetic Disorders

HESC lines that carry specific mutations have been recently suggested as valuable models for the study of hereditary genetic disorders. Two strategies have been developed to obtain mutant cell lines. The first is artificially introducing specific mutations in a preexisting HESC line by homologous recombination. This approach has been used for Lesch–Nyhan syndrome by targeting the HPRT gene in male wild-type HESCs (21, 22). The HPRT-deficient HESCs display the major biochemical defect that characterizes the syndrome, which involves the accumulation of uric acid. These cannot be mimicked by the mouse model due to interspecies genomic differences (23, 24). A similar method was carried out when Xue et al. targeted the Olig2, a basic helix-loop-helix transcription factor that plays an important role in motoneuron and oligodendrocyte development (25). This new knock-in HESC line is a useful tool for the study of neural development, for which other available models are insufficient. One major hurdle in this method is that the efficiency of homology recombination in HESCs is very low (21, 22).

The other approach is to establish an HESC line directly from an affected embryo so that the resulting cell line will carry the inherited mutation (26, 27). In these derived mutated HESC lines, there is no need to genetically manipulate the cells in order to target a gene. In addition, this approach allows a more robust generation of cell lines that harbor genetic modifications that are otherwise inaccessible, such as very small deletions, few base pair substitutions, or triplet repeat expansions. Furthermore, HESC lines which have numerical or structural chromosomal abnormalities can be obtained by this approach, as a model system for the study of specific chromosomal loss and rearrangements.

An alternative to diseased HESC lines has been recently developed by reprogramming fully differentiated cells (such as fibroblasts) extracted from an affected patient into pluripotent ES-like cells, termed inducible pluripotent stem (iPS) cells. Since the differentiated cells subjected to reprogramming are taken directly from affected patients, the resulting iPS cells carry the specific genetic mutation. However, although iPS cells are much easier to attain, they still raise some concerns regarding their degree of similarity to ESCs in terms of gene expression, epigenetic state, and differentiation potential (28–30). In addition, iPS lines cannot be derived for embryonic lethal conditions, such as various structural and numerical chromosomal rearrangements (31, 32). Taken together, although iPS cells may complement HESCs under specific conditions,

HESCs are still considered the gold standard by which all other pluripotent stem cells, including iPS, are assessed.

1.3. Preimplantation Genetic Diagnosis

In contrast to prenatal diagnosis by Chorionic Villus Sampling (CVS) or amniocentesis, which, in the case of an affected fetus, obligatory leads to therapeutic abortion, PGD allows genetic analysis of embryos before their implantation in the uterus (33–35). Thus, PGD prevents the chance for pregnancy with a genetically mutated embryo in families that are carriers of an identified mutation. PGD is currently the state-of-the-art technique for ensuring a disease-free baby for couples at high risk (>25%) of transmitting a genetic disorder to their offspring (36–40). This includes mainly carriers of severe congenital monogenic diseases or chromosomal aberrations, such as translocations. To obtain embryos for PGD, couples have to undergo in vitro fertilization (IVF). Cleavage-stage embryos are biopsied 3 days following fertilization and single blastomeres are genetically analyzed for the specific mutation carried by the parents. This method is based on the concept that all blastomeres are genetically identical, and therefore allow deducing from the analysis performed on the single blastomere on the entire embryo genotype (33, 36–38). PGD is now offered also for carriers of late-onset disease (e.g., Huntington's Disease), as well as for carriers of cancer predisposition mutations, such as APC and BRCA1 (41–45).

PGD protocols are currently available for almost all mutations that can be diagnosed by amniocentesis or CVS during prenatal diagnosis. Two main technologies are used for PGD: polymerase chain reaction (PCR) for monogenic disorders and fluorescence in situ hybridization (FISH) for diagnosing structural or numerical chromosomal aberration, including translocations and sex determination for X-linked disease with unidentified mutation (reviewed by ref. 46).

Due to the fact that diseased embryos are not transferred back for implantation, they are invaluable resources for studying the disease they carry. Approximately 150 PGD-HESC mutation-bearing lines have been derived since 2004 (Table 1), and some have already been used to enhance our knowledge on human pathologies (16, 26, 32, 47–58). This chapter describes some of the most studied disease-bearing HESC lines derived from PGD-affected embryos and their potential use and availability. In addition, we describe the PGD and HESC derivation protocols used in our lab.

1.4. Potential Applications of Mutation-Bearing HESCs

Growth of HESCs in culture and their differentiation into target cells affected by the disease for which they were diagnosed allow modeling a disease in an autonomous cell system. This cell system is independent of external signals of neighboring cells, which are naturally present in the multisystem animal model. HESCs can also serve for therapeutic applications once they are derived in GMP standards and efficient protocols for induced differentiation are established.

Table 1
Disease-associated HESCs derived in different Stem Cell centers worldwide

Stem cell center	Country	Disease	References
Monash Immunology and Stem Cell Laboratories	Australia	Huntington's disease	(48)
Sydney IVF	Australia	Chromosomal aberrations	(49), http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
		Huntington's disease	(47), http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
		Myotonic dystrophy	http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
		Fragile X syndrome	http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
		Von Hippel–Lindau disease	http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
		Charcot–Marie–Tooth Syndrome	http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
		Infantile neuroa-xonal dystrophy	http://www.sydneyivfstemcells.com/AboutUs/Ourstemcells/tabid/648/Default.aspx
Center for Medical Genetics	Belgium	Marfan syndrome	(51)
		Facioscapulohu-meral muscular dystrophy	(51)
		Spinocerebellar ataxia	(51)
		Fragile X syndrome	(51)
		Myotonic dystrophy type 1	(50, 51, 63)
		Charcot–Marie–Tooth type 1A	(51)
		Cystic fibrosis	(50, 51)
		Osteogenesis imperfecta	(51)
		Huntington's disease	(50, 51, 63)

(continued)

Table 1 (continued)

Stem cell center	Country	Disease	References
IGBMC; INSERM	France	Huntington's disease	(52), European Stem Cell Registry http://www.hescreg.eu/
		Fabry syndrome	(52), European Stem Cell Registry http://www.hescreg.eu/
		Fragile X syndrome	(52), European Stem Cell Registry http://www.hescreg.eu/
		Multiple endocrine neoplasia type 2a	(52), European Stem Cell Registry http://www.hescreg.eu/
		Cystic fibrosis	(52), European Stem Cell Registry http://www.hescreg.eu/
		Spinocerebellar ataxia type 2	(52), European Stem Cell Registry http://www.hescreg.eu/
		X-linked myotubular myopathy	(52), European Stem Cell Registry http://www.hescreg.eu/
		Adenomatous polyposis of the colon	European Stem Cell Registry http://www.hescreg.eu/
		Charcot–Marie–Tooth type 1A	European Stem Cell Registry http://www.hescreg.eu/
Clamart Hospital	France	Chromosomal aberrations	(31)
Hadassah University Hospital	Israel	Myotonic dystrophy type 1	(53)
		Cystic fibrosis	(53)
		Male hemophilia A-carrier	(53)
		Female carrier of fragile X permutation	(53)
Tel Aviv Sourasky Medical Center	Israel	Fragile X	(16, 32)
		Saethre–Chotzen syndrome	(32)
		Gaucher	(32)
		Androgen insensitivity carrier	(32)
		Torsion dystonia	(32)
		DMD	(32)
		Myotonic dystrophy	(32)
		Chromosomal aberrations	(32)

Royan Institute for Stem Cell Biology and Technology	Iran	Chromosomal aberrations	(54)
Istanbul Memorial hospital	Turkey	HLA mismatched	(55)
		Beta-thalassemia	(55)
		Chromosomal aberrations	(55)
King's College London	The UK	Huntington's disease	(56)
		Cystic fibrosis	(27)
Cedars-Sinai medical center	The USA	Chromosomal aberrations	(57, 58)
Reproductive genetics institute	The USA	Chromosomal aberrations	(26), http://www.reproductivegenetics.com/docs/hesc_lines2.ppt, European Stem Cell Registry http://www.hescreg.eu/
		Hemoglobin alpha; HBA	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Cystic fibrosis	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Fanconi anemia	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Spinal muscular atrophy type 1; SMA1	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Sandhoff disease	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Albinism, ocular, type 1; OA1	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Adenoleukodystrophy; ALD	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Duchenne and Becker muscular dystrophy	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt
		Fragile X	European Stem Cell Registry http://www.hescreg.eu/, http://www.reproductivegenetics.com/docs/hesc_lines2.ppt

1.4.1. HESC Differentiation into Neurons for the Study of Neurodegenerative Disorders

Since it is difficult to grow mature neurons in culture, it has been proposed that HESC differentiation into neurons can serve as a tool for studying neurodegenerative disorders. There are two advantages in this process: first, the generation of an unlimited and large number of neurons which facilitates the research and bypasses the need for biopsies or cadaver tissue. Second, differentiation in culture allows not only studying neurons as the end-point tissue, but rather allows studying the entire developmental process. This is most important in developmental or pediatric pathologies. The availability of HESC-derived neurons carrying a specific mutation provides the means to study the molecular mechanism affecting nerve system development at the cellular level and will thus shed light on the pathogenesis of the disease.

Huntington's Disease

Huntington's disease is an autosomal dominant neurodegenerative disease caused by expanded CAG repeats in exon 1 of the *Huntingtin* (*Htt*) gene (59). Individuals with less than 35 repeats are healthy, 36–40 repeats may potentially cause HD, and more than 40 repeats demonstrate the characteristics of the affected phenotype with the increasing number of repeats directly correlating to disease severity and age of onset (60). The expanded repeat region causes a negative gain of function of the Htt protein, which forms aggregates within the nucleus of certain neuronal cells. The aggregation of htt protein in the brain causes the classic symptoms of chorea, emotional issues, and cognitive decline seen in HD patients. To date, it is unknown why specific neurons are primarily affected by the toxic-expanded Htt protein (61, 62).

A number of labs have derived Huntington ESC lines for the study of the disease (3). The first showed that the Huntington ESC line has an expansion of CAG repeats, and that this is stable even following differentiation (63). Bradley et al. derived four new Huntington ESC lines and showed that these lines can differentiate into neurons (47) and express the characteristic protein of the Huntington's—the Htt protein. These cells can, therefore, serve as a valuable model for the study of Huntington's disease.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a relatively common autosomal recessive disease with an incidence of 1/6,000 to 1/10,000 and a carrier frequency of 1/40 to 1/50 (64–66). SMA is a neuromuscular disorder caused by the degeneration of motor neurons in the anterior horn of the spinal cord. The degenerative process leads to progressive symmetric proximal muscular atrophy, which, in the most severe form, culminates in respiratory failure and infant death. The gene responsible for the disease is termed *Survival Motor Neuron* (*SMN*), and is located on chromosomal region 5q13 (67, 68). The disease is caused by the deletion or mutation in the *Smn1* gene while the severity might be influenced by the number of *SMN2* copies. About 94% of patients have homozygote deletions of

SMN1 gene. Although there is a mouse model for SMA (69), there are currently no human models. PGD-HESCs derived from SMA-mutated embryos, which are then differentiated into muscle and neurons, can serve as means of understanding this disease (70).

1.4.2. Mutated HESC Lines for Modeling Human Genetic Disorders

Fragile X Syndrome

Fragile X Syndrome is an X-linked genetic disorder, and is the most common form of inherited mental retardation (71). It is caused by the absence of the Fragile X mental retardation protein (FMRP) due to inactivation of the *Fragile X mental retardation 1 gene* (*FMR1*) (16). *FMR1* inactivation is induced by the CGG triplet repeat expansion in the 5′ untranslated region of the gene. Full expansion of the CGG repeat usually coincides with hypermethylation of the repeat region and its upstream flanking CpG island-type promoter. Existing models for Fragile X syndrome include fmr1 KO animals which do not express FMRP, even at early stages of development and are therefore inadequate for examining the various impairments occurring during early neurogenesis (72).

We have published the first report in which a PGD-derived HESC line was used to study a developmentally regulated genetic syndrome—Fragile X (16). We have shown that in undifferentiated fragile X-HESCs the *FMR1* gene is expressed and undergoes transcriptional silencing only following differentiation. When iPS lines were generated from fibroblasts of individuals carrying the Fragile X mutation, the *FMR1* gene remained inactive in the undifferentiated cells and the heterochromatin was inactivated, demonstrating an epigenetic memory of the reprogrammed cells (29). These data highlight the differences between ESCs and iPS lines in modeling the Fragile X syndrome.

Myotonic Dystrophy

Myotonic dystrophy-1 (DM-1) is caused by a trinucleotide repeat expansion (CTG)n in the 3′ untranslated region of the *Dystrophia Myotonica Protein Kinase gene* (*DMPK*) on chromosome 19q13. DM-1 is an autosomal dominant disorder characterized by muscular dystrophy (73). Disease severity varies with the number of repeats: individuals with 5–37 repeats are normal, 50–150 repeats are mildly affected, and patients with congenital onset can have more than 2,000 repeats (73). Similarly to Fragile X syndrome, this pathology is characterized by anticipation, i.e., the increase in repeat numbers and thus severity from generation to generation. A recent study demonstrates that upon differentiation of expansion bearing MD HESC lines a stabilization in expansion is observed. Using PGD-derived cells, this paper shows for the first time a correlation between expansion and DNA mismatch repair systems (63).

Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is one of the most common causes of muscular dystrophy in childhood. It is characterized first by proximal muscle weakness, a waddling gait, and difficulty climbing (74). The disease is rapidly progressive, with most affected children

becoming wheelchair bound by the age of 12. Death usually occurs in the 3rd decade as a result of respiratory failure and cardiomyopathy. The disease is caused by mutation in the *DMD* gene, which encodes for the structural protein, dystrophin. The *DMD* gene is one of the largest human genes spanning 2.3 Mb and composed of 79 exons. About two-thirds of DMD patients have deletions of one or more *DMD* exons. Becker Muscular Dystrophy (BMD) is also caused by *DMD* mutations, and has a milder course and a later age of onset. Both DMD and BMD, collectively referred to as dystrophinopathies, are inherited in an X-linked recessive manner (75). A recent review suggested that differentiation of fetal and muscle-derived myogenic stem cells into muscles can serve for cell therapy in Duchenne patients (76), and demonstrates that gene therapy in these cells could cure this disease. Although DMD HESC lines have been derived (including by us), there is currently no publication utilizing these lines for the study of DMD.

1.4.3. HESC Derivation for the Study of Chromosomal Aberrations

There are a number of chromosomal numerical and structural aberrations, including trisomies, monosomies, and translocations, which are lethal very early during embryo development and can lead to the high rate of spontaneous abortions in human pregnancies (77). Hence, studying the differentiation of aneuploid HESC lines recapitulates, as closely as possible, early processes that might lead to these spontaneous abortions (77, 78). Recent reports described 25 newly derived HESC lines that are aneuploid in chromosomes 13, 16, 17, 21, or X (57, 79). These aberrations represent pathologies found in Patau syndrome (Trisomy 13), Down syndrome (Trisomy 21), and Triple X (Trisomy X-chromosome), XXY (Klinefelter's syndrome). The authors showed that in these trisomies all three chromosomes are transcriptionally active. Another report by this group studied the gene expression profile of a Turner's syndrome (45, XO) HESC line, and compared to WT HESC lines. The largest differences were found in the expression of placental genes (80). This finding suggests that the lack of a single copy of the X chromosome may lead to early lethality in XO embryos due to abnormal placental differentiation. Mainly, normal diploid WT HESC lines are derived when ICM of aneuploid blastocysts are plated for HESC derivation (58, 81). This result supports previously published data on self-correction mechanisms earlier shown to exist within the preimplantaion embryo (82), and that HESC lines can be used to study them (83–85). Monosomic HESC lines would be valuable for studying gene dosage and imprinting effects in humans (86, 87). It was suggested that early mortality of monosomic embryos is caused by imprinting effects rather than by haploid expression of lethal genes, as shown in mice models (88).

Structural chromosomal aberrations, known as chromosomal translocations, are a major cause for implantation failure. It would

be extremely useful to have cellular systems that enable studying the effect of translocations on cell viability, proliferation, and differentiation. Since the first derivation of a translocation-bearing line in 2009 (31), a number of other translocation-bearing HESC lines have been reported (including ours (32) as well as from the HESC banks (55, 57, 58)). However, to date, there have not been any studies regarding the use of these lines for studying these pathologies.

1.4.4. HESCs for the Study of Cancer

Usually, for cancer development, mutations in two alleles of genes, involved in cell cycle regulation or mismatch repair, are needed. Congenital mutation in one allele of such genes (like *FAP*, *BRCA*, *HNPCC*) predisposes for cancer development, since there is a greater chance to lose both alleles during life. HESC lines mutated in these genes can serve as an excellent cellular model for the study of malignant transformation, and allows following the multistep process from the initial mutation to its final stage as a transformed cell (89–92). Since HESCs can form distinct populations of terminally differentiated cells in vitro, they may be used for large-scale screening of new pharmaceutical compounds, optimizing currently available drugs as well as examining their effect on cell toxicity and teratogenicity.

1.5. Summary

HESC lines derived from PGD-affected embryos are important for the study of human genetics, especially in cases where no suitable cellular and/or animal models are available. These mutated HESC lines carry the natural mutations associated with the patients' disease and, thus, serve as a valuable research model. To date, there have been over 150 disease-bearing HESC lines derived. These in vitro systems have great value in the study of human genetics, as well as for the exploration of new therapeutic protocols.

2. Materials

2.1. PCR

1. EDTA Tubes for blood collection (Purple cap) (BD Vacutainer, BD, Plymouth, UK).
2. RBC Lysis buffer (Biological Industries, Beit-Haemek, Israel).
3. Cleavage medium and Blastomere media (Cat# 1526; Sage, Pasadena, CA, USA).
4. Ca^{2+} Mg^{2+} Free Blastomere Medium (Cat# 00234; Sage, Pasadena, CA, USA) supplemented with 10% Serum Substitute Supplement (Cat# 99193; IrvineScientific, Santa Ana, CA, USA).

5. Single cell collection buffer (10 ml):
   1 ml 10× PBS (Amresco Inc., Solon, OH, USA).
   1% PVP (Sigma, St. Luis, MO, USA).
   0.1 mg/ml Phenol Red (Sigma, St. Luis, MO, USA).
6. Glass pipettes (Sigma, St. Luis, MO, USA).
7. PCR tubes (ABgene, Epson, UK).
8. NaOH, 50 mM (Amresco Inc., Solon, OH, USA).
9. TRIS, 100 mM (Amresco Inc., Solon, OH, USA).
10. Lysis buffer (50 mM DTT, 200 mM NaOH in DDW).
    DTT (Sigma, St. Luis, MO, USA).
    NaOH (Sigma, St. Luis, MO, USA).
11. 0.2-ml PCR tubes.
12. PCR I- Mix I solution for a total of 20 μl:

2 μl	10× PCR buffer (Bioline)
2 μl	Additive (Bioline)
1 μl	Neucleotides 5mM (Roche)
1 μl	Mg 50mM (Bioline)
1 μl	DMSO (Amresco Inc., Solon, OH, USA)
0.5 μl	Gelatine 1% (Sigma, St. Luis, MO, USA)
0.5 μl	Tricine 1M (Sigma, St. Luis, MO, USA)
0.4 μl	Primer forward (20 μM)
0.4 μl	Primer reverse (20 μM)
DDW	For a 20-μl reaction

13. PCR I- Mix II:

2.75 μl	10× PCR Buffer (Bioline)
2 μl	MG^{2+} 50 mM (Bioline)
0.25 μl	DNA Polimerase (Bio-X-Act; Bioline)

14. Nested Mix 1 reaction

8.25 μl	DDW
0.7 μl	DMSO (Amresco Inc., Solon, OH, USA)
1 μl	Primer forward (20 μM)*
1 μl	Primer reverse (20 μM)*

*Whenever polymorphic markers are analyzed by GeneScan, one of the primers should be fluorescently tagged. All primers are from Sigma, St. Luis, MO, USA.

15. Nested Mix 2 solution:

12.5 μl	FailSafe buffer (EPICENTRE Biotechnologies, Madison, WI, USA)
0.375 μl	DNA Polymerase (Bio-X-Act; Bioline)
0.125 μl	DDW

2.2. FISH

1. LH Lithium Heparin Tubes for blood collection (green cap BD Vacutainer tubes; Cat #454029).
2. Peripherial Blood Karyotyping medium (Biological Industries, Beit-Haemek, Israel; Cat # 01-198-1B).
3. Phytohemagglutinin-M (PHA-M) (Biological Industries, Beit-Haemek, Israel; Cat #12-006-1 H).
4. T25 flasks.
5. 15-ml conical tubes.
6. Colcemid (Biological Industries, Beit-Haemek, Israel; Cat #12-004-1D).
7. Potassium chloride solution, 0.075 M (Biological Industries, Beit-Haemek, Israel; Cat #12-005-1B).
8. Methanol.
9. Acetic acid.
10. Superfrost Plus glass slide (Kindler, Freiburg, Germany).
11. Spreading buffer:

    5 ml DDW.

    50 μl 1 N HCl (0.01 N HCl).

    5 μl Tween 20 (0.1% Tween 20).
12. Dulbecco's Phosphate Buffered Saline (DPBS) without Ca and Mg (Biological Industries, Beit-Haemek, Israel; Cat # 02-023-1B).
13. Ethanol.
14. Pepsin.
15. Formaldehyde.

2.3. HESC Derivation

1. Blastocyst Medium (Cat# 1529; Sage, Pasadena, CA, USA).
2. Anti-human serum antibody (Sigma, St. Louis, MO; Cat#H-3383).
3. Guinea pig complement, lyophilized (Invitrogen, Rockville, MD; store at –80°C until use).
4. Embryo-quality H_2O (Sigma).
5. Mineral or silicon oil, embryo quality (Cooper Medical).
6. IVF capillary transfer pipette.

7. 4-well non-tissue culture-treated dish.
8. 12-well tissue culture dish.
9. 0.1% (w/v) gelatin in DPBS (Invitrogen, cat. no. 14190-144).
10. Inactivated mouse embryonic fibroblasts (MEFs; Specialty Media, http://www.specialtymedia.com; also available from ATCC, cat. no. SCRC-1040.2).
11. MEF medium.

    Dulbecco's Modified Eagle's Medium, high-glucose formulation with L-Glutamine (Sigma; D-5796) supplemented with:

    10% fetal bovine serum (FBS; Invitrogen cat. no. 16000-044), heat inactivated.

    0.5× Pen/Strep (add from 100× stock; Invitrogen).

    1× nonessential amino acids (add from 100× stock; Invitrogen).

    Filter sterilize using 0.22-μm filter.

    Store up to 1 week at 4°C.
12. HESC medium for derivation:

    80% Knockout DMEM (Invitrogen) supplemented with:

    20% (v/v) Knockout Serum Replacement (Invitrogen).

    0.6× Pen/Strep (add from 100× stock; Invitrogen).

    1× L-glutamine (add from 100× stock; Invitrogen).

    1× nonessential amino acids (add from 100× stock; Invitrogen).

    4 ng/ml basic fibroblast growth factor (bFGF; Invitrogen).

    0.6× Insulin–Transferrin–Selenium (ITS; Gibco).

    0.01× β-mercaptoethanol, 1 M (Sigma M-7522).

    Filter sterilize using 0.22-μm filter.

    Store up to 1 week at 4°C.

    Note: A variety of commercial "ready-to-use" mediums can now be purchased and utilized for the derivation of HESCs and their maintenance. Nutristem (Biological Industries, Beit-Haemek, Israel) is successfully used in our lab; TeSR (StemCell Technologies, Vancouver, Canada) has also been successfully used for HESC derivation (93, 94).
13. Prepare MEF plates.

    (a) At a time point 24 h prior to immunosurgery, prepare a gelatin-coated 12-well plate by placing 2 ml of 0.1% gelatin in PBS into each well and incubating in a sterile environment 1 h at room temperature.

    (b) Rinse wells with MEF medium and plate 200,000 mitotically inactivated MEF cells in 2 ml MEF medium.

(c) Return plates to incubator.

(d) On the day of the immunosurgery, remove the MEF medium and rinse each well with 2 ml HESC medium.

(e) Discard rinse.

(f) Add 2 ml of HESC medium to each well.

(g) Return plates to incubator.

2.4. Equipment

Listed are the equipment required for PGD and HESC derivation in addition to the equipment described in the methods (part 4) below.

Incubators (37°C; 5% CO_2).

Inverted microscope.

Heat block.

Refrigerator (4°C).

Freezer (–20°C).

Centrifuge.

Minifuge.

Slide warmer.

GeneScan (genetic analyzer—ABI Prism 3100).

PCR machine.

Gel electrophoresis analysis equipment.

3. Methods

This chapter contains the methods for carrying out PGD as well as the derivation of HESCs from disease-bearing embryos that are not suitable for implantation (Fig. 1). In general, as described above, there are two molecular methods for PGD, depending on the type of genetic aberration tested. PCR is used when a specific monogenic mutation has to be diagnosed, while FISH analysis is carried out when a structural or numerical chromosomal aberration is analyzed. In both cases, single cell analysis is performed on a blastomere biopsied from a preimplantation embryo.

The entire PGD procedures described here and illustrated in Fig. 1 are performed under the Guidelines for genetic diagnosis approved by the Israeli Ministry of Health (CL15-001/3 and 50/2006) and in compliance with the European Society of Human Reproduction and Embryology (ESHRE; http://humrep.oxfordjournals.org/content/20/1/35.full.pdf).

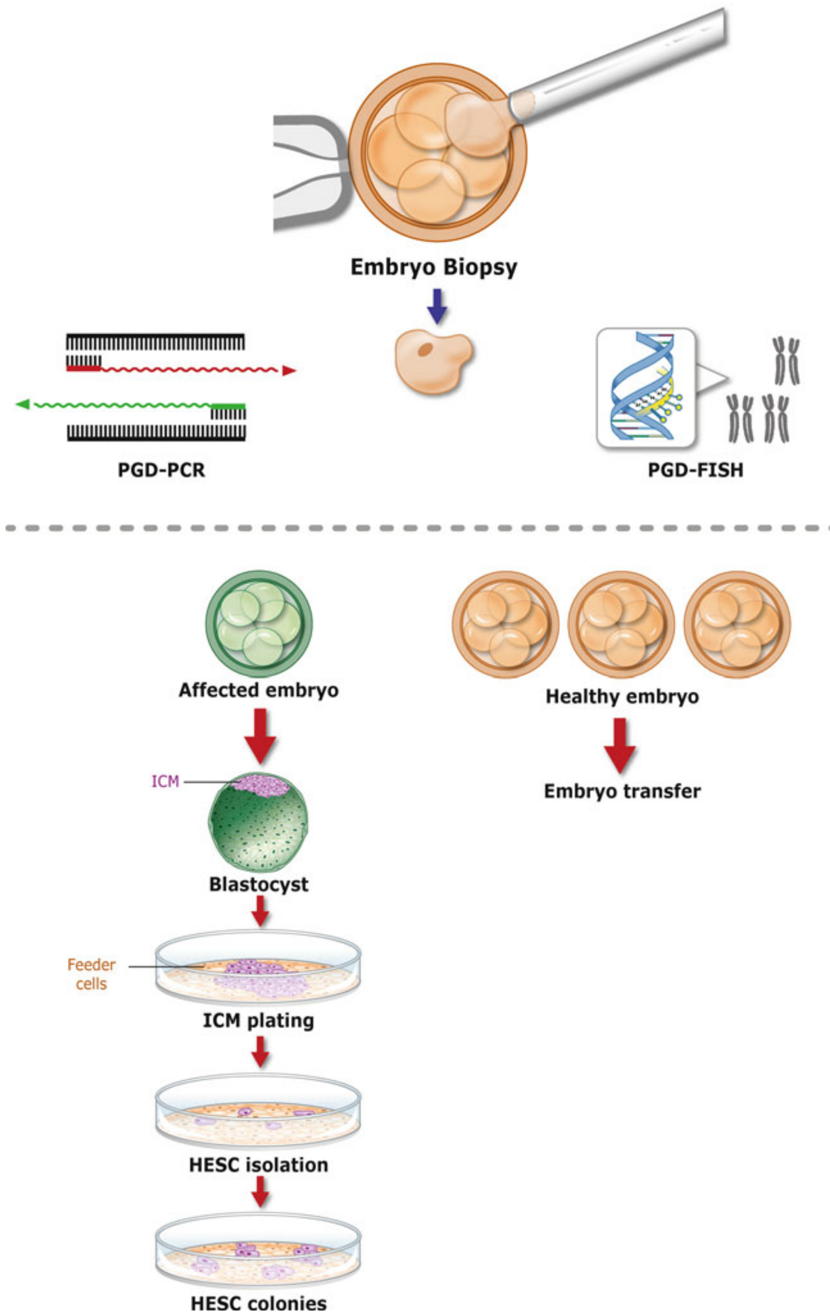

Fig. 1. Derivation of disease-bearing HESC lines from PGD-affected embryos. Preimplantaion embryos consisting of six to eight cells are biopsied on day 3 of development. One/two blastomeres are removed (*top*). These single blastomeres are diagnosed for a specific genetic disease carried by the parents by either PCR or FISH analysis. The resulting healthy embryos are transferred to the uterus for pregnancy, while embryos diagnosed to be affected are further grown for another 2–3 days until they reach the blastocyst stage. The Inner Cell Mass (ICM) of the affected blastocyst is isolated and plated on a feeder layer for HESC derivation. Outgrowths are then propagated into HESC colonies that can be used for the study of the disease they carry.

3.1. Blastomere Biopsy

Single blastomeres are biopsied from good-quality cleavage-stage embryos on day 3 following fertilization. The biopsied embryo should contain at least six blastomeres and less than 50% fragmentation. Embryo biopsy is performed using a micromanipulator (Narishige, Japan, or similar) mounted on an inverted microscope (Nikon eclipse TE 200 or similar).

1. Prior to biopsy, embryos are grown in human cleavage medium (see Note 1).
2. Wash cleaved embryos twice with a Ca^{2+}/Mg^{2+}-free blastomere medium supplemented with 10% synthetic serum supplement.
3. Incubate at 37°C for 5–10 min to facilitate blastomere aspiration.
4. Perforate the zona pellucida (ZP) using an in-contact laser apparatus (ZILOS, Hamilton).
5. Remove one to two blastomeres using a micromanipulator and biopsy pipette.
6. Using pooled glass pipettes, remove blastomeres from the biopsy dish and wash several times in single cell collection buffer.
7. For PCR-PGD, follow Subheading 3.2. For FISH-PGD, follow Subheading 3.5 (see Note 2).

3.2. Monogenic Disorders Diagnosed by PGD-PCR

Monogenic disorders are diagnosed in blastomeres only after family genotyping of affected, carriers, and healthy members. The mutation and flanking haplotype is first determined in genomic DNA and then validated in single cells, usually leukocytes isolated from peripheral blood. In rare occasions, sperm cells or polar bodies can be used for genotyping in cases where no relatives are available or in cases of de-novo mutations.

3.2.1. Leukocyte Isolation

These cells are used for genomic DNA extraction and single cell isolation.

1. Collect blood in Purple-cap BD Vacutainer EDTA tubes.
2. Dilute blood twofold in RBC lysis buffer for erythrocytes lysis.
3. Invert back and forth for 10 min.
4. Spin down leukocytes at $210 \times g$ for 10 min at room temperature.
5. Remove upper supernatant.
6. Resuspend leukocytes in 5 ml RBC lysis buffer for an additional wash.
7. Spin down cells at $210 \times g$ for 7 min at room temperature.
8. Remove upper supernatant, resulting in a leukocyte pellet (see Note 3).

3.2.2. Leukocyte Single Cell Isolation

1. Resuspend leukocyte population in IVF medium supplemented with artificial serum replacement.
2. Transfer small samples to a petri dish that contains drops of leukocyte-collecting media.
3. Dilute cells until single cells can be distinguished.
4. Using an inverted microscope and very-fine-pulled glass pipettes, collect single cells into sterile PCR tubes.
5. Immediately transfer tubes to a heat block and incubate at 65°C for 10 min for DNase inactivation.
6. Tubes can be kept for extended periods of time at –20°C until use.

3.2.3. Genomic DNA Isolation

1. Use pellet from step 8, Subheading 3.2.1.
2. Dissolve cells by adding 300 μl 50 mM NaOH.
3. Mix by aggressive pipetting until all cells are dissolved (about 3 min) (see Note 4).
4. Incubate in heat block at 95°C (or boiling water) for 10 min.
5. Mix completely until solution becomes viscous.
6. Add 40 μl TRIS, 100 mM.
7. Mix and store at 4°C until use.

3.3. Single Cell PCR Analysis

Since isolation of DNA from single cells is not possible, lysis of a single cell is performed in order to expose the DNA to PCR components.

1. Place single cell (leukocyte or blastomere) in 0.2-ml PCR tubes in minimal volume.
2. Place tubes in heat block (65°C) for 10 min (see Note 5).
3. Store at –20°C for at least 30 min or until PCR is carried out.
4. Add 3 μl lysis buffer to each tube containing the single cell.
5. Incubate at 80°C for 10 min.

3.3.1. Multiplex Nested PCR

1. Cool tubes quickly.
2. Add 17 μl of Mix 1 solution for a total of 20 μl.
3. Denature for 8 min at 96°C.
4. Reduce temperature to 75°C.
5. Under these conditions, add 1 unit DNA polymerase (Bio-X-act; Bioline) diluted in 5 μl PCR I- Mix II (Bioline).
6. Our standard protocol is described below. For unique sequences, such as GC-rich sequences and others, appropriate adoptions must be made, as described in ref. (36).

7. Follow the PCR program:

	Time	Temperature	Number of cycles
Denaturation	10′	98	1
Denaturation Annealing Elongation	1′ 1′ 1′	96 60 72	14
Denaturation Annealing Elongation	30″ 45′ 30″	96 60 72	20
Final elongation	8′	72	1

The amount of product resulting from this initial PCR is insufficient for genetic analysis. Therefore, a nested PCR must be carried out.

3.3.2. Nested PCR

1. For each tested locus, take 1 μl from the first PCR reaction.
2. Add 11 μl from Nested reaction tube.
3. Close the tube.
4. Vortex.
5. Spin down.
6. Denature for 8 min at 96°C.
7. Reduce temperature to 75°C.
8. To each tube, add 13 μl of Nested Mix 2 solution.
9. Follow the PCR program:

	Time	Temperature	Number of cycles
Denaturation	10′	97	1
Deraturation Annealing Elongation	1′ 2′ 3′	97 60 72	7
Deraturation Annealing Elongation	45 1′ 1′	97 60 72	10
Final elongation	8′	72	1

10. Characterizing of the genetic mutation is carried out by one (or more) of the following.
    (a) Polymorphic fluorescently labeled sites: Analyze samples using GeneScan (genetic analyzer—ABI Prism 3100) according to the manufacturer's instructions.

(b) Alleles that differ from each other in their sequence are digested with appropriate restriction enzymes and analyzed by gel electrophoresis.

(c) If a large deletion is the reason for PGD, gel electrophoresis can be carried out of the nested PCR directly.

3.4. Chromosomal Aberrations Diagnosed by PGD-FISH

Chromosomal aberrations and sexing are diagnosed in single cells only after karyotyping carriers of the chromosomal aberration and controls. The specific chromosomal aberration is first determined on metaphases derived from the patient's lymphocytes using commercial fluorescent probes when possible. The same set of probes are then used for single blastomere analysis during PGD.

3.4.1. Peripheral Blood Collection

1. Collect peripheral blood in heparin-containing tubes (Green cap BD Vacutainer LH Lithium Heparin tubes).
2. Transfer 0.4 ml peripheral blood to T25 flasks containing 5 ml karyotype medium and 0.2 ml PHA.
3. Culture cells in incubators at 37°C, 5% CO_2, for 72–96 h to insure cell divisions.
4. Add 0.1–0.2 ml of Colcemid to each flask.
5. Incubate the culture for an additional 30 min at 37°C.
6. Transfer to 15-ml conical centrifuge tubes.
7. Spin at $210\times g$ for 10 min.
8. Remove supernatant.
9. Slowly add 5 ml of hypotonic 0.075 M KCL to the pellet.
10. Incubate at 37°C for 12 min.
11. Slowly (drop by drop) add ice-cold 5 ml fixative (3:1 methanol:acetic acid).
12. Spin at $210\times g$ for 10 min.
13. Remove supernatant.
14. Repeat washes three times: for 10, 15, and 20 min, respectively.
15. Spin cells and resuspend in fixative solution between each wash.
16. Incubate overnight at –2°C.
17. Wash cells with fresh fixative solution.
18. Slowly drop cells onto a slide using glass pipettes.
19. Keep overnight at room temperature.
20. Perform FISH analysis on slides as described below.

3.5. FISH Analysis

FISH analysis for PGD is carried out on single cells with probes designed and tested on peripheral blood. This protocol is based on (95).

3.5.1. Preparation of Slides for FISH Analysis

1. Prepare two plates in order to wash cells from oil: one with media that contains serum and one with spreading buffer (0.01 N HCl/0.1% Tween 20).
2. Following biopsy, remove blastomeres from the biopsy dish using pooled glass pipettes.
3. Under a binocular, pass cell to media drop and then to spreading buffer drop.
4. Place cells onto Superfrost Plus slides.
5. Add a drop of spreading buffer using a 50-μm pulled glass pipettes onto the cells until cytoplasm is removed.
6. Watch cells under the binocular continuously to insure that a nucleus is present.
7. Mark location of nucleus to insure proper analysis.
8. Air dry slides.
9. All washes and incubations are carried out in Coplin Jars containing appropriate buffers.
10. Wash slides in phosphate-buffered saline (DPBS) for 1 min.
11. Dehydrate slides by transferring through an ethanol series (70, 85, and 100%) 1 min each.
12. Incubate for 10 min at 37°C with preheated pepsin:

    (98 ml DDW, 1 ml 1 N HCl, 1 ml Pepsin 10 mg/ml).
13. Wash with DPBS for 1 min.
14. Fix slides with formaldehyde (3 ml formaldehyde 37% in 97 ml DDW) at 4°C for 7 min.
15. Wash with PBS.
16. Dehydrate slides with increasing concentrations of ethanol (75, 85, and 100%) 1 min each.

3.5.2. Hybridization

1. Scan all embryonic nuclei under a phase-contrast microscope.
2. FISH is carried out according to the manufacturer's recommendations and as described previously (96).
3. FISH images are captured using a computerized system (FISHView; Applied Spectral Imaging, Migdal HaEmek, Israel) or any other appropriate analysis software.
4. Results are interpreted by two observers.
5. The criterion for signal scoring was that signals had to be a minimum of a signal's width apart to be scored as two separate signals (97) (see Note 6).

3.6. HESC Derivation

For derivation of diseased HESC lines, affected embryos at the blastocyst stage are donated for research after receiving informed consent and under the approved protocol as described in the

International Society for Stem Cell Research (ISSCR) guidelines (http://www.isscr.org/guidelines/ISSCRHESCguidelines2006.pdf).

Every couple undergoing PGD has to be informed that the purpose of the research is to obtain HESCs harboring the genetic disorder for which PGD is performed. The couples' participation in the study should be voluntary without receiving any direct compensation for their participation.

HESC lines are established by isolation and propagation of ICM cells obtained from blastocyst-stage embryos. Blastocysts are graded according to the ICM and trophectoderm appearance according to (98): Briefly—a good score indicates expanded blastocysts with tightly packed ICM cells and well-organized trophectoderm, a lower score indicates that the ICM was less compacted and adhered loosely together, and a poor score indicates that very few cells were visible. At days 6–8 of embryo development, embryos are micromanipulated to remove the zona pellucida and to isolate the ICM using one of the following two technologies:

1. *Immunosurgery*—this method was the original method by which the first HESC lines were derived (6).
2. *Mechanical isolation*—see Note 7.

3.6.1. Inner Cell Mass Isolation

Immunosurgery (see Note 8)

1. Immediately after zona is removed, transfer blastocysts into 3–5 ml HEPES medium.
2. Reconstitute antihuman serum with 2 ml DDW.
3. Reconstitute complement with 5 ml PBS.
4. Prepare a 1:5 dilution of both antihuman serum and complement in blastocysts culture medium (for a final concentration of 20%).
5. In a 4-well non-tissue culture-treated dish, place in one well 50-μl drops of the diluted antihuman serum. In the other 3 wells, place 0.5 ml blactocyst medium.
6. Add just enough embryo-quality mineral or silicon oil to completely cover the drops.
7. Warm to 37°C for at least 15 min before immunosurgery.
8. Prepare a similar 4-well plate for complement.
9. Cover with oil and warm for at least 15 min.
10. Transfer zona-free blastocysts to the drops of diluted antihuman serum and incubate on a 37°C slide warmer for 30 min.
11. Transfer embryos after three washes in blastocyst medium directly into the complement solution.
12. Incubate in the complement for 30 min at 37°C.
13. Wash three times in blastocyst medium.
14. Remove damaged trophectoderm cells by pipetting the blastocyst through a small-bore glass pipette.

Mechanical Isolation of ICM by Laser

The ICM is dissected from the trophectoderm by laser micromanipulation as described previously (53) (see Note 9).

3.6.2. Plate Isolated ICM on MEFs

1. Add 2 ml of human ES medium to each well of a 12-well tissue culture dish containing an MEF feeder layer (see Note 10).
2. Plate one isolated ICM into each well of the dish using a glass pipette.
3. Return plated embryos to the incubator.
4. Do not disturb for at least 24 h and preferably 48 h.
5. Check the wells to determine if the ICM has firmly attached to the substrate. If the ICM is firmly attached, replace 80% of the medium with 2 ml human ES medium that has been prewarmed to 37°C.
6. Every 48 h, replace the medium with fresh human ES medium.
7. Continue for about 10 days, until it becomes necessary to perform the first passage of the putative cell line.
8. Manually passage any cells with proper embryonic stem cell morphology (high nuclei/cytoplasm ratio and prominent nucleoli), cutting the cell masses into pieces containing 10–15 cells with a fine glass needle and transferring the pieces to newly plated MEFs in a 12-well plate, as described above. Also cut and passage cell masses that do not resemble embryonic stem cells, if possible.
9. Maintain the initial culture plates for at least 1 week, changing medium every 48 h, to determine if any other human ES colonies begin to grow.
10. After the initial passage, passage cell lines approximately weekly using manual passaging, being sure to select only cells with proper ES cell morphology (see Note 11).
11. HESC maintenance, freezing, and characterization have been described extensively elsewhere (2, 102) (see Note 12).

4. Notes

1. There are many commercial "ready-to-use" media for IVF, including all stages of preimplantation embryo development like Fertilization medium, Cleavage medium, Blastocyst medium, and Blastomere medium. In our lab, we routinely use media from Sage (Pasadena, CA, USA) and IrvineScientific (Santa Ana, CA, USA).
2. PGD of blastomere biopsied from IVF embryos can be carried out only after PCR or FISH for the diagnosed mutation is tested and calibrated on other single cells (like peripheral blood).

3. This pellet is used both for single cell isolation (Subheading 3.2.2) and genomic DNA isolation (Subheading 3.2.3).
4. Do not vortex since this will result in DNA shredding.
5. Transfer under DNA contamination-free conditions to a clean room: an isolated room into which only a few people enter. All surfaces are cleaned with bleach; every tube is cleaned with 10% bleach. Everything is UV irradiated.
6. Probes can be removed and slides can be reprobed.
7. A comparison between stem cell derivation methods has recently been carried out by others (99).
8. All immunosurgery steps are performed at 37°C on a prewarmed slide warmer.
9. Embryonic stem cells can also be derived by plating intact embryo (morula or blastocysts) on a feeder layer without isolating the ICM (100, 101).
10. We have recently derived HESC lines using Nutristem (Biological Industries, Beit Haemek, Israel) with good efficiency.
11. For proper morphology, please refer to our previous published manuscript (32).
12. We recommend examining the newly derived disease-bearing HESC line for the mutation that was initially tested in the PGD, on a frequent basis. Diagnosis should be carried out in a similar manner to the PGD diagnosis, including the comparison to all familial genotypes.

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Chapter 12

Single-Cell Enzymatic Dissociation of hESC Lines OxF1–OxF4 and Culture in Feeder-Free Conditions

Frances A. Brook

Abstract

Experimental manipulation of hESCs has been hampered by their fragility and susceptibility to apoptosis when dissociated into single cells. The OxF lines are particularly robust and may be successfully passaged as single cells, with the inclusion of ROCK inhibitor in the medium. The protocols here describe the enzymatic dissociation of hESCs into a single-cell suspension and the plating of these cells onto either feeder cells or a protein-coated surface.

Key words: Human embryonic stem cells, OxF1–OxF4, Enzymatic dissociation, Single cell, Fibronectin, Vitronectin, Matrigel

1. Introduction

The stem cell lines OxF1–OxF4 were derived between 2008 and 2009 from surplus embryos donated with informed consent by patients attending the Oxford Fertility Unit. The cells were derived from the mechanically isolated inner cell mass of good-quality blastocysts and cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in medium containing Knockout Serum Replacer (KSR, Invitrogen) and FGF2. The lines all express the pluripotency markers Oct 3/4, Nanog, Sox 2, Tra-1-60, and SSEA 4 as demonstrated by flow cytometry and immunostaining, and in vitro differentiation via embryoid bodies has shown that the lines can differentiate into cells representing all three germ layers. In addition, OxF1 undergoes directed differentiation through haematopoiesis to give rise to macrophages (1).

hESCs are conventionally maintained on MEF feeder layers and passaged by manually cutting colonies, a procedure which is time

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consuming and limits cell production. Dissociated hESCs, unlike mouse embryonic stem cells, are particularly fragile and subject to apoptosis (2), a characteristic that limits their capacity for manipulation. Addition of ROCK inhibitor protects hESC from programmed cell death (2) and is now widely used in culture to enhance survival during thawing and passaging hESC. The OxF lines are particularly robust and withstand enzymatic dissociation well: OxF1 has been shown to maintain the expression of pluripotency markers and differentiative capacity for at least ten passages when seeded as a single-cell suspension onto MEF feeder layers (unpublished observations). Dissociated cells also grow well and express pluripotency markers when passaged on Matrigel-, vitronectin-, or fibronectin-coated surfaces in MEF-conditioned medium. The OxF lines are, therefore, useful for experiments which require significant numbers of cells grown in feeder-free conditions; up to 10^7 cells may be obtained from one 6-well plate.

To minimise any accumulation of karyotypic abnormalities that may occur with repeated passages as a single-cell suspension, stock cultures of the OxF lines are maintained by manual passage on MEF feeder layers.

The following methods describe the maintenance of stock cultures, including the production of feeder layers, and the enzymatic dissociation of colonies for passaging as a single-cell suspension.

The OxF lines are made available through the UK Stem Cell Bank.

2. Materials

2.1. Preparation of Feeder Layers

1. MEFs: Primary cultures may be prepared from mouse embryos at day 14 of gestation using established methods, e.g. (3), or MEFs may be obtained commercially. Aliquots should be frozen at 3×10^6 cells/mL.
2. Feeder medium:180 mL DMEM containing 2.2 mg/mL sodium bicarbonate, 20 mL fetal calf serum (FCS), 2 mL of 200 mM glutamine, 0.2 mL of 50 mM mercaptoethanol solution (Invitrogen). Filter sterilise if necessary.
3. Gelatin: 0.1% in water. Autoclave.
4. Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Sigma).
5. Trypsin solution: 0.25% trypsin/0.02% EDTA (Sigma).
6. Mitomycin C: 1 mg/mL in sterile PBS.
7. Tissue culture flask: T75.
8. Tissue culture plate: 6-well plate.

2.2. Culture of Stock OxF Lines

1. ESC medium: 200 mL DMEM/F12, 50 mL KSR (Invitrogen), 1.25 mL of 200 mM glutamine, 2.5 mL of 100× non-essential amino acids, 0.5 mL of 2 μg/mL FGF-2. (Dissolve 50 μg FGF-2 in 50 μL of 50 mM Tris-HCl, pH 7.6. Add 24.95 mL of sterile PBS + 0.1% BSA. Aliquot and store at −20°C.) Filter sterilise if necessary.
2. Hypodermic needle: Size 25 G, 5/8″ attached to 1-mL syringe, sterile.
3. Glass Pasteur pipette, sterile.

2.3. Additional Components for Dissociation into Single Cells

1. ROCK inhibitor, Y-27632: 1 mM solution in sterile water. Aliquot and store at −20°C. Use at 10 μM (add 10 μL/1 mL medium).
2. TrypLE Express (Invitrogen).
3. P1000 pipettor with extra-long tips.
4. Haemocytometer.
5. MEF-conditioned medium: Add 3×10^6 irradiated MEF feeder cells to a gelatinised T75 flask in feeder medium. Leave overnight, then aspirate the medium, wash in PBS, and add 18 mL ESC medium. Leave for 1 day, collect the medium, and filter using a 0.2-μm syringe filter. Use immediately or aliquot and freeze.
6. Matrigel (BD): Thaw an aliquot on ice. Dilute 1:10 in cold serum-free medium using a pre-cooled pipette. Add 1 mL per well of a 6-well plate and leave at room temperature for 1 h. Aspirate Matrigel, wash with PBS, and use immediately.
7. Fibronectin: 100 nM in PBS. Dissolve 1 mg fibronectin from human plasma in 1 mL sterile water at 37°C for 30 min. Aliquot and store at −20°C. For each well of a 6-well plate, add 23 μL of stock fibronectin to 1 mL sterile PBS and leave at 4°C overnight. Aspirate fibronectin and wash in PBS before use.
8. Vitronectin: 125 nM in PBS. Dissolve purified human vitronectin at 0.5 mg/mL in 0.1% NaCl, 3 mM Hepes at pH 7.4. Aliquot and store at −20°C. For each well of a 6-well plate, add 20 μL of stock vitronectin to 1 mL PBS and leave at 4°C overnight. Aspirate vitronectin and wash in PBS before use.

3. Methods

3.1. Preparation of MEF Feeder Layers

1. Thaw a frozen aliquot of MEFs into a T75 flask containing 25 mL feeder medium (see Note 1). Place in a gassed (5% CO_2) incubator at 37°C for 2 days, by which time the cells should be barely confluent (see Note 2).
2. Prepare a 6-well plate by adding 1 mL/well gelatin. Leave at room temperature while the following steps are carried out.

3. Aspirate medium from the cells, wash with approximately 4 mL PBS, and replace this with 2 mL trypsin. Incubate at 37°C for 2–3 min until the cells detach upon gentle shaking. Add 8 mL feeder medium and transfer to a sterile 15-mL tube.
4. Mitotically inactivate the cells in a ^{60}Co source with 3,000 rads of γ-irradiation (see Note 3).
5. Count the cells using a haemocytometer. Aspirate the gelatin from the 6-well plate and add 1.7×10^5 cells/well in a volume of 2 mL (= 1×10^6 cells per plate).
6. Leave in the incubator overnight before use (see Note 4).

3.2. Maintenance of Stock hESC

Stock cultures of OxF lines should be passaged when colonies are large but have not yet begun to differentiate. Normally, one well is split into three new wells, but this ratio should be modified according to the number and quality of colonies.

1. Aspirate the medium from the well to be passaged and replace with 2.5 mL fresh warmed ESC medium.
2. Take the plate to a class II microbiological cabinet fitted with a dissecting microscope. Use a hypodermic needle to score across the colonies, three or four lines in one direction and then three or four lines perpendicular to these to give a grid. Select only the best colonies or the undifferentiated parts of colonies that are differentiating.
3. Heat a Pasteur pipette in a gentle flame. Pull to a small diameter, break it, and seal the tip in the flame. Using this tool, detach the cut sections of colony from the dish. Leave behind any differentiated sections.
4. When all sections are detached, remove the medium plus cells and place in a 15-mL tube. Wash the well with a further 2.5 mL of medium and add to the tube. If splitting into three wells, add an additional 2.5 mL medium.
5. Aspirate the medium from the feeder wells, wash with 2.5 mL PBS/well, and then add the medium containing the clumps of cells, 2.5 mL/well (see Note 5). Replace in the incubator.
6. Medium should be changed after 2 days and then daily. Cells should be passaged after 4–6 days, either as above for continuance of stock or as follows for experimental work.

3.3. Passage of hESCs as Single Cells

1. Aspirate the medium from feeder wells, wash with PBS, and add 2 mL per well ESC medium containing ROCK inhibitor (Y-27632). Equilibrate in the incubator for at least 2 h (see Note 6).
2. Aspirate the medium from one well of stock hESC, wash with PBS, and add 0.6 mL TryPLE Express (see Note 7). Place in the incubator for 4 min.

3. Check, using an inverted microscope, that the cells are starting to lift off, and then add 0.4 mL ESC medium. Disaggregate the cells by gentle trituration using a P1000 pipettor with an extra-long tip. Check under the microscope for a single-cell suspension (see Note 8), then transfer to a 15-mL tube, and add ESC medium to an appropriate volume for counting (e.g. 3 mL for 1 well).
4. Count cells using a haemocytometer (see Note 9).
5. Seed each new feeder well with 1×10^5 cells. Replace in the incubator.
6. Change medium on days 2, 4, 5, and 6. Do not include ROCK inhibitor in the medium changes.
7. By day 7, there should be approximately 2×10^6 cells per well. Cells may be passaged as above onto fresh feeder layers or into wells coated with Matrigel, fibronectin, or vitronectin using MEF-conditioned medium in the absence of feeders (Fig. 1).

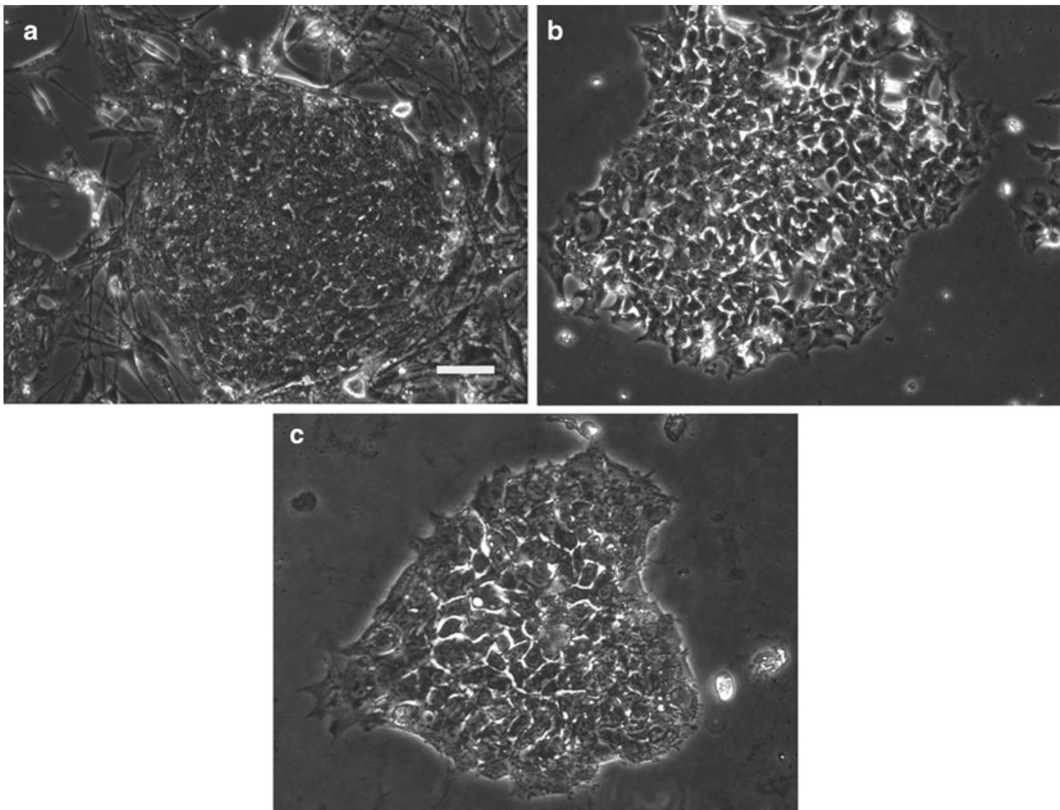

Fig. 1. Morphology of hESC colonies after five to eight passages by single-cell dissociation. (**a**) OxF1 growing on MEF feeder cells in ESC medium. (**b**) OxF2 growing on human vitronectin in conditioned medium. (**c**) OxF1 growing on Matrigel in conditioned medium. Scale bar is 50 μm.

In all cases, add ROCK inhibitor to the medium at the time of passaging but not to subsequent medium changes.

8. For experiments in feeder-free conditions, it is recommended that hESCs are grown on Matrigel, fibronectin, or vitronectin for one passage beforehand to remove all feeder cells. It is not recommended that hESCs are grown continuously on matrix substrates for, although the cells express markers of pluripotency after ten passages on vitronectin (unpublished observations), it is not known what chromosomal changes may be brought about.

4. Notes

1. Thawing of cells in a water bath at 37°C is a potential source of contamination, all the more relevant as the media used here do not contain antibiotics. To avoid this, thaw cells by holding the vial in the hand, roll it around until all the ice crystals have gone, and immediately transfer the cells into the flask. MEFs are not centrifuged; the volume of medium is sufficient to dilute the freezing mix.
2. Do not let the cells become overconfluent; there should be approximately $3–4 \times 10^6$ cells in total. If cell density becomes too great, the quality of any subsequent feeders is poor and they do not support hESCs well. Do not use MEFs beyond passage four, as higher passages also make poor feeders.
3. An alternative method of mitotic inactivation is to use mitomycin C. Aspirate medium from the flask of MEFs and replace with 5 mL feeder medium to which has been added 50 μL of 1 mg/mL mitomycin C stock solution (to give a final concentration of 10 μg/mL). Incubate for 2–3 h. Aspirate the mitomycin-containing medium and wash the cells at least three times with PBS. Dispose of the mitomycin solution according to local regulations. Trypsinise the cells as in step 3, count, and plate as in step 5.
4. Feeder plates may be used for up to a week after preparation, but are best used within 2–3 days.
5. The cut colonies settle out rapidly; therefore, the suspension should be continually mixed while adding the cells to the wells. Before replacing in the incubator, rock the plate gently from side to side in each direction to distribute clumps evenly. Failure to do this results in colonies clustering in the centre and merging together.

6. The addition of ROCK inhibitor improves hESCs' survival. ROCK inhibitor may be omitted with OxF1, but the yield of cells after 7 days will be reduced to less than 1×10^6 per well.
7. TryPLE Express should be at room temperature.
8. Repeat the trituration once more if there are still clumps. When cells are grown on feeders, strings of undissociated cells are often encountered, even after repeated trituration. These are best left behind when removing the suspension.
9. Count all cells, and do not attempt to distinguish between hESC and feeders.

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Chapter 13

Protocol for Expansion of Undifferentiated Human Embryonic and Pluripotent Stem Cells in Suspension

Hossein Baharvand, Mehran Rezaei Larijani, and Maryam Yousefi

Abstract

Human embryonic and induced pluripotent stem cells (hESCs and hiPSCs) offer a platform technology with the potential for developmental biology and cell-based therapy. Therefore, robust and cost-effective ways for mass production of them is necessary. Here, we have presented a protocol to grow pluripotent hESCs and hiPSCs in suspension by using a simple, inexpensive, microcarrier-free method. Under this condition, the cells maintained stability during freeze/thaw cycles without the loss of pluripotency markers for extended periods (>1 year). The cells maintained a stable karyotype and showed very similar expression profiles when compared to the adherent culture. The combination of this system with a bioreactor culture system will allow scale up culture of hESCs and hiPSCs needed for clinical and translational applications.

Key words: Human embryonic stem cells, Human-induced pluripotent stem cells, Suspension culture

1. Introduction

Unlimited capacity of self-renewal and differentiation into all cell types makes human embryonic and induced pluripotent stem cells (hESCs and hiPSCs) (1, 2) an invaluable tool to elucidate molecular mechanisms that determine adult cell fate, generate cellular models for discovery of new drugs and create populations of differentiated cells for novel transplantation therapies. However, to realize the therapeutic potential of these cells, it is necessary to understand underlying mechanisms of self-renewal, pluripotency, and differentiation to various cell types and find robust and cost-effective ways for mass production of these cells to satisfy clinical applications' demands. Since culture as monolayer colonies in the presence or absence of feeder cells does not provide cells in clinically relevant amounts and their three dimensional culture as embryoid bodies (EBs) leads to differentiation, thus mass production of these

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human pluripotent stem cells (hPSCs) requires single hPSCs seeding and the development of large-scale expansion protocols.

By providing large and adjustable attachment areas, microcarriers allow the culture of anchorage-dependent cells in suspension. Long-term propagation of hESCs on microcarriers has been reported while maintaining their self-renewal potential (3, 4). A homogenous and more controllable environment and less susceptibility to contamination make this approach advantageous in scaling up the production of hPSCs (5, 6). Additionally, hESCs were cultured in alginate microbeads for 260 days without passaging while maintained expression of pluripotency markers and the ability of differentiation to three germ layers (4). Poly(lactic-co-glycolic acid)/poly (L-lactic acid) scaffolds, agarose hydrogels, synthetic semi-interpenetrating polymers and hyaluronic acid are other useful polymers for the encapsulation of hESCs with the ability of supporting pluripotency and self-renewal (7, 8). However, in all of these examples, dissociating a large number of cells from beads for passages is challenging. In this regards, to overcome the problem of single cell passaging, Watanabe et al. (9) have shown that, it is already possible to passage hESCs as single cells without losing their pluripotency using Rho-associated kinase (ROCK) inhibitor Y-27632.

On the other hand, recently, suspension culture of hPSCs without using microcarriers was reported in the presence of IL6RIL6 chimera (interleukin-6 receptor fused to interleukin-6) and basic fibroblast growth factor (bFGF) while maintaining their pluripotency and stable karyotype (10). Upscaling propagation of hESCs up to 50 ml in stirred bioreactor in the presence of ROCK inhibitor and/or rapamycin in nonconditioned mTeSR medium was also reported (3, 11–13). Additionally, Steiner et al. (14) reported the possibility of derivation, expansion, and differentiation of hESCs in neurobasal medium with serum replacements (knockout serum replacement and Nutridoma-CS) supplemented with extracellular matrix components, neurotrophic (NT-3, NT-4, and BDNF) and growth factors (bFGF and activin A). However, the media and additives used in these reports were relatively expensive. We compared these protocols before (15).

Recently, we have described a protocol that resulted in a simple, inexpensive platform for the culture of hESCs and hiPSCs in suspension aggregates without losing karyotype stability during freeze/thaw cycles, and altering pluripotency and self-renewal. The cells are cultured in a microcarrier-free environment in the presence of conditioned medium (CM) without the addition of ECM proteins (laminin, fibronectin, or Matrigel) or other growth factors, excluding bFGF (15).

The two major drawbacks of this protocol were the presence of a low expansion rate of human PSCs in suspension culture compared to adherent colonies and the mouse embryonic fibroblast

(MEF)-CM. Recently, we successfully overcame these problems by a xeno-free expansion condition and the application of stirred-suspension bioreactors to increase fold expansion of hPSCs (Abbasalizadeh S, Larijani MR, Samadian A, Baharvand H, unpublished data). Therefore, our protocol presents a novel culture system in terms of its potential use for future therapeutic applications. Here, we report the protocol of published report (15) step-by-step for the culture of undifferentiated hPSCs (hESCs and hiPSCs) in suspension.

2. Materials

2.1. Chemicals

bFGF (Sigma-Aldrich, cat. no. F0291). Here, we used our produced bFGF (Royan Institute).

1. DMEM/F12 (Invitrogen, cat. no. 21331-020).
2. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, cat. no. D2650).
3. Fetal bovine serum (FBS, Hyclone, cat. no. SH30071.03).
4. Fetal bovine serum (FBS, Hyclone, cat. no. SH30070.03).
5. Insulin–transferrin–selenium (ITS, Invitrogen, 41400-045).
6. Knockout serum replacement (KOSR, Invitrogen, cat. no. 10828-028).
7. L-Glutamine (L-Gln, Invitrogen, cat. no. 25030-024).
8. β-Mercaptoethanol (Sigma-Aldrich, cat. no. M7522).
9. Mitomycin C (Sigma-Aldrich, cat. no. M0503).
10. Nonessential amino acid solution (Invitrogen, cat. no. 11140-035).
11. PBS without Ca^{2+}/Mg^{2+} (PBS^{-}, Invitrogen, cat. no. 21600-010).
12. Penicillin/streptomycin (Invitrogen, cat. no. 15070-063).
13. ROCK inhibitor Y-27632 (Sigma-Aldrich, cat. no. Y0503).
14. Trypsin/EDTA (0.05%/0.53 mM, Invitrogen, cat. no. 25300-054).
15. Accutase (Invitrogen, cat. no. A11105-01).
16. Accumax (eBioscience, cat. no. 00-4666-56).

2.2. Disposables

1. 60 mm tissue culture dish (Falcon, cat. no. 353004).
2. 5 ml tube, 5 ml test tube (Falcon, cat. no. 352003).
3. Syringe (2, 5, 20, and 50 ml).
4. Cell Scraper (TPP, cat. no. 99002).
5. Pipette (TPP 5, 10 ml, cat. no. 94005, 94010).
6. Flask T-75 (TPP, cat. no. 90075).

7. 0.22-μm pore size filter (Orange, cat. no. 1520012).
8. Vacuum filtration (TPP 500 ml, cat. no. 99500).
9. Filter pipette tips (0.5–10, 5–100, and 50–1,000 μl).
10. 60 mm nonadhesive bacterial plate (Griner, cat. no. 628102).
11. Cryo tube (TPP 2 ml, cat. no. 89020).

2.3. Equipment

1. Inverted phase contrast microscope (4, 10, 20, and 40× objectives, Olympus, CKX41).
2. Stereomicroscope (Olympus, SZX12).
3. Micropipette (Eppendorf, 1–10, 10–100, and 100–1,000 μl).
4. Pipettor (Boeco, Germany).
5. Laminar flow hood (Class I and II, Jal Tajhiz, Iran).
6. Hemocytometer (Neubaur, HBG, Germany).

2.4. Reagent Setup

1. *bFGF solution*: Dissolve bFGF in Tris-base to 10 μg/ml (1,000×). Divide solution into 100 μl aliquots and freeze at –70°C. Aliquots can be stored for more than a year but must be used within 1 week after thawing.
2. *β-Mercaptoethanol*: Dissolve 70 μl in 10 ml PBS^- to 100 mM (1,000×). Sterilize through a 0.22-μm filter. It should be maintained in a tube with dark cover.
3. *hPSC medium*: To one 500 ml bottle of DMEM/F12, add 132 ml KOSR supplement, 6.6 ml NEAAs, 6.6 ml L-Glu, 6.6 ml Pen/Strep, 6.6 ml ITS and 0.66 ml β-mercaptoethanol. Prior to use, add bFGF to give final a concentration of 100 ng/ml.
4. *ROCK inhibitor (ROCKi), Y-27632*: Add 5 mg Y-27632 to 14.75 ml cooled, sterilized, distilled water to 1,000 μM (100×). Sterilize through a 0.22-μm filter. Divide into 100–500 μl aliquots and store at –20°C. After thawing, maintain solution at 2–8°C. Y-27632 is light sensitive; therefore, it should be handled in subdued "yellow" lighting.
5. *Feeder cells*: Prepare MEFs and human foreskin fibroblast feeder layers as previously described (16) (see Note 1).
6. *Conditioned medium*: Incubate 15 ml hESC medium (including 4 ng/ml bFGF, 200 μl/cm^2) with a confluent MEF feeder layer (75,000 cells/cm^2) from the NMRI strain overnight in 75 cm^2 T flasks that were inactivated by mitomycin C.
7. *CM-hPSC medium*: Add 100 ng/ml bFGF to hESC medium, which was conditioned on MEFs.
8. *Freezing medium*: Mix 10% DMSO and 90% FBS or KOSR. Always prepare fresh on ice.

3. Methods

3.1. Transfer from Adherent Culture

1. Prepare a 60 mm diameter cell culture dish containing feeder-free hPSCs cultured for 6-7 days.
2. Examine the plate by inverted phase contrast microscopy to evaluate morphology, microbial contamination, and confluency.
3. Add 10 μl/ml of ROCKi Y-27632 to the culture medium 1–2 h before detaching the cells from the plate.
4. Remove the differentiated area completely by using the pipette tip (10–100 μl) under a dark-field stereomicroscope (Fig. 1).
5. Aspirate the medium and rinse the plate using PBS^{-}.
6. Add 1 ml of prewarmed trypsin/EDTA and incubate the cells for 2 min.
7. Gently remove trypsin/EDTA and continue the treatment for 3 min. Check cell dissociation under the inverted-phase contrast microscope.
8. Add 2 ml CM-hPSC medium containing ROCK inhibitor.
9. Detach hPSC colonies by cell scraper.
10. Pipette medium over the tilted plate 3–5 times until the solution becomes cloudy and the cells completely disperse.
11. Check cell dissociation under the inverted-phase contrast microscope (see Note 2).
12. Count the cells using a hemocytometer. Assess cell viability by the trypan blue exclusion method.
13. Transfer cells onto 60 mm nonadhesive bacterial plates that contain 5 ml of CM-hPSC medium at 15×10^4 viable cells/ml (see Notes 3 and 4).
14. Gently move the plate several times, horizontally and vertically, to distribute the cells evenly. Do not swirl, as it will bring all the cells to the center of the plate.
15. Incubate the cells under standard conditions (37°C, 5% CO_2, saturated humidity) in suspension culture.
16. After 2 days, swirls the plate until hPSC spheres collect in the center of plate. Gently remove 80–90% of the old medium from the sides and add 5 ml new CM-hPSC medium that does not contain ROCK inhibitor.
17. Renew the medium every other day, up to 7 days, the optimum day for passaging, according of sphere diameter (Fig. 2) and hPSC line (15).

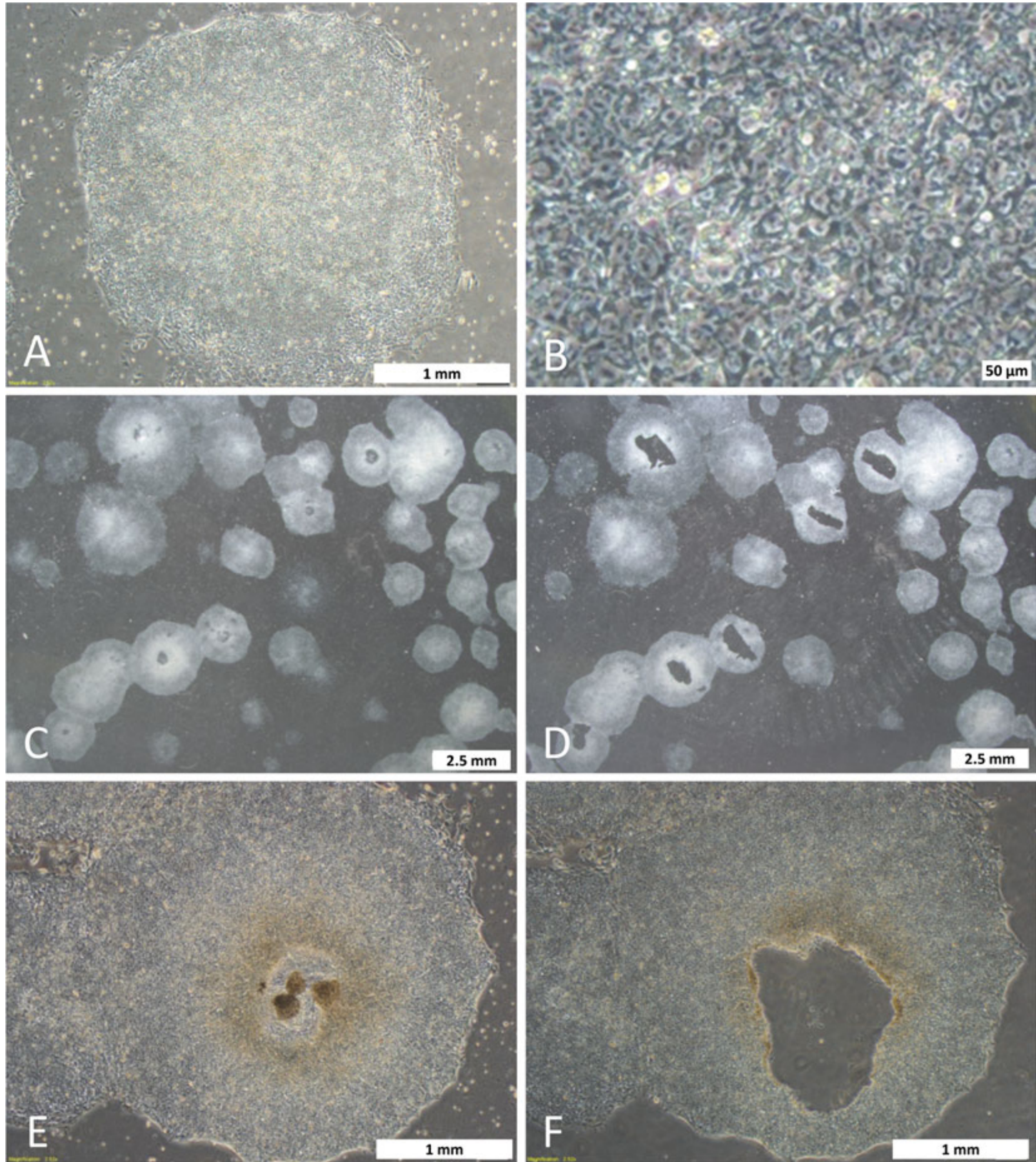

Fig. 1. Transfer of hPSCs from adherent culture. Morphology by phase contrast microscopy (**a**) and higher magnification (**b**) of hPSCs. Remove the differentiated area using the pipette tip under a dark-field stereomicroscope (C and D). Higher magnification under phase contrast microscopy (**e**, **f**).

3.2. Expansion of hPSCs

18. Add 10 µl/ml of ROCKi Y-27632 to the culture medium 1–2 h before cell dissociation.
19. Swirl plate until hPSC spheres collect in the center of the plate (Fig. 3a).
20. Transfer the hPSC spheres into a 5 ml tube that contains 2 ml PBS⁻ (Fig. 3b).

Fig. 2. Morphology and growth of hPSCs cultured in suspension. Phase contrast microscopy (**a**1–12); growth curve related to the diameters of the spheres (**b**).

Fig. 3. Expansion of hPSCs. Collect hPSC spheres in the center of the plate (**a**). Harvest spheres in 5 ml tube and wash the spheres in PBS$^-$ (**b**). Trypsinize the spheres and check cell dissociation under the inverted-phase contrast microscope (**c**).

21. Wash the spheres with 2 ml PBS⁻.
22. After 2 min, gently aspirate supernatant and add 0.5 ml trypsin/EDTA (see Note 5).
23. Incubate the cells for 4–5 min and pipette them 10–30 times to have dissociated single cells (Fig. 3c).
24. Add 2 ml CM-hPSC medium that contains ROCK inhibitor.
25. Repeat steps 11–17 (see Notes 6 and 7).

3.3. Freezing/Thawing of hPSCs

Cryopreservation of hPSCs is frozen/thawed as previously described (17).

3.4. Anticipated Results

To evaluate morphology of hPSCs and expression of markers, we analyzed the cells expanded in CM-hPSC medium over multiple passages for markers of pluripotency and early stages of differentiation. The cells proliferated for 14–73 passages and maintained their pluripotency over long-term culture as determined by the expression of pluripotency markers, OCT4, NANOG, ALP, SSEA3, and TRA-1-81 (Fig. 4). Furthermore, we assayed gene expression during spontaneous and directed differentiation in vitro. Cultures of hESCs and hiPSCs maintained a stable karyotype after 43–57 weeks of cultivation in suspension. Microarray gene expression analysis of the three lines showed similarity of the cells in adherent and suspension culture conditions. After thawing, hPSCs retained their undifferentiated characteristics. We believe that protocol presented here offers simple handling and passaging, in addition to less susceptibility to contamination and low-cost procedure for prolonged expansion of hESCs and hiPSCS in suspension with karyotype stability (Fig. 4).

4. Notes

1. MEF-derived conditioned hPSC medium can be replaced by human foreskin fibroblast-derived CM (Abbasalizadeh S, Larijani MR, Samadian A, Baharvand H, unpublished data).
2. Passaging time (6–8 days) of hPSCs is variable.
3. Seeding of low cell density does not give rise to well spheres and high cell density leads to clumping of all cells during the first 2 days or later.
4. 60 mm nonadhesive bacterial plates are better for maintaining hPSC spheres compared with 35 mm nonadhesive bacterial plates that cause cell clumping.
5. Application of Accutase or Accumax, a formulated mixture of digestive enzymes, instead of trypsin/EDTA improves cell viability with no need for FBS or trypsin inhibitor.

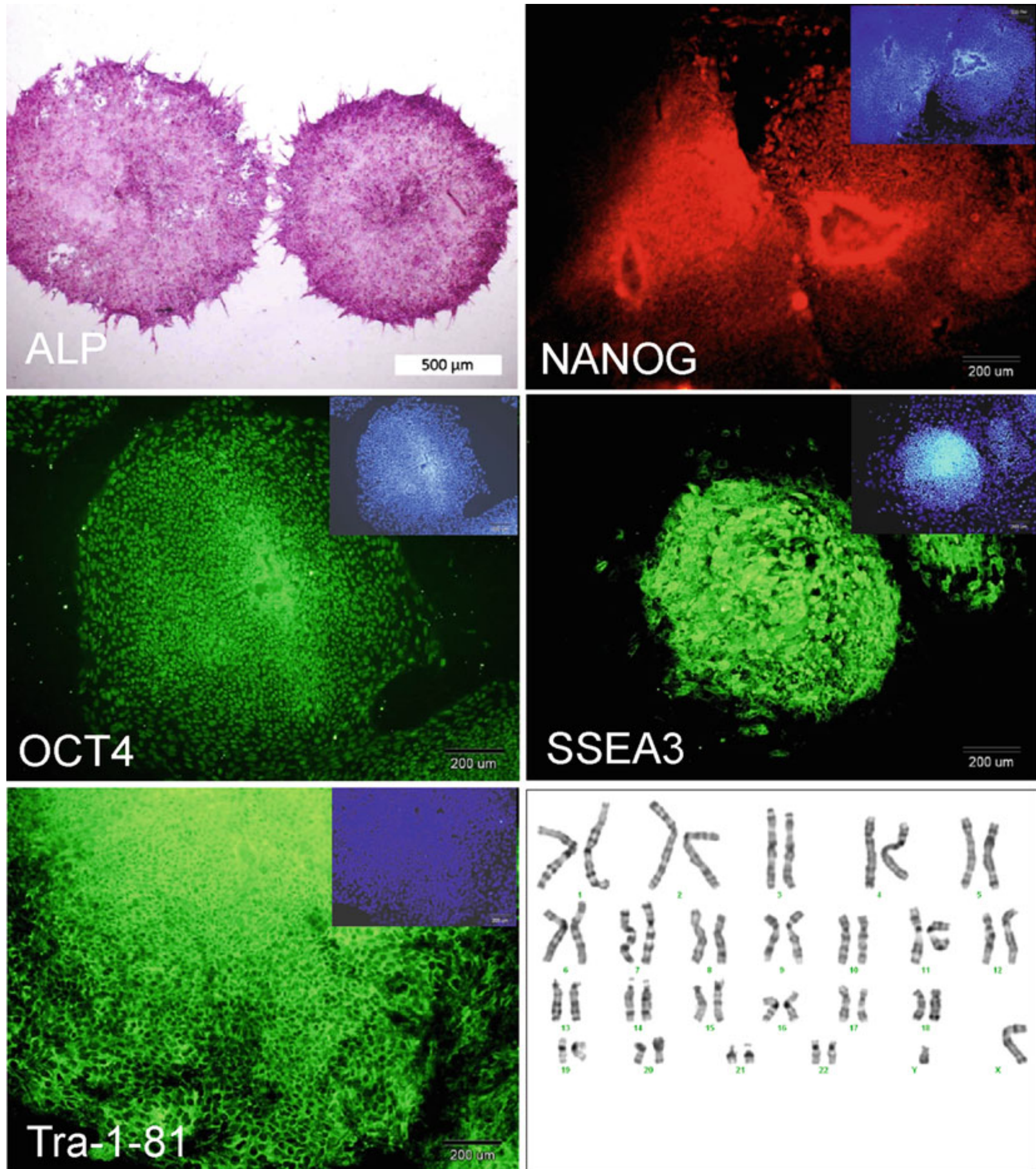

Fig. 4. Characterization of pluripotency markers in suspension culture. The spheres were replated on Matrigel-coated plates to show the characteristics. Immunofluorescence staining for expression of pluripotency markers ALP, NANOG, OCT4, SSEA3, and TRA-1-81. Nuclei were stained with DAPI (*blue*, *inset box*).The karyotypes of hPSC were normal.

6. In our experience, the hPSCs can be routinely passaged at a split ratio of 1:2–1:3 once weekly to 60 mm nonadhesive bacterial plates that contain 5 ml CM-hPSC medium.
7. The expansion ratio of hPSCs can increase six- to eightfold as aggregates in suspension in a stirred spinner while maintaining their self-renewal capabilities (Abbasalizadeh S, Larijani MR, Samadian A, Baharvand H, unpublished data).

Acknowledgment

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Chapter 14

Suspension Bioreactor Expansion of Undifferentiated Human Embryonic Stem Cells

Roman Krawetz and Derrick E. Rancourt

Abstract

Embryonic stem cells (ESCs) are unique cells, which have the ability to differentiate into all cell types that comprise the adult organism. Furthermore, ESCs can infinitely self-renew under optimized conditions. These features place human ESCs (hESCs) in a position where these cells can be exploited for tissue engineering and regenerative medicine approaches in treating human degenerative disorders. However, cell therapy approaches will require large amounts of clinically useable cells, not typically achievable using standard static cell culture methods. Here, we describe a method wherein clinically relevant numbers of hESCs can be generated in a cost and time effective manner.

Key words: Embryonic stem cells, Bioreactor, Pluripotency, Cell therapy, Y-27632, Rapamycin

1. Introduction

Stem cells were first discovered in the mouse in the early 1960s, initially observed as cells with unique differentiation and renewal capacity (1), the first embryonic stem cell (ESC) lines were derived in the early 1980s (2). Through further study it was discovered that cells derived from the inner cell mass (ICM) of an early embryo retained the ability to differentiate into all cell types that comprised the adult organism (pluripotency). These ICM cells were also able to undergo "infinite" self-renewal in static cell culture when cultured in the presence of leukemia inhibitory factor (LIF), whereas typically primary cells undergo cellular senescence within the first ten passages. Unlike mouse ESCs, hESCs do not respond to LIF, but instead rely on a basic fibroblast growth factor (bFGF): insulin growth factor 2 (IGF2) signaling niche to maintain pluripotency in culture (3, 4).

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hESCs are typically grown under feeder or feeder-free conditions in the presence of high concentrations of bFGF to maintain pluripotency and karyotype stability (3, 5) Under standard feeder conditions, either human or mouse fibroblasts are used as a feeder cell layer, which allows for proper adherence of the hESCs, as these cells do not adhere to untreated plastic culture surfaces. Under feeder-free conditions an extracted or artificial extra-cellular matrix (ECM) is added to the culture ware and the hESCs will normally thrive when a sufficient concentration of bFGF is added. However, the cost, cell culture experience and hands on time required to maintain hESCs under these standard static culturing practices is not trivial and can limit the number of cells generated each passage. In standard static cell culture, the surface areas of the flask/plate coupled with oxygen diffusion is limiting to the number of cells that can be generated in a single piece of culture ware.

Bioreactors have been employed to expand both mouse and human pluripotent stem cell population (6–9). Stirred suspension bioreactors use a design wherein a magnetic stir bar within the culture vessel is driven by an external magnetic stir plate, which can control the agitation speed within the bioreactor (Fig. 1).

Fig. 1. Stirred suspension bioreactor setup in 37°C CO_2 incubator. The 100 ml bioreactor is placed on the magnetic stir plate and the speed is set and confirmed (see Note 8) at 100 rpm.

Because of the design of the suspension bioreactor and the lack of an ECM, cell expansion within the bioreactor is not limited to the available surface area, but is influenced by the agitation rate, oxygen concentration, and nutrient availability. Therefore, this technology is ideally situated to aid in translating bench-top therapeutics into the clinic in regards to the sheer number of stem cells that will be required in any given treatment.

2. Materials

Prepare all solutions under sterile conditions and use cell culture tested reagents whenever possible. If nonsterile reagents are to be utilized filter all reagents using 0.2 μm filters before use. It is strongly recommended to batch test any/all culture reagents for adverse effects of hESCs in static culture.

2.1. hESC Culture Reagents

1. hESC media: mTeSR™ feeder-free hESC media (Stem Cell Technologies, Vancouver Canada) is recommended. This media comes in 500 ml bottles with separate base media and growth factor components. Each 100 ml suspension bioreactor will require 200 ml of mTeSR™ media per passage.
2. Extracellular matrix: Matrigel™ a nonzeno-free ECM (BD Biosciences, Franklin Lakes, NJ) is recommended for static culture and when using mTeSR™ media. Resuspend and coat the culture dishes with Matrigel™ according to the manufacture's instructions.
3. Dissociation enzymes: Accutase™ (Stem Cell Technologies) is recommended for single cell dissociation of hESCs in static and suspension culture systems. Other enzymes, such as Collagenase and Trypsin, are not recommended for dissociation of suspension culture-generated hESC aggregates. It is strongly recommended that the Accutase™ is aliquoted and stored at −20°C as repeated freeze-thaws and/or extended period at 4°C will lower the potency of the enzymes.
4. ROCK inhibitor: 10 μM Y-27632 (Sigma, St. Louis, MO) treatment is recommended for single cell dissociation steps.
5. Rapamycin: 0.1 nM Rapamycin (Sigma) is required for successful generation and maintenance of hESC aggregates in suspension. Concentrations less than 0.1 nM will not be effective and values above 1 nM may start to impact on the hESC expansion rate.

2.2. Cell Counting and Viability Assessment

1. Trypan Blue: Trypan Blue (Bio-Rad, Hercules, CA) is used with the Bio-Rad cell counting system to calculate the total and viable cell densities in static and suspension culture.

Alternatively, any cell counting and viability assessment method should be acceptable.

2.3. Pluripotency Assessment

1. Flow cytometry: Fluorescently labeled antibodies to Oct-4, Nanog, SSEA-3, SSEA-4, and REX-1 (all BD) are incubated with fixed, permeabilized and blocked hESCs according to the manufacture's instructions. The labeled cells are then analyzed using a flow cytometer (BD) registering at least 10,000 events.
2. PCR: Both RT (reverse-transcriptase) and Q (quantitative) PCR can be utilized to determine if the hESCs are expressing mRNA transcripts of pluripotency-related genes to Oct-4, Nanog, SSEA-3, SSEA-4, and REX-1.
3. Teratoma analysis: One million dissociated hESCs should be resuspended in 1× DPBS (Invitrogen) and injected into the thigh muscle of an SCID mouse (Taconic Farms, Hudson, NY). Tumor cell masses should be generated in 4–6 weeks and the resultant tissues should be analyzed with histology to determine if tissues derived from all three germ layers are present.

3. Suspension Bioreactor Materials

1. Bioreactor vessel: 100 ml suspension bioreactors (NDS Technologies, Vineland, NJ) with magnetic impellers should be pretreated with Sigmacoat (Sigma) following manufacture's instructions to prevent sticking of the hESC aggregates to the glass surface. This process should be repeated every 6 months or as required.
2. Magnetic stir plate: Variable speed magnetic stir plates are available from a number of suppliers (VWR International, Radnor, PA). However, make sure that the stir plate can (A) maintain a speed of 100 rpm and (B) be housed in a cell culture incubator. If your unit is not designed to be housed at 37°C, the temperature in the incubator may reach 90–100°C effectively killing all mammalian cells.

4. Methods

4.1. Preparation of hESCs in Static Culture

1. hESCs can either be cultured under feeder-dependent or feeder-free conditions. Under feeder-dependent conditions either inactivated (see Note 1) human or murine fibroblasts can be utilized. Under feeder-free conditions Matrigel™ is recommended, used according to the manufacture's instructions.

mTeSR™ hESC media is recommended (see Note 2) for static culturing of hESCs, as this media will be used within the suspension bioreactor.

2. For single cell passaging under static culture conditions, it is recommended that Accutase™ and the ROCK inhibitor Y-27632 be utilized. Normally, hESCs are exposed to 10 μM Y-27632 1 h before dissociation and 24 hrs after replating the cells (see Note 3).
3. One million viable hESCs are required to seed each 100 ml suspension bioreactor vessel. Therefore, it is recommended to have at least doubled that amount in static culture per bioreactor, since a number of cells will undergo apoptosis during single cell dissociation even in the presence of Y-27632.

4.2. Transfer of hESCs from Static to Suspension Culture

1. One hour before single cell dissociation (see Note 4) add 10 μM Y-27632 to the culture media of static hESCs.
2. Remove the culture media and add a sufficient amount of Accutase™ to cover the bottom of the dish/plate. Incubate the hESCs at 37°C, checking on the dissociation every 1–2 min (see Note 5).
3. Stop the reaction with the addition of fresh media and break up any cell clumps with gentle agitation. Pellet the hESCs and discard the supernatant.
4. Resuspend the cells at a density of one million cells per milliliter in a sterile 15 ml conical tube containing fresh mTeSR™ with 10 μM Y-27632. Incubate the cells in suspension for 1 h at 37°C at 5% CO_2 (see Note 6).
5. Prepare a 100 ml suspension bioreactor with 100 ml of fresh prewarmed (see Note 7) mTeSR™ media with 10 μM Y-27632 and 0.1 nM Rapamycin.
6. Add one million hESCs to each 100 ml suspension bioreactor and culture the cells at 37°C, 5% CO_2 and 100 rpm (see Note 8).

4.3. Culturing of hESCs in Suspension

1. Twenty-four hours after initial seeding (referred to as day 1 of culture) the hESCs in the suspension bioreactor, it is essential to change the media (see Note 9).
2. Collect all 100 ml of media with hESCs in two sterile 50 ml conical. Spin the conicals at 800 × *g* for 5 min. Carefully remove as much of the supernatant as possible with aspiration. Do not pour off the old media as the small hESC aggregates will not adhere to the bottom of the conical and will be lost. We recommend leaving the last 1–5 ml of old media per conical so not to accidently remove the hESCs.

3. Prepare the bioreactor with 100 ml of prewarmed mTeSR™ media and 0.1 nM Rapamycin. It is not recommended to add Y-27632.
4. Place the bioreactor vessel at 37°C, 5% CO_2, and 100 rpm. The media usually does not need replacing until day 6 of culture; however, it can be replaced as often as desired.

4.4. Passaging of hESCs in Suspension

1. On day 6 of bioreactor culturing, remove all the media and cells from the bioreactor and place into 2 × 50 ml sterile conical tubes. Spin down and remove the old media leaving 1–2 ml. Add 10 μM Y-27632 to the media and cells and incubate at 37°C, 5% CO_2 for 1 h.
2. Transfer the hESCs to a 15 ml sterile conical and spin down at 800 × *g* for 5 min. Remove as much as the old media as possible and add 1 ml of Accutase™. Incubate the cells at 37°C, 5% CO_2 checking occasionally under a dissecting microscope to observe dissociation of aggregates (Fig. 2).

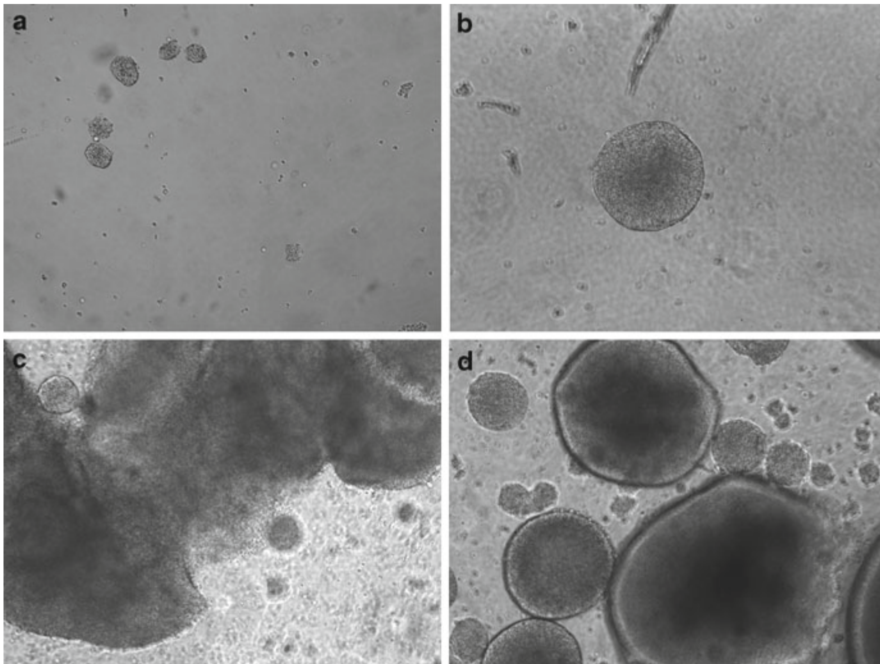

Fig. 2. Typical hESC aggregates seen during one passage. Small aggregates can be observed at 24 h after initial seeding (**a**). Larger aggregates can be observed at day 4 of passaging (**b**). Aggregates with evidence of spontaneous differentiation (**c**). If aggregates are not passaged every 5–6 days or cultured at lower rpm, then very large and unmanageable hESC aggregates may form (**d**). All images taken at ×10 magnification.

3. Once the majority of the aggregates have been dissociated into single cells, use gentle agitation to break up any small clumps.
4. Repeat steps 4–6 of Subheading 3.2.

4.5. Expansion Rate Calculation and Assessment of Pluripotency

1. During each day of bioreactor culture, it is recommended to count the cells and determine the viability.
2. Take a 5 ml sample from the bioreactor and repeat steps 1–3 of Subheading 3.4 (minding only to take 5 ml and not the entire volume). Add 5 ml of fresh media to the bioreactor.
3. Using Trypan Blue and an automated cell counter or hemocytometer, record the percent viability and total cell count.
4. At the conclusion of your experiment, it is recommended to analyze the pluripotency of the hESCs. Normally, we assess this through FACS, PCR, and Teratoma analysis (see Note 10).

5. Notes

1. Many previous papers have described methods for the static culturing of hESCs (3, 5, 10, 11). However, currently, only culturing methods that use mTeSR™ are suitable for suspension culture. Therefore, hESCs should be adapted to mTeSR™ before attempting to grow the cells in suspension.
2. The method described here and alternative methods to grow hESCs in suspension have utilized mTeSR™ hESC medium. Other culture mediums may also work but culturing conditions and growth factor compositions will need to be optimized.
3. Y-27632 and other ROCK pathway inhibitors (C3 toxin, HA-100) allow human ESCs (hESCs) to be dissociated into single cells with adverse apoptosis (12, 13). However, for the successful generation of hESC aggregates in suspension only Rho/ROCK pathway inhibitors that target ROCK or are upstream of ROCK are effective.
4. Single cell dissociation is vital to the successful generation of undifferentiated hESC aggregates. If partial collagenase dissociation is utilized, the risk of generating EBs is greatly increased.
5. Normally, in static culture hESC colonies are tightly packed with well-defined borders. With enzymatic dissociation, individual cells will be identifiable within the colony. It is recommended not to allow single cell dissociation to proceed until all cells are released into the media, as this is unnecessarily harsh on the hESCs. Instead wait until most of the cells are identifiable within the hESC colonies, stop the reaction with fresh

media and break up any leftover cell clumps with gentle mechanical dissociation.

6. This 1 h incubation with Y-27632 allows small “seed” aggregates (1–5 cells) to form. This greatly enhances the efficiency of aggregation and expansion when the cells are transferred to the stirred bioreactor.
7. Prewarm all hESC culture media to 37°C to avoid shocking the cells.
8. To calculate rpm of the bioreactor, do not rely on the digital/analog settings on your stir plate. Each bioreactor design with behave differently when placed on the magnetic stir plate. Verify your rpms by counting the number of impeller rotations in 10 s and multiply by 6 to get your revolutions per minute. One hundred rotation per minute is optimal for hESC aggregate formation and expansion, if significantly slower speeds are used (60–80 rpm) aggregates will grow quickly because of reduced shear stress and may develop either differentiated or necrotic centers. At higher speeds (120+) cells do not effectively aggregate, any resultant small aggregates will normally die.
9. If the Y-27632 is not removed from the bioreactor after 24 h, the aggregates will still remain viable and undifferentiated; however, the expansion rate will be significantly negatively impacted.
10. The following reference describes commonly used methods to analyze pluripotency of hESCs (14–16).

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Chapter 15

Derivation, Propagation, and Characterization of Neuroprogenitors from Pluripotent Stem Cells (hESCs and hiPSCs)

Khun-Hong Lie, Henry C.Y. Chung, and Kuldip S. Sidhu

Abstract

The differentiation of human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) towards functional neurons particularly hold great potential for the cell-based replacement therapy in neurodegenerative diseases. Here, we describe a stepwise differentiation protocol that mimics the early stage of neural development in human to promote the generation of neuroprogenitors at a high yield. Both the hESCs and hiPSCs are initially cultured in an optimized feeder-free condition, which offer an efficient formation of aggregates. To specify the neuroectodermal specification, these aggregates are differentiated in a defined neural induction medium to develop into neural rosettes-like structures. The rosettes are expanded into free-floating sphere and can be further propagated or developed into variety of neuronal subtypes.

Key words: Pluripotent stem cells, Differentiation, Neuroprogenitors, Neural rosettes, Neurospheres, Embryoid bodies

1. Introduction

The study of human development, aging and disease is limited by a lack of model systems that can reproduce the precise sequence and timing of cellular and molecular events. In response to proper signals, hESCs are capable of self-renewal and producing differentiated progenies, which make them good candidates for cell replacement therapy (1–3). Other pluripotent cells, including the recent induced pluripotent cells that now can be derived from adult skins (4–17) also have the inherent ability to recapitulate human development in vitro. Developing a robust protocol for the derivation of neuroprogenitors from well-defined sources of pluripotent cells is therefore an essential requirement for devising cell therapy in the field.

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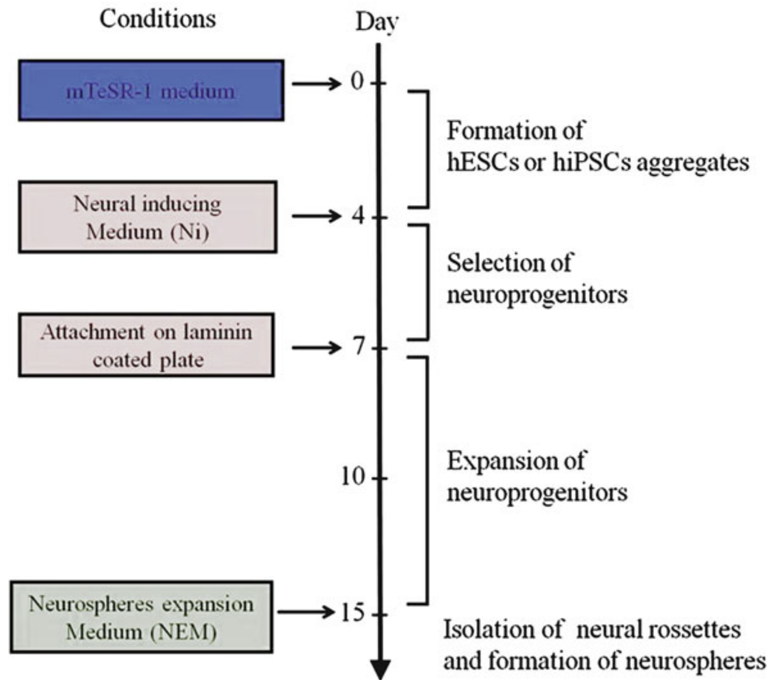

Fig. 1. Schematic illustration of differentiation of hESCs/hiPSCs into neuroprogenitors and DA neurons. The differentiation encompasses the formation of hESCs/hiPSCs aggregates in mTeSR-1 medium, followed by selection and expansion of neuroprogenitors in Ni medium. After the isolation of neural rosettes and formation of neurospheres structure in NEM medium, cells were differentiated into DA neurons through the addition of multiple signaling factors within 21 days (phase 1–3).

We have developed a differentiation protocol (Fig. 1) towards neuroprogenitors starting with a homogenous pluripotent stem cell population cultured under feeder-free conditions in a chemically defined medium, mTeSR™1. This approach has considerable clinical and practical advantages compared to earlier differentiation protocols which required the use of feeder cells to maintain pluripotency (18, 19). Importantly, feeder-free hESCs and hiPSCs transcriptomically resembles feeder hESCs and hiPSCs in many biological functions and appear more robust and follow differentiation pathways more reliably in our hands. Our differentiation is initiated by the generation of compact cell aggregates that are cultured in suspension for 4 days using the mTeSR™1 medium. At this stage, the cell aggregates are uniform in size and morphologically bear resemblance to the egg-cylinder stage of an embryo. At the next stage, the uniform-sized aggregates are placed in the neural induction medium to induce neuroectoderm specification. To observe substantial changes in morphology, aggregates are allowed to attach onto a tissue culture dish to form monolayer

colonies. Prolonged incubation of these monolayer colonies in neural induction medium gives rise to neuroepithelia and neural rosettes structures that resemble the radial arrangements of columnar cells in the neural tube (20). Despite the similarity, the level to which neural rosettes can model neurogenesis in vivo is not clear.

Notably, the neural rosette structure signifies the development of neuroprogenitors in cultures of differentiating pluripotent stem cells and is commonly characterized by the increasing expression of neuroectodermal transcription factors, such as PAX6, SOX1, and OTX2 (21, 22). The neural rosette structure can be selected out from the cultures to form free-floating spheres resembling neurosphere-like structures that are generated during suspension of adult neural stem cells (23). These free-floating spheres are expandable and can be passaged multiple times without losing its differentiation capacity.

2. Materials

2.1. Cell Culturing Supplies

1. Polystyrene multidishes: 6-, 12-, or 24-well.
2. Nontissue culture-treated multidishes: 6-well.
3. Polystyrene conical tube: 15- and 50-ml.
4. 1.5-ml microtube.
5. Serological pipettes: 5- and 10-ml.
6. 20-, 200-, and 1,000-p micropipette.
7. 20-, 200-, and 1,000-p micropipette tip.

2.2. Stock Solutions

1. mTeSR™1 media solution (Stemcell Technologies, Inc., Vancouver, Canada): make aliquots of 50 ml and store at 4°C.
2. Matrigel™ (BD Biosciences, Bedford, MA, USA): make aliquots of 500 μl and store at −20°C.
3. MEM nonessential amino acids solution (Invitrogen, Carlsbad, CA, USA): 10 mM (100×).
4. Dulbecco's modified eagle medium: nutrient mixture F-12 1: 1 (DMEM/F12) (Invitrogen).
5. Basic fibroblast growth factor (Invitrogen): human basic FGF is dissolved in 0.1% BSA/PBS at a final concentration of 2 ng/μl. Aliquot 100 μl into sterilized tubes and store at −80°C. A final concentration of 20 ng/ml is used to expand the neurospheres.
6. Dispase solution (5 mg/ml) (Stemcell Technologies, Inc.): dilute 2 ml of dispase in 8 ml of DMEM/F12 to give a final concentration of 1 mg/ml.

7. Accutase (Millipore, Billerica, MA, US): make aliquots of 10 ml and store at −20°C.
8. Heparin (Sigma, St. Louis, MO, USA): dissolve 5 mg heparin in 5 ml DMEM/F12 medium to give a final concentration of 1 mg/ml. Aliquot 0.2 ml into sterilized tubes and store at −80°C.
9. 100× N2 supplement (R&D Systems, Minneapolis, MN, USA): make aliquots of 0.5 ml and store at −20°C.
10. 50× B27 serum-free supplement (Invitrogen): make aliquots of 1 ml and store at −20°C.
11. Natural Mouse Laminin (Invitrogen): make aliquots of 100 μl (final concentration 1 mg/ml) and store at −20°C.
12. 1× Dulbecco's PBS Mg^-/Ca^- (Invitrogen).

2.3. Media

1. Human ESC growth medium. Sterilely combine 50 ml of mTeSR™1 supplement (5×) into 450 ml of mTeSR™1 basal medium. Medium can be stored at 4°C for up to 2 weeks (see Note 1).
2. Neural induction medium (Ni). Sterilely combine 489 ml of DMEM/F12, 5 ml N2 supplement, 5 ml MEM nonessential amino acids solution, and 1 ml of 1 mg/ml heparin. Medium can be stored at 4°C for up to 2 weeks.
3. Neurospheres expansion medium (NEM). Sterilely combine 479 ml of DMEM/F12, 10 ml B27 supplement, 5 ml N2 supplement, 5 ml MEM nonessential amino acids solution, and 1 ml of 1 mg/ml Heparin. bFGF supplement (final concentration 20 ng/ml) should be added freshly before changing the medium. Medium can be stored at 4°C for up to 2 weeks.

3. Methods

3.1. Feeder-Free Culturing and Propagation of hESCs and Human iPSCs

1. Both hESCs and human iPSCs (hiPSCs) are cultured under feeder-free conditions using mTeSR™1 medium on Matrigel-coated tissue culture 6-well plates: (Fig. 2a, b).
2. Prior to subculturing hESCs/hiPSCs, precoat 6-well tissue culture plate with hESC-certified Matrigel diluted in DMEM/F12 for at least 1 h (see Note 2).
3. Warm mTeSR™1 medium and dispase solution in a 37°C water bath.
4. Aspirate medium from one well of the 6-well containing the hESCs/hiPSCs and wash twice with 1 ml PBS to dilute away culture medium.

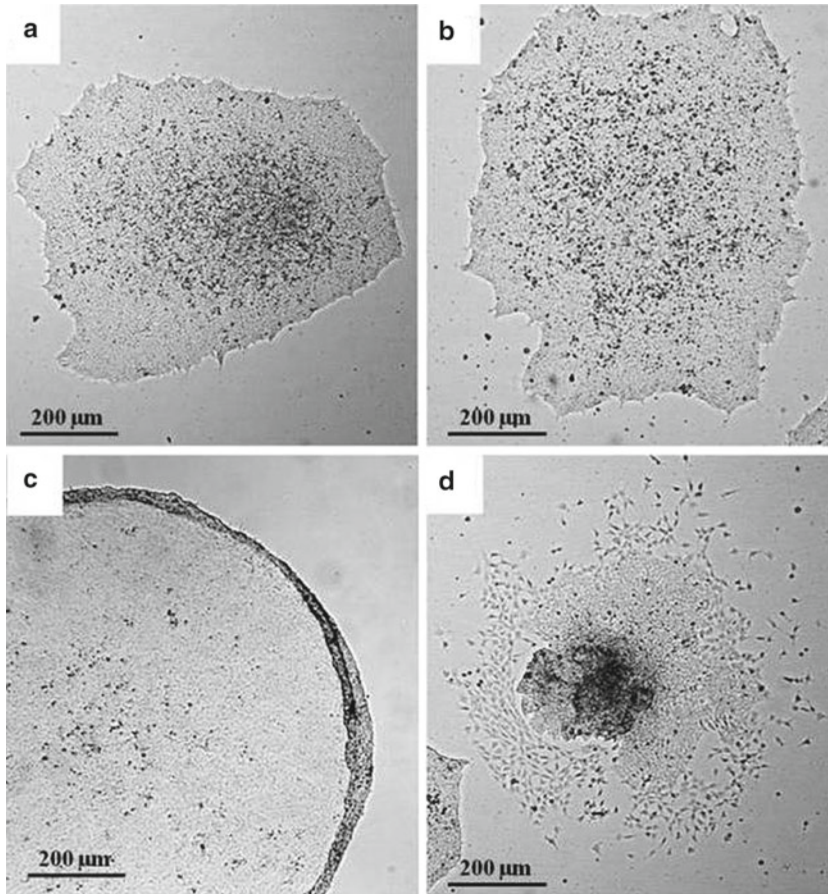

Fig. 2. Morphologies of hiPSCs cultured under feeder-free condition in Matrigel-coated plate. (**a**) Phase-contrast image showing an hESC colony cultured for 5 days. (**b**) Phase-contrast image showing an hiPSC colony cultured for 5 days. (**c**) Phase-contrast image showing a dispase-treated hiPSC colony before subculturing. (**d**) Phase-contrast image showing a spontaneously differentiated hiPSC colony.

5. Add in 1 ml of 1 mg/ml dispase solution to the well and incubate in the CO_2 incubator for 5 min; after 5 min, observe the plate every 2–3 min under the microscope for slightly "curled" edges of the stem cell colonies (Fig. 2c). This is the sign to stop dispase treatment.
6. Aspirate dispase solution and gently wash well twice with 1 ml PBS to dilute away dispase. (Traces of dispase solution will affect cell attachment).
7. Aspirate PBS and add 1 ml of prewarmed mTeSR™1 medium to the well.
8. Using a 5 ml/10 ml pipette tip, gently scrape the well horizontally and vertically to collect the colonies. Gently blow off the remaining attached colonies until the well is clear.

9. Using a 1,000-p micropipette tip, triturate suspension once to break up the colonies into even small 50–150 cells clusters; around 50–100 μm.
10. Observe under the microscope to determine if more triturations are necessary.
11. Transfer cell suspension into a 15 ml tube with 11 ml of mTeSR™1 medium and aliquot into Matrigel-precoated 6-well tissue culture plate.
12. Just before returning the plate to the CO_2 incubator, gently agitate the plate in both horizontal and vertical motions to evenly disperse the clusters across the well.
13. Daily media changes are required until the culture reaches approximately 80% confluency, which typically occurs on a 5–7 day basis. The culture needs to be subcultured to promote cell growth and to prevent spontaneous differentiation (Fig. 2d) (see Note 3).

3.2. Generation of hESC/hiPSC Aggregates

1. Warm mTeSR™1 medium and dispase solution in a 37°C water bath.
2. Wash each well of the 6-well plate twice with 1 ml PBS to dilute away culture medium.
3. Add in 1 ml of 1 mg/ml dispase solution to each well and leave in the 37°C incubator for 15–30 min until intact colonies can be gently "swirled" off. Repeated gentle tapping of the tissue culture plate can also help detachment of intact colonies.
4. Gently collect all intact colonies suspended in dispase solution into a 15 ml tube. Rinse each well with 1–2 ml PBS to collect any remaining colonies and pool together in the 15 ml tube. (It is important to use either 5 ml/10 ml pipette tips to collect intact colonies as the opening of 1,000-p micropipette tip will break the colonies into small clusters).
5. Centrifuge the tube at 300 rpm for 2 min.
6. Aspirate supernatant and wash colonies with 5 ml PBS.
7. Centrifuge the tube at 300 rpm for 2 min and repeat step 6 once.
8. Aspirates PBS and gently resuspend colonies in 1 ml of prewarmed mTeSR™1 medium.
9. Using a 1,000-p micropipette tip, gently break intact colonies into smaller clusters (~200 μm). This helps achieve a homogenous size of hESC/hiPSC aggregates.
10. Add in 5 ml of mTeSR™1 medium and aliquot cluster suspension onto three wells of the nontissue culture treated 6-well plates.
11. One day after, clusters would round up to form spherical structures (Fig. 3a), which we call aggregates. (If the culture

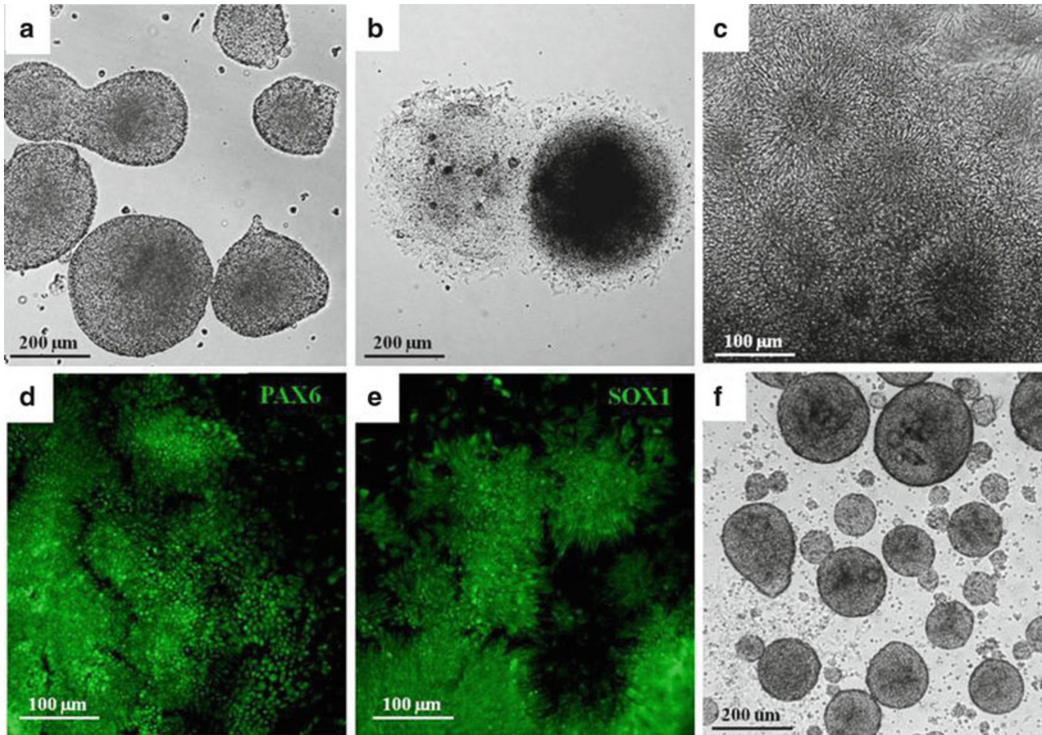

Fig. 3. Morphologies of neural structures observed during hiPSCs differentiation towards neuroprogenitors. (**a**) The shape of hiPSC aggregates formed at day 1 in mTeSR-1 medium. (**b**) Phase-contrast image showing the attachment of hiPSC aggregates on laminin-coated plate at day 8 of differentiation. (**c**) Phase-contrast image showing the neuroepithelial structure consisting of elongated neural tube-like rosette with surrounding flat cells in the periphery by day 15 of differentiation. (**d**, **e**) Immunocytochemical analyses showed the expression of neuroectoderm marker PAX6 and SOX1 in neural rosette structures formed at day 15. (**f**) Phase-contrast image showing the expanded and purified neurospheres.

contains a lot of single floating cells, change half the medium with fresh prewarmed mTeSR™1 medium).

12. Culture the aggregates in mTeSR™1 medium for 4 days with medium change every other day.

3.3. Selection of Neuroprogenitors

1. On day 4, collect all hESC/hiPSC aggregates in a 15 ml tube and centrifuged at 300 rpm for 2 min.
2. Aspirate media and wash once with 5 ml of prewarmed neural induction (Ni) media. Centrifuge for another 2 min at 300 rpm and aspirate media.
3. Resuspend aggregates in 6 ml of Ni media and aliquot into three wells of the nontissue culture treated 6-well plates.
4. Culture the aggregates in Ni medium for 3 days with medium change every other day.
5. On day 7, precoat tissue culture plates with half the appropriate volume of 20 µg/ml laminin diluted in Ni medium for at least 1 h (i.e., 1 ml for a 6-well plate, 0.5 ml for a 12-well plate, etc.).

6. Collect all aggregates into a 15 ml tube and centrifuge at 300 rpm for 2 min.
7. Aspirate Ni media and resuspend in fresh Ni media.
8. Aliquot appropriate volumes into laminin-precoated tissue culture plates. Aspiration of laminin solution is not required.
9. Aspirate the medium containing laminin and add fresh Ni medium on the next day. Aggregates should attach and show signs of spreading (Fig. 3b).
10. Culture the aggregates in Ni medium for a further 8 days with partial media change every other day.
11. By day 15, neuroepithelial structure consisting of elongated neural tube-like rosette with surrounding flat cells in the periphery should be apparent (Fig. 3c).
12. At this point, numerous neural tube-like rosette structures can be characterized through immunostaining with PAX6 (Fig. 3d) and SOX1 (Fig. 3e) antibody.

3.4. Formation and Expansion of Neurospheres

1. On day 15, aspirate Ni medium and add in 1 ml of 1 mg/ml dispase solution to each well and leave in the 37°C incubator for 5–10 min.
2. Rinse each well twice with 1–2 ml of PBS and add NEM supplemented with 20 ng/ml bFGF.
3. Using a 1,000-p micropipette tip, gently blow off the neural rosette structures and triturate several times to break "stringy" aggregates into evenly sized visible clumps.
4. Transfer the clumps into a tissue culture 6-well plate and incubate overnight.
5. Overnight incubation allows attachment of "unwanted" epithelial-like cells (see Note 4).
6. Aspirate half the media and add in equivalent amount of fresh NEM media and transfer the floating cell/cluster suspension into a new well and incubate overnight
7. Partial media changes are performed every other day with NEM media supplemented with 20 ng/ml bFGF.
8. Culturing of the neuroepithelial aggregates leads to the formation of floating neurospherical structure (neurospheres) that will grow larger in size (Fig. 3f).
9. When the neurospheres attain a certain size (500 μm in diameter) after 10–15 days, passage by triturating into smaller aggregates (100 μm in diameter).
10. The neurospheres can be passaged for many times, depending on the number of cells required for the differentiation process.

4. Notes

1. If small amounts of mTeSR™1 medium are used, one can make 50 ml aliquots by initially aliquoting 10 ml 5× supplement into 50 ml tubes and refreeze at −20°C. Thaw aliquots as required and add 40 ml basal medium; 50 ml media can be stored for up to 2 weeks.
2. Dilution of Matrigel is dependent on the batch received. Dilution factors are listed on the specification certificates and generally range from 1:80 to 1:100.
3. Five to ten percent spontaneously differentiated colonies is normal. If possible, remove spontaneously differentiated colonies prior to subculturing.
4. Cystic and irregular spherical structures tend to attach in subsequent days. All floating structures should be transferred to a new well 3–4 times for the first week to negatively select out epithelial cells/structures. After 1–2 weeks, a homogenous population of neurospheres will be retained.

Acknowledgment

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Chapter 16

Comparison of Neural Differentiation Potential of Human Pluripotent Stem Cell Lines Using a Quantitative Neural Differentiation Protocol

Dezhong Yin, Tahereh Tavakoli, Wei-Qiang Gao, and Wu Ma

Abstract

Neural differentiation of human embryonic (ES) and induced pluripotent (iPS) stem cell lines has been used for research in early human development, drug discovery, and cell replacement therapies. It is critical to establish generic differentiation protocols to compare the neural specification potential of each individually derived pluripotent stem cell line and identify the efficacious lines for research and therapeutic use. Here, we describe a reproducible and quantitative protocol to assess the neural progenitor (NP) generation of human pluripotent stem cell lines. This method includes a robust and well-defined neural inducing platform for $Pax6^{+}$ neural rosette (neuroectodermal cells) generation, propagation, and subsequent differentiation into $nestin^{+}$ NPs. A side-by-side comparison under common culture conditions among three human ES cell lines, TE03, TE06, and BG01V, and one iPS cell line, HD02, showed highly variable efficiency in their differentiation into NPs.

Key words: Human embryonic stem cell, Induced pluripotent stem cell, Neural progenitor, Neural differentiation, Embryoid body, Immunocytochemistry

1. Introduction

Human embryonic and induced pluripotent stem cells (hESCs/iPSCs) have enormous potential as a source of cells for cell replacement therapies and as a model for early human development and drug discovery. An increasing number of hES and iPS cell lines have been derived worldwide. Although there have been considerable efforts to characterize some pluripotent stem cell lines, for many lines, the effectiveness of neural differentiation is unknown. It is critical to establish methods to compare the reliability and variability of each individually derived cell line as to neural differentiation potential and to identify the efficient lines for research and therapeutic use.

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Methods for neural differentiation of hESCs have been extensively studied (1–10). But direct comparisons of neural differentiation between independently derived pluripotent stem cell lines under common culture conditions are not well documented (11–13). Our side-by-side comparison confirms the general finding that hESC lines share the properties of self-renewal, expression of "stemness," and pluripotency markers, and the ability to differentiate, but many distinctions remain among cell lines (11). These include the ability to maintain an undifferentiated state, to self-renew, and to differentiate. In addition to inherited variations in the sex, stage, quality, and genetic background of embryos used for hESC line derivation, these different qualities may be associated with considerable diversity in derivation methods and changes acquired during passaging that may influence the differentiation capacity of cell lines. To this end, it is important to set up standards shared by multiple laboratories for routine analysis of neural differentiation testing under common culture conditions. However, the information regarding common culture conditions permitting neural differentiation comparison is lacking. It is also challenging to determine reliable readouts to quantify the differentiation effectiveness of cell lines. In the present article, hES and iPS cell lines were maintained routinely on mouse embryonic fibroblast (MEF) feeder layers or on Matrigel using enzymatic passaging. We assessed the neural differentiation potential of both hESC and iPSC lines via embryoid body (EB) formation. We established a dynamic process to generate robust Pax6^{+} neural rosettes (neuroectodermal cells) and to further differentiate them into nestin^{+} neural progenitors (NPs) in a defined neural inducing (N2) medium. The differentiation effectiveness was analyzed by a simple measurement of the distance from the edge of the EB sphere to the far edge of the rim of nestin-positive neurospheres.

As a sample, we evaluate and compare the efficiency of neural differentiation under standardized conditions from three hESC lines, TE03 (NIH Registry), TE06 (NIH Registry), and BG01V (NIH Registry), and one iPSC line, HD02. TE03 and TE06 were derived using rabbit antihuman whole antiserum with a normal XX and a normal XY karyotype, respectively (14). BG01V is a variant cell line with abnormal karyotype derived from the karyotypically normal cell line BG01 (NIH Registry Name hESBGN-01) (15, 16). The iPS cell line HD02 was derived from a patient with Huntington's disease. The standardized conditions include chemically defined neural inducing (N2) medium, uniformed EB size selection, robust neuroectodermal cells' propagation, and Poly-L-ornithine/laminin substrate.

2. Materials

2.1. Reagents

1. BG01V hESC line (ATCC, SCRC-2002).
2. I3 hESC line (WiCell, TE03).
3. I6 hESC line (WiCell, TE06).
4. iPS cell line HD02.
5. 1× Phosphate buffered saline (ATCC).
6. DMEM/F12 (ATCC).
7. ES culture certified fetal bovine serum (ATCC; cat. no. SCRR 30-2020).
8. Knockout Serum Replacement (Invitrogen).
9. L-Alanyl/L-glutamine (100×) (ATCC).
10. β-Mercaptoethanol (Sigma).
11. Non-essential amino acids (100×) (ATCC).
12. Penicillin–Streptomycin (100×) (ATCC).
13. Basic fibroblast growth factor (bFGF) (R&D Systems).
14. N-2 supplement (100×) (Invitrogen).
15. B-27 serum-free supplement (50×) (Invitrogen).
16. Poly-L-ornithine (Sigma).
17. Mouse laminin (1 mg/ml, 200×) (Invitrogen).
18. Collagenase IV (Invitrogen).
19. Cell dissociation buffer (Invitrogen).
20. DMEM (ATCC; cat. no. 30-2002).
21. F12 (ATCC).
22. Neurobasal medium (Invitrogen).
23. Rabbit anti-human nestin antibody (Millipore).
24. Mouse anti-human Tuj1 antibody (Millipore).
25. Rhodamine-conjugated donkey anti-rabbit IgG (H+L) (Jackson Immunoresearch).
26. Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen).
27. Saponin (Sigma).
28. 4′-6′-Diamidino-2-phenylindole (DAPI, Sigma).
29. Paraformaldehyde (EMS).
30. 1× PBS (ATCC).
31. Irradiated or mitomycin C-treated CF-1 MEFs (ATCC, SCRC-1040.1, SCRC-1040.2a).

32. mTeSR™1 5× Supplement (StemCell Technologies).
33. mTeSR™1 Basal Medium (StemCell Technologies).
34. KO DMEM (Invitrogen).
35. Plasmanate (Talecris).
36. Glutamax (Invitrogen).
37. KO-DMEM (Invitrogen).
38. Mouse anti-human Pax6 IgG1 (Abcam).
39. Alexa Fluor 488 conjugated goat anti-mouse IgG1 (Invitrogen).

2.2. Media and Stock Solutions

1. hESC culture medium: In a 500-ml unit, combine 384 ml DMEM/F12, 75 ml ES culture-certified FBS, 25 ml knockout serum replacement, 5 ml L-alanyl/L-glutamine, 5 ml nonessential amino acids (100×), 0.91 ml of 55 mM β-mercaptoethanol, 5 ml penicillin–streptomycin (100×), and 0.2 ml of 10 μg/ml bFGF. Media may be stored at 4°C for up to 1 week.
2. Human EB culture medium: In a 500-ml unit, combine 390 ml KO DMEM, 50 ml Knockout Serum Replacement, 50 ml Plasmanate, 5 ml nonessential amino acids (100×), and 5 ml Glutamax (100×). Media may be stored at 4°C for up to 1 week.
3. Human NP culture medium: In a 500-ml unit, combine 321 ml DMEM, 163 ml F12, 5 ml N-2 supplement, 5 ml non-essential amino acids, 5 ml penicillin–streptomycin, and 1 ml of 20 μg/ml bFGF. Media may be stored at 4°C for up to 1 week.
4. Mouse Embryonic Fibroblast culture medium: In a 500-ml unit, combine 449.5 ml DMEM, 50 ml ES-FBS, and 500 μl β-Mercaptoethanol.
5. Poly-L-ornithine (10 mg/ml, 500×): Dissolve 50 mg poly-L-ornithine in 5 ml sterile water, make aliquots of 1 ml, and store at −20°C.

2.3. Equipment and Supplies

1. Sorvall Legend™ T/RT Table Centrifuge.
2. Boekel Grant water bath.
3. Biological Safety Cabinet.
4. CO_2 incubator.
5. Accujet pipettor.
6. Pipetman and tips.
7. Serological pipettes (1, 5, 10, and 25 ml).
8. Conical tubes (15 and 50 ml).
9. Tissue culture plates and dishes.

10. T25, T75, and T225 flasks.
11. 35- and 100-mm dishes.
12. 6-Well plates.
13. Ultra-low attachment dishes (10 cm, Corning).
14. Syringes and syringe filters.

3. Methods

hESC lines, TE03, TE06, and BG01V, and iPS cell line, HD02, are maintained and passaged weekly on mitomycin C-treated mouse CF-1 embryonic fibroblasts. First step is to remove the colonies of pluripotent stem cells from feeders, triturated and replated in low-attachment dishes with hESC medium without bFGF for 5 days to obtain spontaneously differentiating EBs (Fig. 1). Neural differentiation is induced in floating EBs in the neural differentiation N2 medium for 10 days and then plated on poly-L-ornithine/laminin-coated 35-mm dishes. Emerging neuroectodermal cells in neural rosettes can be visualized either in suspended EBs or after plating the EBs on the substrate. Neuroectodermal cells are immunostained for Pax6 and further differentiated into nestin^{+} NPs and their progeny. The differentiation effectiveness among hESC/hiPSC lines was analyzed by a simple measurement of the distance from the edge of the EB sphere to the far edge of the rim of nestin-positive neurospheres.

3.1. Maintenance and Expansion of Human ES Cells

1. Seed irradiated or mitomycin C-treated MEFs in 10-cm dishes at the seeding density of 55,000 cells/cm^2 in MEF medium 1 day before thawing hESCs (see Note 1).
2. Thaw a vial of hESCs (BG01V, TE-03, and TE-06) rapidly in a 37°C water bath.

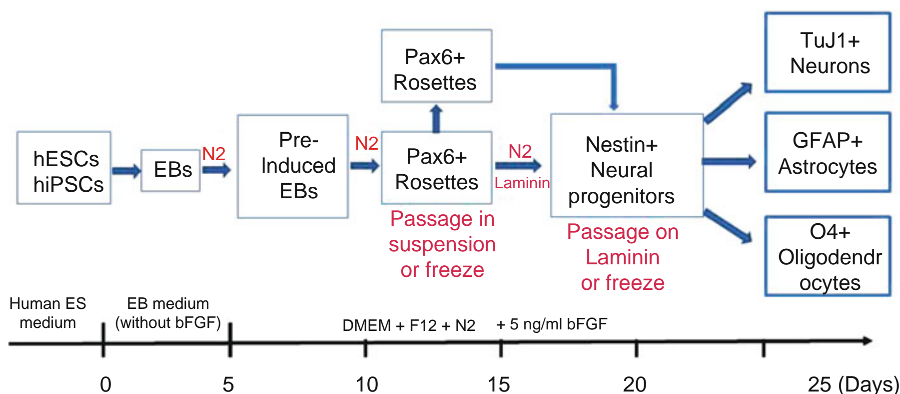

Fig. 1. Schematic representation of the protocol for direct differentiation of hES and iPS cells into neural progenitors.

3. Transfer vial contents to 15-ml tubes and add 10 ml hESC culture medium to each tube.
4. Centrifuge the cells at 200 × *g* for 5 min.
5. Aspirate the supernatant and resuspend the cell pellet into small clumps by pipetting up and down gently several times in 5 ml of hESC culture medium (see Note 2).
6. Remove medium from previously prepared MEF monolayers.
7. Dispense hESC aggregates into one 10-cm dish containing the MEF monolayers.
8. Add hESC culture medium to equal 15 ml per dish and incubate cells at 37°C, 5% CO_2.
9. Do not change the medium for the first 48 h. However, add an additional 4 ng/ml of bFGF to the culture medium 24 h post seeding.
10. Perform daily medium change since day 2.
11. Observe colony formation of hESCs on MEFs under a microscope (see Note 3).
12. When hESC cultures are confluent on irradiated MEFs, remove media, wash cells with PBS, and then add 4 ml prewarmed Collagenase IV solution (400 U/ml)/10-cm dish.
13. Incubate cells with the Collagenase IV solution at 37°C for 10–20 min, checking dishes every 10 min thereafter, until colonies lift off the MEF monolayer. Dissociate colonies by gently agitating and triturating.
14. Collect hESC colony suspension to a 50-ml conical tube and centrifuge for 5 min at 200 × *g*.
15. Carefully aspirate off the supernatant, resuspend cell pellet in 15 ml hESC culture medium, and break the colonies into small clumps by pipetting up and down eight to ten times with a P1000 tip (see Note 2).
16. Transfer hESC colony suspension of each hESC lines into three 10-cm dishes with MEF feeders and add hESC medium to 15 ml per dish.
17. After 24 h, add an additional 4 ng/ml bFGF to the medium in the dishes.
18. Change the medium after the first 48 h with hESC culture medium and then change the medium daily thereafter.

3.2. Maintenance and Expansion of Human iPS Cells

1. Seed 10-cm tissue culture dishes with 55,000 MEF/cm^2 in MEF medium 1 day before thawing hiPSCs.
2. Thaw a vial of HD02 iPSCs from human Huntington's disease cells and H7 iPSCs from normal control cells by immersing the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.

3. Spray the vial with 70% ethanol or isopropyl alcohol to sterilize the outside of the tube. Briefly (30–60 s) air dry the vial in the sterile biosafety cabinet.
4. Transfer the cells gently into a sterile 15-ml conical tube using a 5-ml pipette with 4 ml warm mTeSR. Dispense 3 ml of collected cells into a labeled 15-ml conical tube. Use the remaining 2 ml to rinse cryovial and add to the same 15-ml conical tube.
5. Centrifuge the cells in a 15-ml conical tube at 200×*g* for 5 min.
6. Aspirate and discard the supernatant and gently resuspend pellet in 2 ml mTeSR. Take care not to break cell clumps into single cells.
7. Remove the MEF medium from 10-cm dishes, and add 5 ml hESC media to the dish.
8. Aspirate the hESC media from the MEF dish prior to plating.
9. Add the iPS cell suspension dropwise on MEF dishes and add hESC media to 12 ml per dish.
10. Place dishes in incubator and do not disturb for 24 h.
11. On day 2, feed hiPSC by aspirating off media and replacing with 12–15 ml fresh hESC medium. Repeat the procedure daily until cells require passaging.
12. Prepare MEFs the day before you plan to passage cells.
13. Aspirate the hESC media from the culture to be split. Wash the wells with 10 ml of 1× phosphate-buffered solution (PBS) per dish.
14. Add 4 ml prewarmed Collagenase IV Solution (400 U/ml) to each passaged dish. Incubate for 10–20 min at 37°C. Examine cells under microscope to confirm colony separation from the plate. Tap dish gently to help cells detach.
15. Pool cells in 10× the volume of Collagenase IV Solution of hESC media prepared in the 50-ml conical tube. Wash plate with 3–8 ml of fresh hESC media and add to a conical tube.
16. Centrifuge cells at 200×*g* for 5 min.
17. Resuspend cell pellet in fresh hESC media by pipetting up and down gently to break the colonies to small aggregates. How much additional medium is required to cell suspension is dependent on the split ratio and the number of dishes used. There should be a total of 12 ml of medium and cells in each of the new dish.
18. Aspirate MEF medium off the MEF dish. Plate cells on fresh MEF dishes. Label with date, cell line, and passage number.
19. Return the dish to incubator. Do not disturb for 24 h.

3.3. Human ESC- and iPSC-Derived Embryoid Body Formation and Expansion

1. On day 0, when hES/iPS cell cultures are confluent on irradiated MEFs, remove media, wash cells with PBS, and then add 4 ml prewarmed Collagenase IV solution (400 U/ml)/10-cm dish. Incubate at 37°C for 10–20 min, checking dishes every 10 min thereafter, until colonies lift off the MEF monolayer. Dissociate colonies by gently agitating and triturating.
2. Pool cells with Collagenase IV solution into 50-ml conical tubes with 5x volume of EB media. Wash dishes two times with EB media and collect cells in the same tube.
3. Centrifuge cell suspension for 5 min at $200\times g$.
4. Remove Collagenase IV/media and gently resuspend cell pellet in a small volume of EB medium (for example, 5 ml/10-cm dish of starting cells) for cell number evaluation.
5. Seed ES/iPS cells in 10-cm ultralow-attachment dishes at a density of about 4.5 million cells per dish with a total volume of 12 ml EB media per dish.
6. Incubate the cells in a 37°C incubator. Culture the EBs in suspension for another 2 days without medium change.
7. At day 3, add an additional 10 ml of EB media per dish.
8. At day 5, pool EBs in a 50-ml falcon tube and allow settling by gravity for 10 min.
9. Aspirate the media carefully, and resuspend EBs with NP medium in the absence of bFGF (see Note 4) in a volume to allow replating of 10 ml EB suspension per dish.
10. Aliquot 10 ml EB suspension per dish in N2 medium to new 10-cm ultralow-attachment dishes.

3.4. Maintenance and Expansion of Neuro-Induced EBs

1. N2 medium containing NP culture medium is used for the maintenance and expansion of neuro-induced EBs. Feed EB cultures every 2–3 days with NP medium. Place 10-cm dishes on the flat surface of the biological safety cabinet. Swirl the dish in a circular motion (see Note 5). Carefully remove about 50% of NP medium per dish without removing floating EBs. Replace the NP medium with 50% fresh NP medium.
2. Passaging the expanded neuro-induced EBs involves breaking up individual EB clusters and expanding the broken clusters to new 10-cm ultralow-attachment dishes. The EBs are broken up three times every 10–14 days using a P200 tip and P200 pipetman. This is executed by aspirating EBs into the bore of the tip and then expelling the EBs until the individual EB breaks into two to three sections. Expand half of the newly broken EBs into a new 10-cm ultralow-attachment dish with fresh NP medium when there are approximately a hundred EB pieces in a dish (see Note 6).

3. For assessment of growth rates of EBs derived from different hESC lines, phase-contrast photographs of EBs cultured in EB medium for 10 days are taken and total areas of EBs can be measured by imaging software (see Note 7).
4. EB cultures can be maintained in the EB medium for up to 10 months. Neuro-induced EBs aged 1–10 months are ready for derivation of human NPs described in Subheading 3.5 (see Note 8).
5. Neural differentiation in EBs can be assessed by the generation of neural rosettes (neuroectodermal cells) stained with Pax6 antibody.
6. Fix EBs cultured in the EB medium for 10 days in 1 ml of 4% paraformaldehyde prepared in 1× PBS per 35-mm dish for 15 min at room temperature (RT).
7. Rinse cells with 2 ml of 1× PBS per well once.
8. Permeabilize cells with 1 ml of 0.5% saponin prepared in 1× PBS per well for 15 min.
9. Incubate EBs with 0.5 ml of mouse anti-Pax6 IgG1 (1:100 dilution in 1× PBS) overnight at 4°C.
10. Aspirate primary antibodies and rinse cells twice with 1× PBS.
11. Add 0.5 ml of Alexa Fluor 488 conjugated goat anti-mouse IgG1 (1:50 dilution in 1× PBS).
12. Place dishes for 1 h at RT in the dark.
13. Aspirate secondary antibodies and rinse cells twice with 2 ml of 1× PBS.
14. Counterstain cells with DAPI (1:1,000 dilution in 1× PBS) for 15 min at RT.
15. Wash EBs with 2 ml of 1× PBS twice.
16. Observe the generation of neural rosettes stained with anti-Pax6 antibody under a fluorescence microscope.

3.5. Assessment of the Neural Differentiation Potential of hESCs and hiPSCs

After plating on poly-L-ornithine/laminin substrates, new NPs are constantly generated and migrated radially away from the differentiating EBs, resulting in a rim of cells around the EB sphere (Fig. 2). To quantify the neural differentiation potential of different pluripotent stem cell lines, pluripotent stem cell-derived EBs within individual experiments are matched for size prior to plating onto laminin substrates. EB-derived neurospheres at 3 days after plating are fixed, and immunofluorescence staining for nestin is carried out. NP generation and expansion are quantified by measuring the distance from the edge of the EB sphere to the far edge of the rim of nestin-positive neurospheres. Figure 2 shows a significant difference in the percentage increase in the number of Nestin^{+} cells differentiated from the three cell lines after 3 days post plating.

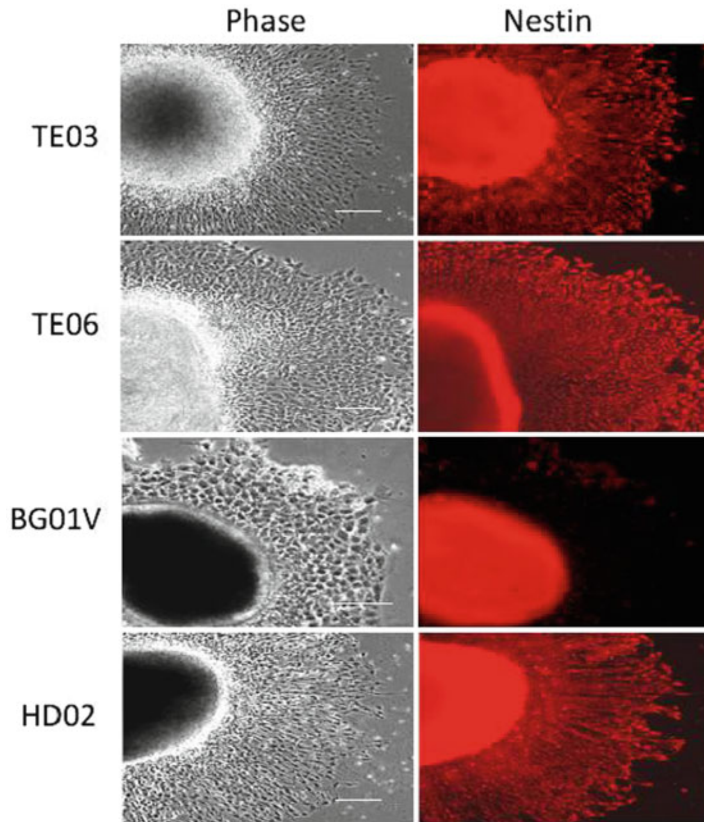

Fig. 2. Variation in neural differentiation effectiveness between hESC lines, TE03, TE06, and BG01V, and iPS cell line, HD02. Three days after transferring EBs to a poly-L-ornithine/laminin substrate, parallel immunofluorescent staining for Nestin was performed in hESC- or iPSC-derived cell populations. The measurement of neural progenitor expansion distances shows a difference in neural differentiation effectiveness: TE06 > HD02 > TE03 > BG01V (bars in phase-contrast images = 100 μm).

The percentage increase in Nestin+ cells generated from the TE03 and HD02 cells was greater than that of the TE06 cells. The BG01V generated an insignificant number of NPs, which were lightly scattered among differentiated cells.

1. Prepare working solution of poly-L-ornithine at 20 μg/ml by making 1:500 dilution of poly-L-ornithine stock solution (10 mg/ml) in sterile water.
2. Prepare working solution of mouse laminin at 5 μg/ml by making 1:200 dilution of mouse laminin stock solution (1 mg/ml) in 1× PBS.
3. Coat 35-mm dishes with 2 ml of the poly-L-ornithine working solution per dish at 37°C for 1 h.
4. Rinse coated plates once with sterile water.

5. Add 2 ml of the mouse laminin solution to each well of the poly-L-ornithine-coated plates and incubate them at 37°C for 1 h.
6. Aspirate the mouse laminin solution and rinse the plates once with 1× PBS (see Note 9).
7. Add human NP culture medium to 35-mm dishes at 3 ml the NP medium per dish.
8. Seed newly passaged and similar sized EBs derived from different hESC or hiPSC lines to 35-mm dishes at 100 μl EB suspension per dish by using a 200-μl tip (see Note 10).
9. Incubate the plate at 37°C with 5% CO_2 for 2 days without medium change.
10. Change the NP medium on day 3 and every other day thereafter if for further expansion of NPs (see Note 11).
11. Newly derived NPs are generated and migrated radially away from EBs, resulting in a rim of cells around the EB spheres. The EB-derived neurospheres cultured at 3 days after plating are fixed with 1 ml of 4% paraformaldehyde prepared in 1× PBS per dish for 15 min at RT.
12. Rinse cells with 2 ml of 1× PBS per dish once.
13. Permeabilize cells with 1 ml of 0.5% saponin prepared in 1× PBS per well for 15 min.
14. Incubate cells with 0.5 ml of rabbit anti-nestin (1:200 dilution in 1× PBS) primary antibody overnight at 4°C.
15. Aspirate primary antibodies and rinse cells twice with 2 ml of 1× PBS per dish.
16. Add 0.5 ml of rhodamine-conjugated donkey anti-rabbit (1:50 dilution in 1× PBS) secondary antibody.
17. Place dishes for 1 h at RT in the dark.
18. Aspirate secondary antibodies and rinse cells twice with 1× PBS.
19. Counterstain cells with DAPI (1:1000 dilution in 1× PBS) for 15 min at RT.
20. Wash cells with 1× PBS twice.
21. Observe immunofluorescence signals under a fluorescence microscope and take photos of EB-derived neurospheres.
22. To estimate differences in neural differentiation effectiveness between different pluripotent stem cell lines, the expansion distances of NPs from the edge of the EB sphere to the outside edge of the rim are measured from neurospheres cultured at 3 days post plating. Neural differentiation potential can be quantitatively compared using the mean of neural cell expansion distances from at least ten EB-derived neurospheres for each pluripotent stem cell line (see Note 12 and Fig. 2).

4. Notes

1. MEF feeders may be used for hESC culture within a week.
2. Take care not to break cell clumps too much. Otherwise, hESCs will not recover well if they are broken into single cell suspensions. Fifteen to thirty cells per clump are ideal.
3. It may take 3–7 days for hESCs to form colonies. BG01V colony formation is earlier than TE03 and TE06. The first passage should occur 2–3 days after colonies are visible.
4. No bFGF should be in the N2 medium for the first 5 days of initiation of culture.
5. EBs are very lightly attached to the dish. Swirling the dish can move the floating EBs to the center of the dish. Care must be taken to avoid aspirating of EB when removing the medium from the edge of the dish.
6. Broken up EBs may also be transferred to new ultralow-attachment dishes at 1:2–3 split ratio according to the density of EBs.
7. Monitoring EB growth provides a unique tool to investigate in vitro differentiation of hESCs. The percent increase in the size of cell spheroids is significantly different among hESC lines examined. For example, the relative EB growth is greater in TE03 and BG01V cells compared to that in TE06 cells.
8. EBs may also be cryopreserved according to standard procedures of hESC cryopreservation.
9. Laminin-coated plates or dishes may be stored at 4°C for up to 3 weeks in mouse laminin working solution.
10. To improve the efficiency of human NP production, EBs should be evenly distributed throughout the entire well and try to seed EBs derived from hESCs or hiPSCs with similar sizes.
11. EB-derived NPs can be subcultured in the NP medium for up to eight passages without changing characterization of NPs and differentiated into neurons or glia cells under appropriate culture media.
12. Three days after transferring EBs to poly-L-ornithine/laminin substrates, both TE06 and TE03 hESCs along with HD02 iPS generate significantly more Nestin^{+} NPs than BG01V hESC line based on the measurement of neural cell expansion distances.

Acknowledgment

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Chapter 17

Array-Comparative Genomic Hybridization Characterization of Human Pluripotent Stem Cells

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Abstract

During culture adaptation, human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) tend to acquire chromosomal aberrations. Generally, stem cell lines are screened for large-scale chromosomal changes using low resolution karyotype analysis. Recent studies characterizing human stem cells using array-comparative genomic hybridization (aCGH) suggests most abnormalities acquired during culture are under the resolution of karyotype analysis and therefore are routinely missed. Here, we describe a custom-designed stem cell focused microarray utilizing 44K probes, with increased resolution in relevant stem cell-associated and cancer-related genes.

Key words: Array-comparative genomic hybridization, Embryonic stem cells, Induced pluripotent stem cells, Genome stability, Karyotype

1. Introduction

It is well established that human stem cell lines frequently accumulate genomic alterations in tissue culture. Aberrations can range from whole chromosome aneuploidies to single gene duplications and deletions (1–3). Evidence suggests these mutations provide a selective advantage to the cells resulting in better growth properties, increased cell survival, larger colonies, and slower differentiation (4). This adaptive process is similar to that observed during malignant transformation. Therefore, maintaining the genetic integrity of human stem cell lines during culture is critical for reliable and reproducible experimental results. The most common methods for characterizing the genomic stability of stem cells include G-banding metaphase karyotyping and array-comparative genomic hybridization (aCGH). Karyotype analysis is a low resolution technique, only detecting aneuploidies over 5 Mb in size (5). aCGH has much

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higher resolution than karyotyping and is routinely used to detect structural variants under 1 kb (6). We have developed a stem cell-focused microarray which includes increased probe coverage in over 60 stem cell-associated genes and 195 cancer-related genes (7). The StemArray™ was developed on both the Agilent platform (44K probes) and the Roche NimbleGen platform (135K probes). Here, we describe its use on the Agilent platform.

2. Materials

Materials for labeling and hybridization can be purchased from both Agilent and Roche NimbleGen for the aCGH protocol. Here, we describe our workflow using the Agilent protocol.

2.1. Human Stem Cell Culture

1. Human stem cell culture media: KODMEM with 20% knock-out serum replacement, 1% GlutaMax, 1% nonessential amino acids, 1 mM 2-mercaptoethanol, and 8 ng/ml bFGF (all from Invitrogen).
2. Cells grown on PMEF-CFs (Millipore) or Matrigel (BD Biosciences).
3. Cell isolation: 1 mg/ml collagenase IV (Invitrogen). Add 1 mg of collagenase IV to 1 ml base medium (KODMEM).

2.2. Genomic DNA Isolation and Restriction Digestion

1. DNA isolation: PureGene DNA Purification Kit with RNase A (Qiagen).
2. 100% Isopropanol (Sigma).
3. 70% Ethanol (Sigma).
4. 500 ng pooled sex-matched reference DNA (Promega) (see Note 1).
5. Restriction digestion: 10× buffer C, acetylated BSA (10 μg/μl), Alu I (10 U/μl), and Rsa I (10 U/μl) (all from Promega).

2.3. Sample Labeling and Clean-up

1. Labeling: Random primers, 5× buffer, 10× dNTPs, cyanine 3-dUTP (1.0 mM), cyanine 5-dUTP (1.0 mM), Exo-Klenow fragment (all from Agilent Technologies) (see Note 2).
2. Clean-up of labeled DNA: Amicon 30 kDa filters (Millipore), 1× TE (pH 8.0).

2.4. DNA Hybridization

1. 44K StemArray™ Microarray slide (Ambry Genetics/Agilent Technologies).
2. Gasket slide and hybridization chamber (Agilent Technologies).
3. 10× Blocking Agent (Agilent Technologies).
4. 2× Hi-RPM Buffer (Agilent Technologies).
5. Human Cot-1 DNA (1.0 mg/ml) (Invitrogen).

2.5. Washing

1. Three slide-staining dishes.
2. Slide staining rack.
3. Two magnetic stir plates with heating element and two stir bars.
4. 1.5 L glass dish.
5. Wash buffers: Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2 (Both from Agilent Technologies).

3. Methods

3.1. Genomic DNA Isolation and Restriction Digestion

1. Culture human iPSCs or ESCs in 6- or 12-well plates for 5–6 days. Generally, one well of a 12-well plate will produce 500 ng of genomic DNA. If cells are cultured on a mouse feeder layer, treat the cells with 1 mg/ml collagenase and place at 37°C for 10 min. Stem cell colonies should lift off the plate while most feeder cells should stay attached (see Note 3). Place cells in 15 ml conical tube and wash with PBS.
2. Lyse the cells and isolate the genomic DNA following the PureGene DNA Purification protocol from Qiagen (see Note 4). After addition of RNase A, place at 37°C for 10 min and then immediately place on ice. Quantitate the resulting DNA on a Nanodrop spectrophotometer to determine the purity and concentration. For 44K aCGH, the DNA needs to be at a concentration ^{3}25 ng/µl with a 260/230 ≥ 1.5 and 260/280 ≥ 1.8 (see Note 5).
3. Remove 500 ng of human-purified stem cell DNA and bring volume up to 20.2 µl with nuclease-free water in a PCR tube. On ice add 2.0 µl nuclease-free water, 2.6 µl 10× buffer C, 0.2 µl acetylated BSA (10 µg/µl), 0.5 µl Alu I (10 U/µl), and 0.5 µl Rsa I (10 U/µl) per reaction. Repeat in a separate tube with 500 ng sex-matched pooled genomic reference DNA (Promega) (see Note 6).
4. Place restriction digestion reactions in a PCR machine with the following conditions: 2 h at 37°C, 20 min at 65°C, and hold at 4°C. To assess the digestion, remove 2 µl of digested DNA and run on a 0.8% agarose gel stained with ethidium bromide (Fig. 1). Samples can be stored at this point up to a month at –20°C.

3.2. Sample Labeling and Clean-up

1. Centrifuge samples for 1 min at 6,000 × *g* and add 5 µl of Random Primers to each reaction tube. Heat denature samples by placing in a PCR machine for 3 min at 95°C. Immediately place in ice-water bath for 5 min (see Note 7).
2. Prepare labeling master mix on ice. For each reaction, add 2 µl nuclease-free water, 10 µl 5× buffer, 5 µl 10× dNTPs, 3 µl of cyanine 5-dUTP for sample DNA, cyanine 3-dUTP for reference

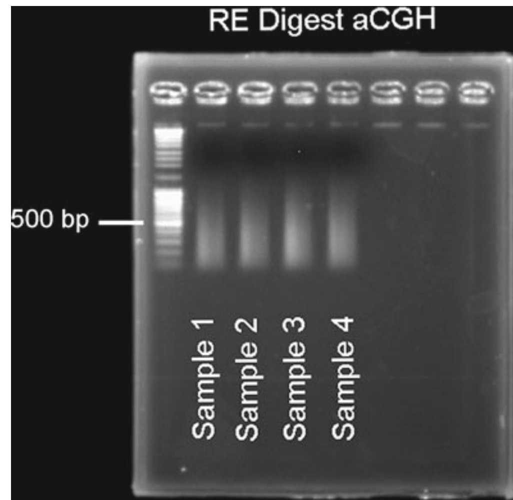

Fig. 1. Example of agarose gel electrophoresis for restriction digested genomic DNA. The majority of DNA should be between 200 and 500 bp in length.

DNA, and 1 µl Exo-Klenow fragment. Add 21 µl of labeling master mix to sample and mix by pipetting up and down.

3. Place labeling reaction in PCR machine with the following conditions: 2 h at 37°C, 10 min at 65°C, and hold at 4°C. Remove samples from PCR machine and centrifuge samples for 1 min at 6,000 × *g*. Transfer samples to 1.5 ml tube and add 430 µl of 1× TE (pH 8.0) to each reaction tube.
4. To purify samples using Amicon 30 kDa filter columns load samples diluted in 1× TE into filter and spin for 10 min at 14,000 × *g* and discard flow-through. Add 480 of 1× TE (pH 8.0) and spin again for 10 min at 14,000 × *g*. To collect the sample, invert the filter into a new 1.5 ml tube and spin 1 min at 1,000 × *g*. The resulting volume should be ~21 µl. If less, add 1× TE (pH 8.0) up to 21 µl and if volume is more dry the sample down to 21 µl in a vacuum concentrator.
5. To determine labeling efficiency, remove 1.5 µl and load onto a Nanodrop Spectrophotometer using the MicroArray Measurement module. Cy3-labeled DNA should have a yield of 5–7 µg and a specific activity ≥25. Cy5-labeled DNA should have a yield of 5–7 µg and a specific activity ≥20 (see Note 8).

3.3. Hybridization and Washing

1. Combine the purified Cy5-labeled test sample with the Cy3-labeled reference sample. For each hybridization, add 5 µl of human Cot-1 DNA (1.0 mg/ml), 11 µl of 10× blocking agent and 55 µl 2× Hi-RPM Buffer. Mix the sample and incubate in a heat block at 95°C for 5 min and then transfer to 37°C for 30 min. Centrifuge samples for 1 min at 6,000 × *g* and immediately load sample onto gasket slide. Once all samples are loaded,

place microarray active side down and clamp into the hybridization chamber. Place the chamber in a 65°C hybridization oven rotating at 20 rpm for 18–24 h.

2. To wash slides, place the gasket-slide into slide-staining dish containing wash buffer 1 (see Note 9). Using forceps, carefully remove gasket slide while continuing to hold the microarray slide by the barcode. Place the slide in the slide-rack located in wash buffer 1, stirring with medium agitation for 5 min. Transfer slide rack into wash buffer 2 preheated to 37°C and stir with medium agitation for 1 min (see Note 10). Slowly remove slide rack out of wash buffer 2 solution so that no liquid remains on the slide.

3. Load slide into scanning holder and scan in a high resolution scanner at 5 μM. Extract data from image and use data analysis software of choice. Derivative log ratio spread (DLRS), an indicator of noise, should be ≤0.2. Data should be of good quality with aberrations clearly discernable (Fig. 2a). When

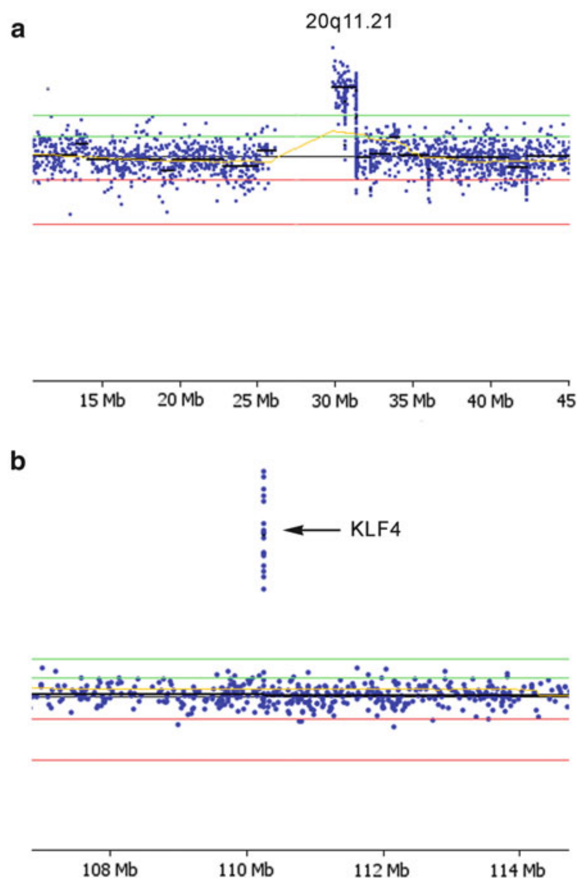

Fig. 2. Duplications detected in stem cell-associated regions. (**a**) Common duplication of 20q11.21 found in an iPSC line after extended culture. (**b**) Numerous copies of the KLF4 gene, one of the reprogramming factors which were used to transform fibroblast cells into iPSCs, can easily be detected by aCGH.

analyzing data from iPSCs, the reprogramming factors, such as SOX2, MYC, OCT3/4, and KLF4, make good internal controls as they should be highly amplified (Fig. 2b).

4. Notes

1. It is best to dilute the DNA stock concentration down to 100 ng/μl to avoid pipetting small volumes into the restriction digestion reaction.
2. After initial thawing of cyanine dyes place them at 4°C. Repeated freeze thawing of the dyes results in a decrease in labeling intensity.
3. Some mouse cell contamination is ok. However, try to limit the contamination to below 10% as homologous mouse DNA will also bind to the probes on the array which could confound results.
4. Other DNA purification methods and kits can be used as long as the DNA is high quality and has a 260/230 ≥ 1.5 and 260/280 ≥ 1.8.
5. Low 260/230 ratios generally result in inefficient dye labeling. If the 260/230 or 260/280 ratios do not meet the criteria following isolation, repurify the DNA using the DNA Clean and Concentrator Kit (Zymo Research).
6. Although restriction digestion of DNA is recommended, high quality data can be obtained by replacing this step with a 10 min incubation at 95°C during the addition of the random primers at labeling.
7. Quick chilling in an ice-water bath after denaturing DNA is essential for high-efficiency labeling. Poor cooling can result in increased dye bias in GC rich regions.
8. To determine specific activity, use the following formula: Specific Activity = (pmol/μl dye)/(μg/μl DNA).
9. All washing and scanning steps should be done in an ozone protected environment as the Cy5 dye is very sensitive to ozone levels.
10. At least 4 h prior to washing place an empty slide-staining dish with magnetic stir bar into 1.5 L glass dish filled with water and place in a hybridization oven at 37°C. Also place wash buffer 2 container into a 37°C water bath.

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Chapter 18

Chromatin Immunoprecipitation-Based Analysis of Gene Regulatory Networks Operative in Human Embryonic Stem Cells

Marc Jung and James Adjaye

Abstract

Chromatin immunoprecipitation (ChIP) followed by microarray-based (ChIP-Chip) or next-generation sequencing-based (ChIP-Seq) analysis has been established as a powerful and widely used method to investigate DNA–protein interactions relative to a genomic location in vivo. Here, we present a ChIP-Chip protocol, which utilizes an alternative, easier amplification protocol and when using high-quality ChIP-grade antibodies, will generate enough material for hybridization or sequencing with negligible enrichment bias due to amplification.

Key words: Embryonic stem cells, Chromatin, Protein–DNA interaction, Immunoprecipitation, Transcription factor, OCT4, NANOG, Next-generation sequencing

1. Introduction

1.1. Chromatin Immunoprecipitation

The basic principle underlying chromatin immunoprecipitations (ChIPs) is that proteins (transcription factors) are cross-linked to the DNA double helix by using cross-linking agents like formaldehyde. Formaldehyde is a tight (2Å) cross-linking agent that efficiently produces both protein–nucleic acid and protein–protein cross-links in vivo. Formaldehyde is a very reactive dipolar compound in which the carbon atom acts as a nucleophilic center. Amino and imino groups of amino acids (lysines, arginines, and histidines) and of DNA (primarily adenines and cytosines) readily react with formaldehyde leading to the formation of a Schiffbase. This intermediate can further react with a second amino group and condense to give the final cross-link. These reactions take place in vivo within minutes after the addition of formaldehyde to living

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cells or embryos (1). Although other cross-linking reagents have been employed (2), formaldehyde remains the most widely used as the reaction can be reversed by heat. This is achieved primarily by protonation of imino groups at low pH in aqueous solution. After cross-linking the chromatin, the cells are either directly lysed or the nuclei are extracted. The chromatin is sheared into fragments of the desired size by sonication or through micrococcal nuclease digest to a size of usually 0.2–1.0 kb. For ChIP-Chip or ChIP-Seq applications, a smaller size is essential if a higher resolution of the subsequent analysis is desired. The fragments bound to the protein of interest are usually enriched by immunoprecipitation (IP) with an antibody against the respective protein under investigation. Protein-specific antibodies require optimizing for their application in IP conditions. Additionally, using different polyclonal antibodies for the same protein may show a different preference for epitopes, resulting in a possible distinct selection of cross-linked loci. Monoclonal antibodies would be preferable due to their specific epitope selection, but it is more difficult to obtain functional ChIP-grade monoclonal antibodies by this approach. The control cell is processed with the pre-immune serum from the host organism of the specific antibody used for the IP. This control identifies unspecific fragments enriched, e.g., by adhesion to the samples tubes. The formaldehyde cross-links are then reversed and the precipitated DNA fragments are purified. Yields from ChIP are usually low but sufficient for subsequent PCR or qPCR analysis. In this chapter, we describe a protocol which we adapted and expanded with a different amplification method from an earlier publication, focusing on the key pluripotency-associated transcription factors OCT4, NANOG, and SOX2 (3) and which we used for the analysis of OCT4-mediated gene regulatory networks necessary for maintaining self-renewal in embryonic stem cells and embryonal carcinoma cells (4).

1.2. Chromatin Immunoprecipitation Followed by Microarray Hybridization (ChIP-Chip) or Deep Sequencing (ChIP-Seq)

As the traditional methods had failed to create high-resolution, genome-wide maps of the interaction between a DNA-binding protein and DNA, the combination of ChIP and whole-genome promoter microarrays (ChIP-Chip) and next-generation sequencing-based (ChIP-Seq) circumvented these limitations by creating high-resolution genome-wide maps of the in vivo interactions between DNA-associated proteins and DNA.

The ChIP-Chip technique was first used to identify binding sites for individual transcription factors in *Saccharomyces cerevisiae* (1, 5, 6). More recently, a c-Myc epitope protein tagging system was used to map the genome-wide positions of 106 transcription factors in yeast (7).

For microarray- and next-generation sequencing-based detection of immunoprecipitated DNA, amplification of the DNA is generally necessary, as the DNA yield, obtained after the pulldown

is not sufficient for hybridization or sequencing. Ideally, the ChIP reactions are scaled up and amplifications are avoided. Three amplification methods have so far been widely used: randomly primed (8), ligation-mediated PCR (9) as well as amplification on the basis of T7 DNA polymerase (10). Before adding the antibodies for the pulldown reaction, a part of the fragmented chromatin will be retained as total genomic reference DNA. Although these samples usually give enough material for microarray hybridization, they should also be amplified to avoid any amplification bias. The enriched and the reference DNA are then fluorescently labeled. Although one color platforms, where both samples have the same label, e.g., Cy3 are hybridized on separate arrays the use of two color platforms is often preferred, as this minimizes the influence of microarray batch effects on the experimental results. In this case, the ChIP DNA is labeled with different fluorescent dyes and the samples are combined and hybridized to a single DNA microarray. The relative intensities of the two dyes allow the detection of the fragments that are enriched in the IP, thereby enabling the identification of protein–DNA interaction sites (see Fig. 1). For a comprehensive analysis, microarrays used in ChIP-Chip applications represent ideally the entire genome of the organism in the form of overlapping fragments. In this case, the limitation will be the obligatory selection of preferred probe sequences for optimal hybridization, which in turn defines the maximal resolution of the tiling array. Furthermore, for larger genomes such as for higher eukaryotes these are not available or only at very high monetary cost. Therefore, arrays are often custom designed for specific applications. The resolution of the identified binding sites depends on the size of the sheared DNA and the size and spacing of the probes on the arrays. For example, typical yeast experiments achieve a resolution of about 1 kb, which is sufficient to assign binding to the regulation of a single gene. Once the bound regulatory region is identified, the exact binding site can often be inferred by computational methods. In comparison, the ChIP-Seq approach offers an unbiased analysis regarding genomic loci, which have not been predefined by tilling arrays. This advantage will still come with a higher cost for deep sequencing projects.

2. Materials

All solutions should be prepared using ultrapure deionized water and analytical grade reagents. Prepare and store all solutions at room temperature unless indicated otherwise. For ChIP reaction:

1. NaCl, 137 mM; KCl, 2.7 mM; Na_2 HPO_4 , 10 mM; and $KHPO_4$, 2 mM of pH 7.2.
2. PBS/2% FBS/PMSF, 1 mM.

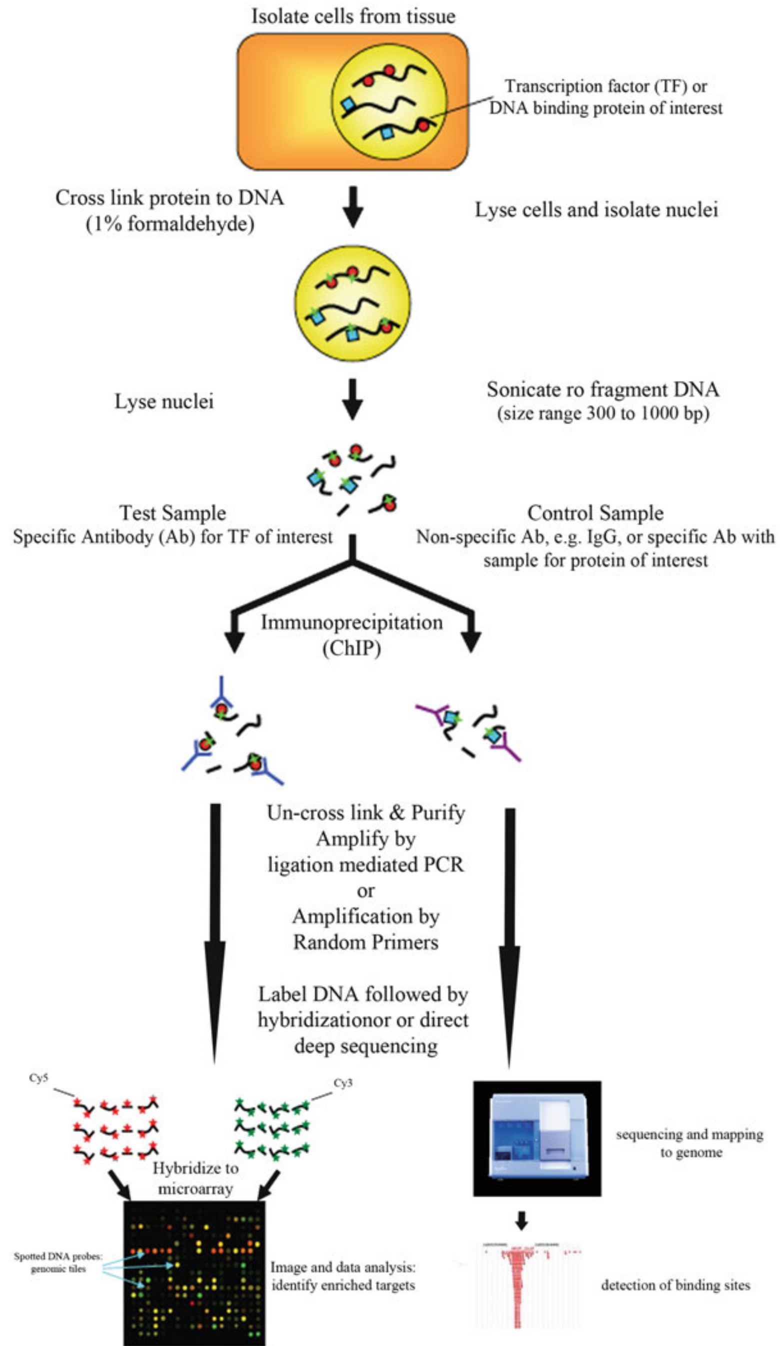

Fig. 1. An illustration of the principle of a ChIP-Chip or ChIP-Seq experiment (Adapted from Peter White, Ph.D.).

3. Formaldehyde 11% (25 ml): 7.45 ml 37% formaldehyde, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, and 50 mM HEPES.
4. Glycine 2.5 M: 18.767 g in 100 ml sterile water.

5. Lysis buffer 1 (LB1): 50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X-100.
6. Lysis buffer 2 (LB2): 10 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA.
7. Lysis buffer 3 (LB3): 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% *N*-lauroylsarcosine.
8. Wash buffer (RIPA): 50 mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate.
9. Elution buffer: 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% SDS.
10. TE: 10 mM Tris–HCl of pH 8.0, 2 mM EDTA. Supplemented with protease inhibitors prior to using.
11. Bovine serum albumin-Fraction V (BSA).
12. MiniElute purification Kit (Qiagen).
13. Wizard SV Gel and PCR clean-up System (Promega, USA).
14. Tris–hydrochloride (Merck, Germany).
15. KCl (Merck).
16. Tween20, nuclease free (Sigma-Aldrich, USA).
17. $MgCl_2$ (Merck).
18. Antibodies used here: Anti-OCT3/4 (H134), sc-9081-x (Santa Cruz, USA).
19. Dynabeads Protein G (Invitrogen, USA).
20. Dynabeads Protein A (Invitrogen).

For random PCR-based amplification:

21. For random_primer_mix:
    Make stocks of 100 pmol/ml of each Primer.
    random_primer_a: GTT TCC CAG TCA CGA TCN NNN NNN NA
    random_primer_t: GTT TCC CAG TCA CGA TCN NNN NNNNT
    random_primer_g: GTT TCC CAG TCA CGA TCN NNNNNN NG
    random_primer_c: GTT TCC CAG TCA CGA TCNNNN NNN NC
    where N can be any base. Dilute each of these to 40 pmol/ml working stock and store at 20°C. Pool in equal amounts (each primer 10 pmol/ml, total 40 pmol/ml). 10× PCR buffer (500 mM KCL, 150 mM Tris pH 8.3, 1% Tween, 15 mM MgCl).
22. Sequenase T7 DNA Polymerase Version 2.0, 13 U/μl (Amersham, UK).
23. Sequenase buffer (Amersham).

24. BSA (molecular biology grade) 500 μg/ml (Biolabs).
25. DTT 0.1 M, RNase free (Promega).
26. dNTPs (Promega).
27. Taq polymerase (Promega).
28. Pfu DNA Polymerase (Fermentas, USA).

3. Methods

3.1. Chromatin Immunoprecipitation

We recommend using approximately 5×10^7–1×10^8 cells (70–90% confluency for adhesion cells from two 15 cm^2 plates or 175 cm^2 flasks) for each IP reaction (see Note 1).

3.1.1. Formaldehyde Cross-linking of Cells

1. Add 1/10th volume of freshly prepared 11% formaldehyde solution to the plates (see Note 2).
2. Swirl plates briefly and allow to stand at room temperature for 10 min.
3. Add 1/20th volume of 2.5 M glycine to the plates to quench the formaldehyde.
4. Rinse cells twice with 5 ml 1× PBS. Harvest cells using a silicon scraper.
5. Pool cells in 50 or 15 ml conical tubes and spin at $1,350 \times g$ for 5 min at 4°C (using a swinging bucket rotor). Discard supernatant and resuspend pellet in 10 ml 1× PBS per 10^8 cells.
6. Transfer 5×10^7–1×10^8 cells to a 15 ml conical tube and spin at $1,350 \times g$ for 5 min at 4°C (using a swinging bucket rotor). Discard the supernatant.

3.1.2. Preblocking and Binding of Antibodies to Magnetic Beads

1. Add 100 μl Dynal magnetic beads to a microfuge tube. Add 1 ml of blocking solution. Set up one tube per IP reaction.
2. Collect the beads using a magnetic stand. Remove supernatant.
3. Wash the beads in 1.5 ml blocking solution two more times.
4. Resuspend the beads in 250 μl blocking solution and add 10 μg of antibody.
5. Incubate overnight at 4°C on a rotating platform.
6. On the next day, wash the beads (three times in 1 ml block solution).
7. As described above in step 3.
8. Resuspend the beads in 100 μl blocking solution.

3.1.3. Sonication of Cells

1. Resuspend each pellet of 5×10^7–10^8 cells in 5 ml of lysis buffer LB1. Place the tube on ice, turning the tubes every 2 min for a period of 10 min. Spin at $1,350 \times g$ for 5 min at 4°C using a tabletop centrifuge.

2. Resuspend each pellet in 5 ml of lysis buffer LB2. Rock gently at room temperature for 10 min. Pellet nuclei using tabletop centrifuge by spinning at 1,350 × *g* for 5 min at 4°C.
3. Resuspend each pellet in each tube in 3 ml of lysis buffer LB3.
4. Transfer cells to a, 15 ml polypropylene conical tube, cut into two pieces at the 7 ml mark.
5. Sonicate suspension with a tapered microtip with a 6.5 mm diameter, attached to a BRANSON 250 and sonicate at power 3 for 11 min with 30% duty cycle at 4°C while samples are immersed in an ice bath. Sonication is a critical step of the whole experiment and should be planned with care (see Note 3).
6. Add 300 μl of 10% Triton X-100 to sonicated lysate. Split into two 1.5 ml centrifuge tubes. Spin at 20,000 × *g* for 10 min at 4°C to pellet debris.
7. Combine supernatants from the two 1.5 ml centrifuge tubes in a new 15 ml conical tube for IP.
8. Save 50 μl of the cell lysate from each sample as whole cell extract (WCE) DNA. Store at −20°C.

3.1.4. Preblocking and Binding of Antibody to Magnetic Beads

1. Add 100 μl Dynal magnetic beads to a microfuge tube. Add 1 ml blocking solution.
   Set up one tube per IP reaction (see Note 5).
2. Collect the beads using the magnetic stand. Remove the supernatant.
3. Wash beads in 1.5 ml blocking solution twice.
4. Resuspend beads in 250 μl blocking solution and add 1–10 μg of antibody (see Note 4).
   The exact amount of antibody needs to be tested first and can vary extensively between different antibodies, but also between different batches of the same antibody (see Note 7).
5. Incubate overnight at 4°C on a rotating platform.
6. Next day, wash beads as described above (three times in 1 ml blocking solution).
7. Resuspend in 100 μl blocking solution.

3.1.5. Reversal of Cross-links After Chromatin Immunoprecipitation

1. Add 100 μl of antibody/magnetic bead mix to cell lysates.
   With the OCT4 antibody used here, we could scale down the volume to a fifth of the total lysate and could still obtain sufficient enrichment signals.
2. Gently mix on rotator or rocker at 4°C overnight. Make sure that there is enough liquid in the tube to enable efficient rotation.

The following steps should be carried out in a 4°C cold room. Prechilled tubes should be used.

1. Transfer half the volume of an IP to a 1.5 ml microfuge tube (see Note 6).
2. Place tubes in the magnetic stand to concentrate and localize the beads. Remove supernatant and add the remaining IP. Repeat this step once.
3. Add 1 ml of wash buffer (RIPA) to each tube. Remove tubes from the magnetic stand and shake or agitate tube gently to resuspend the beads. Place the tubes again in the magnetic stand to concentrate the beads. Remove supernatant. Repeat this washing step 3–7 times.
4. Wash once with 1 ml TE containing 50 mM NaCl.
5. Spin at 1,000 × *g* for 3 min at 4°C and remove any residual TE buffer.
6. Add 200 μl of elution buffer.
7. Elute at 65°C for 20 min. Resuspend beads every 2 min with brief vortexing.
8. Spin down beads at 16,000 × *g* for 1 min at room temperature.
9. Remove 190 μl of supernatant and transfer to a new tube. Reverse cross-link the immunoprecipitated DNA by incubating at 65°C overnight.
10. Thaw 50 μl of WCE reserved after sonication, add 150 μl of elution buffer, and mix. Reverse cross-link of this WCE DNA by incubating at 65°C overnight.

3.1.6. Purification of DNA

Isolate IP and input DNA using the Qiagen PCR purification kit and incorporating these modifications:

1. Add 500 μl of PB buffer and allow to stand for 10 min.
2. After column purification, elute with 50 μl of elution buffer prewarmed to 60°C (see Note 8).

3.2. Amplification of ChIP and Input DNA (PCR-Based Amplification)

1. Linear amplification of ChIPed DNA and input control is based on a random primer amplification described by Bohlander et al. (8) and subsequently modified for ChIP applications (7) (see Note 9 and 10). The protocol is laid out in Table 1.
2. Amplified samples can be purified using the Wizard SV PCR purification kit following the manufacturer's instructions.
3. For assessing the quality of the DNA and possible amplification bias which might have occurred after the randomized amplification, the range of the distribution after the amplification has to be tested, as illustrated in Fig. 2. Furthermore, nonamplified DNA samples should be compared with amplified DNA samples employing qPCR. Figure 3 illustrates a typical comparison between the distribution of nonamplified

Table 1
Reagents and cycling parameters for the PCR-based amplification of ChIP-derived genomic DNA

Round A reaction	
1	Per reaction (µl)
ChIP DNA	7
5× Sequenase buffer	2
Random_primer_mix (40pmol/µl)	1
Total volume	*10* µ*l*
Program for thermocycler	
2 min at 94°C	
5 min at 10°C	
Then, add	
5× Sequenase buffer	1
dNTP (3 mM)	1.5
DTT (0.1 M)	0.75
BSA (500 µg/µl)	1.5
Sequenase (13 U/µl)	0.5
Total volume	*15.25* µ*l*
Program for thermocycler	
Ramp from 10 to 37°C over 8 min	
Hold at 37°C for 8 min	
Rapid ramp to 94°C	
2 min 94°C	
Rapid ramp to 10°C	
Hold 5 min at 10°C	
Add	
1.2 µl sequenase diluted 1:4 with buffer	
Ramp from 10 to 37°C over 8 min	
Hold at 37°C for 8 min	
Add H_2O to a total volume of 60 µl	
Round B template	(µl)
Round A template	15
10× PCR buffer	10
dNTP (25 mM each)	1
random_pimer_2 (100 pmol/µl)	1
Taq polymerase/Pfu (10:1)	1
H_2O	72
Total volume	*100* µ*l*
Add 85 µl of a premixed Mastermix, containing the PCR buffer, the dNTPs, random primers and the Taq polymerase/Pfu mixture to each 15 µl aliquoted reaction from Round A	
Program for thermocycler	
20 Cycles	94°C 30 s 40°C 30 s 50°C 30 s 72°C 2 min

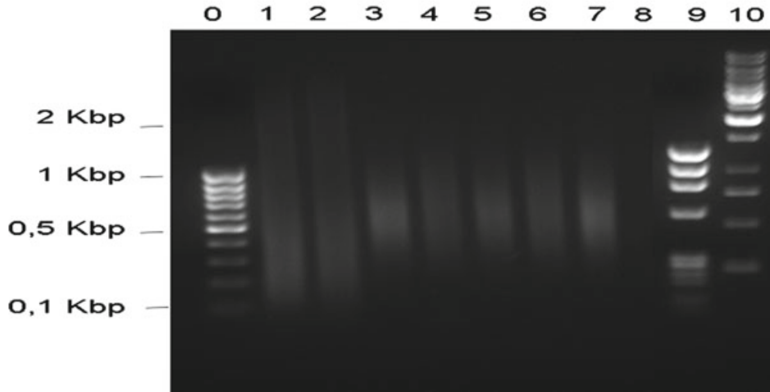

Fig. 2. An illustration of the distribution of DNA fragments before and after a random PCR-based amplification. The lanes are loaded with 300 ng of DNA. *Lanes 1* and *2*: DNA from whole cell extracts (WCE). *Lane 3*: input-antibody control, *Lane 4*: enrichment-antibody control, *Lane 5*: input OCT4-N19, *Lane 6*: enrichment OCT4-N19, *Lane 7*: input OCT4-H134, *Lane 8*: enrichment OCT4-H134, *Lane 9*: 1 kbp ladder (Fermentas), *Lane 0*: 100 bp ladder (Fermentas).

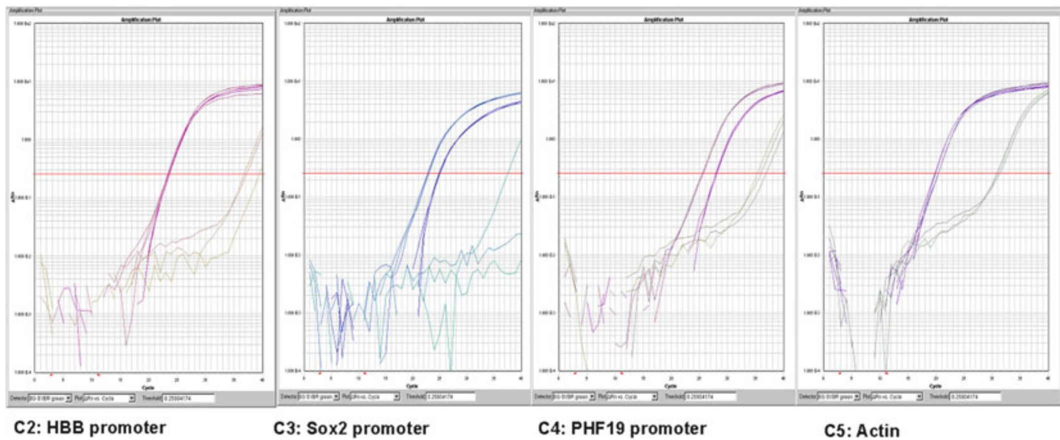

Fig. 3. Quantitative real-time PCR after random PCR-based amplification. *SOX2* and *PHF19* promoters harbor a binding motif for OCT4, whereas control promoter regions from hemoglobin beta (HBB) and *ACTIN* do not show enrichment after amplification.

and amplified DNA. This figure is based on the analysis carried out on our previously reported OCT4 binding sites and negative control loci (4).

4. Based on a comparative result as shown in Figs. 2 and 3, the DNA samples should now be ready for downstream processing for array-based hybridization (ChIP-Chip) or next-generation sequencing (ChIP-Seq).

4. Notes

1. Keep cell numbers constant in each experiment as well as incubation and sonication conditions. Chromatin can be stored at 4°C for up to 4 months.
2. The time needed for cross-linking proteins to chromatin as well as to other proteins should be constant for several samples. For optimal IP, each protein of interest might require different formaldehyde concentrations or reaction times.
3. Sonication conditions need to be optimized. Shearing varies greatly depending on cell type, growth conditions, quantity, volume, cross-linking, and equipment. In general, it is recommended to look for the lowest settings that result in sheared DNA that ranges from 100 to 600 bp in size, in order to prevent over shearing and still guarantee an optimal resolution for the tiling array or sequencing. We recommend a BRANSON 250 model but of course newer models, such as the DIGITAL Sonifier® UNITS, which can be programmed can be used. We adapted our method using a 1.7 microcentrifuge tube and 500 μl total volume with a program, using 20% sonification time for a total of 11 min. Important here is to prevent the sample from foaming and to make sure that the tube is sufficiently cooled during the whole duration of the sonication.
4. Antibodies need to be carefully selected and tested by Western blotting, immunofluorescence, or similar assays to guarantee specificity.
5. The exact type of Dynal beads depends on the antibody being used. Each type has preferences for different species and must be taken into account.
6. The exact number of washes depends on the quality of the antibody and may need to be optimized for each antibody.
7. For the immuno-enrichment and the washing steps use always siliconized tubes to be used for IPs. This is necessary if one aims to reduce nonspecific adherence of the antibodies to the reaction tubes.
8. The prediction of an optimal amount of antibody used is not possible. However, different ranges of concentrations can be tested and the enrichment can be subsequently analyzed for known binding sites by quantitative real-time PCR.
9. After elution, purified DNA can be stored in TE buffer and stored −20°C for long periods.
10. An alternative to random PCR amplification can be ligated mediated PCR. The advantage of the random PCR approach is that the ligation step can be skipped, without increasing bias.

To assess the reproducible amplification of enriched sequences, quantitative real-time PCR analysis should be employed (4). Here, negative genomic controls for PCR amplification should be used, for example a set of primers should be designed within a genomic sequence, adjacent to the binding site at which you would not expect an interaction with the protein under investigation. This reaction should not produce an amplicon. Re-check the specificity of the signal, when using SyberGreen-based real-time PCR, by analyzing the reaction on an agarose gel for the presence of a single amplicon.

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Chapter 19

Analysis of the Methylome of Human Embryonic Stem Cells Employing Methylated DNA Immunoprecipitation Coupled to Next-Generation Sequencing

Christina Grimm and James Adjaye

Abstract

The analysis of DNA-methylation on a genome-wide scale by next-generation sequencing techniques is an invaluable tool towards the understanding of the epigenetic basis of cellular differentiation. Methylated DNA immunoprecipitation (MeDIP) is an immunocapturing method using an antibody targeting 5-methylcytidine (5mC) and thereby enriching methylated DNA. MeDIP combined with next-generation sequencing (MeDIP-seq) provides a powerful tool for the analysis of genome-wide DNA-methylation profiles. Here, we describe a protocol for the preparation of MeDIP samples suitable for next-generation sequencing on a Genome Analyser (Illumina).

Key words: Epigenetic, DNA-methylation, MeDIP, 5-mCytosine

1. Introduction

Methylation of Cytosine at the carbon-5-position of the pyrimidine ring to generate 5-methylcytosine (5mC) is an epigenetic modification present in most eukaryotic organisms and is catalysed by DNA methyltransferases (DNMT). In mammals, methylation occurs preferentially at CpG sites, although in human embryonic stem cells about a quarter of all methylation is detected in a non-CpG context (1). The human (mouse) haploid genomes harbour about 28 (21) million CpGs and according to the definition used by UCSC, 28.691 or 16.026, CpG islands were detected in the hg19 version of the human or mm9 version of the mouse genome, respectively (http://genome.ucsc.edu). Recently, another cytosine modification was identified: 5-hydroxy-methylcytosine, 5hmC (2), its occurrence is dependent on the cell type analysed. For example,

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it occurs within 0.01 (HeLa or HEK293FT) to 0.4% (adult mouse cerebellum) of all nucleotides (3).

Since DNA-methylation is not maintained during PCR amplification, amplification steps prior to bisulfite (BS)-conversion, restriction digest, or enrichment are not possible. The available techniques for the analysis of methylated cytosines can be grouped into three main classes, viz:

1. BS-based techniques where the DNA is treated with BS leading to a conversion of unmethylated cytosine to uracil while leaving methylated cytosines intact. During a subsequent PCR amplification step uracil is then replaced by thymine. Sequencing of BS converted DNA (MethylC-seq) (1) requires the resequencing of the whole genome and it is very costly when applied to mammalian genomes. Reduced representation bisulfite sequencing (RRBS) reduces the complexity of the genome by an initial restriction digest of the genomic DNA followed by a size-selection, thereby minimising costs, but also the number of CpG sites analysed (4). The same holds true for an array-based enrichment of genomic regions prior to BS-treatment and sequencing.
2. Restriction enzyme-based approaches using methylation sensitive restriction enzymes (MRE-seq) which selects for nonmethylated DNA (5), methylation-specific restriction enzymes (e.g., *McrBC*) or methylation sensitive and insensitive isochizomeres (e.g., *HpaII* and *MspI*).
3. Enrichment-based methods using a methylated DNA binding domain (MBD-seq) (5–7) or an antibody against 5-methylcytidine (MeDIP-seq) for the enrichment of methylated sequences (5, 7–11). Of these three techniques, only the BS-based techniques provide a base-wise resolution though at a high cost. The restriction enzyme-based techniques are restricted to the recognition sites of the enzymes used, e.g., for *MspI* about 1.5 million CpG sites of the 21 million CpGs in the mouse genome are localised in an *MspI* site. The enrichment-based methods MeDIP-seq and MBD-seq do identify methylated fragments but cannot assess the methylation status of individual CpGs within the fragment as it is achieved by BS-based techniques. However, these techniques provide a good compromise between resolution and cost for the genome-wide analysis of DNA-methylation profiles. In addition, the BS-based methods do not discriminate between 5mC and 5hmC, whereas the two enrichment-based methods are specific to 5mC and do not detect 5hmC (12). A commercial antibody targeting 5hmC became recently available. Recent comparisons of BS and enrichment-based methods coupled with next-generation sequencing concluded that all techniques produce accurate methylation data, although differing in their ability to detect differentially methylated regions (5, 7).

Here, we describe MeDIP-seq: methylated DNA immunoprecipitation (MeDIP) is an immunocapturing approach whereby genomic DNA is fragmented, denatured and immunoprecipitated by a monoclonal antibody targeting 5′ methylcytidine and was first applied by Weber et al. (13). When combined with next-generation sequencing (Fig. 1), we prepare the library between the DNA-fragmentation and the immunoprecipitation step since T4 DNA ligase prefers double-stranded DNA as a template, the risk of introducing contaminations which will be amplified during the PCR amplification step is minimised and if desired, it is possible to introduce methylated and unmethylated spike-in DNAs as controls for MeDIP-efficiency. A set of lambda-DNA controls is described in ref. 14. Although most published protocols (5, 7, 8, 10, 11, 14) prepare the library before MeDIP, one protocol prepares a second strand synthesis and the library preparation after the immunoprecipitation (9). We successfully applied the MeDIP-seq protocol presented here to unveil methylation changes accompanying the differentiation of human embryonic stem cells into endoderm derivatives (11).

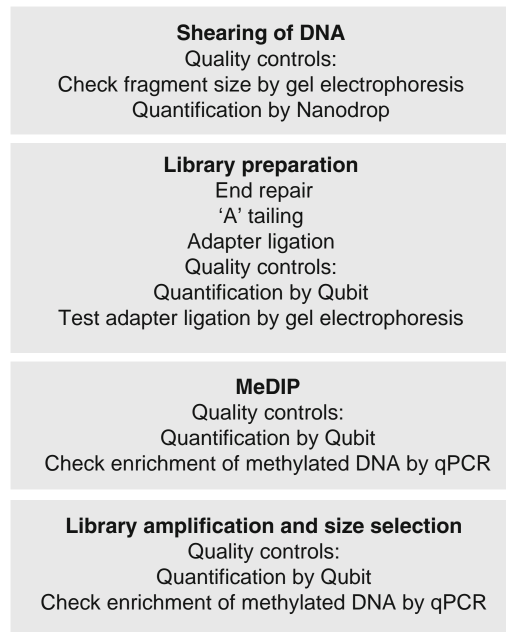

Fig. 1. MeDIP-seq experiment. The different experimental steps and quality controls are shown. Library preparation is performed before MeDIP since the DNA is denatured during the experiment and T4 ligase preferentially ligates double-stranded DNA. The size evaluation steps can also be analysed using a Bioanalyser (Agilent) instead of gel electrophoresis.

2. Materials

2.1. General

1. 1.5 and 2 ml low-binding DNA tubes (Eppendorf) for library preparation and MeDIP (see Note 1).
2. QIAquick Gel Extraktion Kit (Qiagen).
3. Collection tubes (Qiagen) or 2 ml tubes with the lids cut.

2.2. Shearing of Genomic DNA

1. Hielscher UTR2000 sonicator with continuous waterbath cooling. Other devices may also be used, e.g., BioRuptor (Diagenode), Covaris, Branson.
2. Orange dye (Sigma-Aldrich) used as tracking dye in the loading buffer (see Note 2).

2.3. Library Preparation

2.3.1. End Repair

1. T4 polynucleotide kinase, 10 U/μl (NEB).
2. T4 DNA polymerase, 3 U/μl (NEB).
3. Polymerase I, large (Klenow) Fragment, 5 U/μl (NEB).
4. T4 DNA ligase reaction buffer 10× (NEB).
5. 25 mM dNTP Mix (GE Healthcare).

2.3.2. A-tailing

1. Klenow Fragment (3′→5′ exo-), 5 U/μl (NEB) with buffer NEB2.
2. 1 mM dATP (GE Healthcare).
3. MinElute PCR Purification Kit (Qiagen).

2.3.3. Adapter Ligation

1. Adapters included in the Genomic Adapter oligo mix for single-reads or paired-reads (Illumina).
2. Quick Ligation Kit (NEB) containing Quick T4 DNA Ligase and Quick Ligation Reaction Buffer (2×).

2.4. Methylated DNA Immunoprecipitation

1. 5-Methylcytidine antibody, mouse monoclonal (Eurogentec, #BI-MECY-1000). A 5-methylcytidine antibody supplied by Diagenode works comparably well. Aliquot the antibody and store at −20°C, avoid freeze-thaw cycles.
2. Dynabeads M-280 Sheep anti-Mouse IgG (Invitrogen).
3. Albumin, bovine serum, Fraction V, and BSA (Sigma-Aldrich).
4. 0.5% BSA/PBS (*w*/*v*).
5. Magnetic rack for collecting the magnetic beads.
6. 100 mM sodium phosphate, buffer solution pH 7.0 for HPCE (Sigma-Aldrich).
7. 5 M NaCl.
8. Triton X-100 (Sigma-Aldrich).
9. 2× Immunoprecipitation buffer (280 mM NaCl, 20 mM sodium phosphate buffer pH 7.0, 0.5% Triton X-100).

10. SDS elution buffer (50 mM Tris–HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1% SDS).
11. Proteinase K (Roche Applied Science), dissolve in H_2O to 20 mg/ml, aliquot, and store at −20°C, do not refreeze a thawed proteinase K aliquot.
12. 1× TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).
13. 2 ml Phase Lock Gel Heavy tubes (VWR International GmbH).
14. Phenol:CHCl3:Isoamyl alcohol, 25:24:1 (Sigma-Aldrich).
15. Polyacryl carrier (Molecular Research Center Inc., Fermentas).
16. 10 mM Tris–HCl, pH 8.5.

2.5. Amplification and Size Selection

1. Primers included in the Genomic Adapter oligo mix for single-reads or paired-reads (Illumina).
2. Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB).
3. 0.2 ml thin-walled PCR tubes.
4. Ultra low range agarose (BioRad).
5. Clean scalpels for excision of the gel fragment.

2.6. Quantitative PCRs for Control of MeDIP Enrichment

1. SYBR Green PCR Master Mix (Applied Biosystems).
2. Oligonucleotides for the amplification of a methylated and an unmethylated control region (Table 1).

2.7. Quantification of the DNA

1. Nanodrop spectrophotometer (Nanodrop).
2. Quant-iT ssDNA kit (Invitrogen) for quantification after MeDIP.
3. Quant-iT dsHSDNA kit (Invitrogen) for quantification of the size selected amplified library.

Table 1
PCR primers used for evaluating the enrichment of methylated fragments

Name	Forward primer	Reverse primer	species	Methylated	Reference
4994	GGGAATATAAG GAGCGCACA	TCGGTTAAAAC GGTCAGGTC	Human	+	Butcher and Beck (14)
8804	CGAGGCGTGA GTTATTCCTG	CTCTTGTGGCT GAGCTCCTT	Human	–	Butcher and Beck (14)
Xist	CGCGGATCAG TTAAAGGCGT	AACCACGGAAGA ACCGCAC	Mouse	+	Weber et al. (13)
Csa	TGGTTGGCATTTT ATCCCTAGAAC	GCAACATGGCAA CTGGAAACA	Mouse	–	Weber et al. (13)

4. QUBIT device (Invitrogen) for quantification using the Quant-it kits.
5. 0.5 ml reaction tubes suitable for the use with a Qubit device (e.g., Axygen PCT-05C, VWR # Q10212).

3. Methods

3.1. DNA Isolation

DNA isolated by a standard procedure is suitable [phenol-chloroform, or using commercially available kits, e.g., AllPrep DNA/RNA Mini Kit (Qiagen)]. However, it is recommended not to change the DNA isolation method within an experiment. The DNA has to be RNAse free (see Note 3). If RNA digestion was not performed during the DNA isolation step, then perform RNAse digest prior to shearing by incubating 1 μl of RNase (10 U/μl) with 4–10 μg of DNA in a 50 μl volume for 1 h at 37°C.

3.2. Fragmentation of DNA

DNA is sheared to fragment sizes of 100–400 bp. We use a Hielscher UTR2000 sonifier with continuous water bath cooling set to an amplitude of 1,000 and a duty cycle of 0.5. 4–8 μg of DNA is sheared four times for 30 min in 50 μl of 10 mM Tris–HCl pH 8.5 (or in TE) in a 0.5 ml reaction tube. After each 30 min sonication cycle, vortex the DNA and spin down the contents to collect any droplets that may have formed during sonication (see Note 4).

After sonication, 100 ng of the fragmented DNA is subjected to gel electrophoresis on a 2% agarose/1× TAE gel (Fig. 2a). If the DNA is not sheared sufficiently, shear for an additional 30 min (see Note 5). Other shearing devices may also be used [e.g., Bioruptor (Diagenode), Covaris]. Conditions need to be adjusted for each shearing device. After sonification, the sheared DNA is purified using Qiaquick columns (up to 10 mg DNA can be purified using one column).

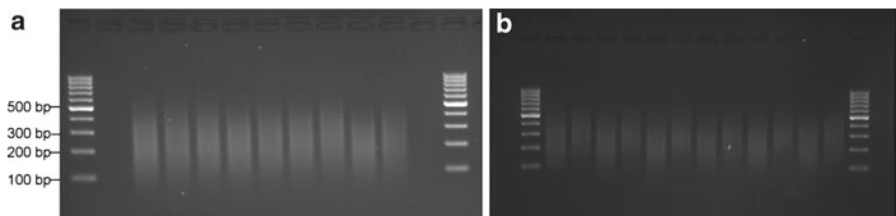

Fig. 2. Size distribution of sheared DNA (**a**) and of sequencing libraries prior to MeDIP (**b**). 160 ng of sheared DNA was separated on a 2% agarose/1× TAE gel stained with ethidium bromide. The size range of most fragments is between 100 and 400 bp with a mean fragment size of 200 bp (**a**). To monitor library preparation 100 ng of sheared DNA and sequencing library produced from the same DNA were loaded side by side on a 2% agarose/1× TAE gel. The size of the library increases by the size of the ligated adapter sequences (approx. 70 bp) (**b**). Marker: Gene Ruler 100 bp DNA ladder (Fermentas). If the amount of DNA is low, 50 ng of DNA is sufficient for size evaluation by gel electrophoresis.

3.2.1. Purification Using Qiaquick Columns

1. Add four volumes QG buffer to your sample (i.e. 50 μl DNA-sample, add 200 μl QG) and mix by vortexing.
2. Incubate for 3 min.
3. Add 1.3 volumes isopropanol (i.e. 50 μl DNA-sample, add 65 μl of isopropanol) and vortex.
4. Transfer the solution to a Qiaquick column.
5. Centrifuge 1 min at >10,000 × *g*.
6. Change collection tube.
7. Aspirate the QG from the inner plastic rim of the column.
8. Pipette 700 μl PE to the column and also to the lid and the outer rim of the column to remove all remaining QG since QG may inhibit subsequent enzymatic reactions.
9. Centrifuge 1 min at >10,000 × *g*.
10. Change collection tube.
11. Repeat steps 8–10.
12. Centrifuge 1 min at >10,000 × *g*.
13. Change collection tube.
14. Aspirate the remaining PE from the inner rim of the column (ca. 2 μl).
15. Centrifuge for 2 min at >10,000 × *g*.
16. Change collection tube.
17. Pipette 16 μl EB-buffer (10 mM Tris–HCl pH 8.5) to the membrane.
18. Incubate 5 min.
19. Centrifuge 1 min at >10,000 × *g*.
20. Pipette 16 μl EB to the membrane.
21. Centrifuge for 2 min.
22. 30 μl of DNA will be obtained, since the dead volume of the column is approximately 2 μl.

    Snap freeze your sheared DNA in liquid nitrogen and store at −20°C or proceed with the library preparation (see Note 6).

3.3. Library Preparation

Library preparation follows in principle the protocol suggested by Illumina with some adjustments as described in http://molbiol.ru/wiki/Next-generation_sequencing and applied in ref. 11. The Web site was set up by the sequencing group from our department and contains useful protocols and tips on second-generation sequencing. Use low-DNA-binding reaction tubes for the library preparation and the immunoprecipitation (see Note 6). After library preparation, 100 ng of the library and 100 ng of the sheared DNA may be separated on a 2% agarose/1× TAE gel in order to analyse the quality of the library. The size of the library should be shifted by the length of the ligated adapters (approx. 70 bp, Fig. 2b).

3.3.1. End Repair

1. Set up a 20°C water bath.
2. Set up for one reaction:

50 µl	DNA (5 µg)
31.7 µl	10× T4 DNA ligase buffer
12 µl	T4 DNA polymerase (3 U/µl)
12 µl	T4 polynucleotide kinase (10 U/µl)
2.4 µl	Polymerase I, large (Klenow) Fragment (5 U/µl)
3.2 µl	dNTP Mix (25 mM)
205.7 µl	H_2O
317 µl	*Total volume*

Incubate for 30 min in a 20°C water bath.

Purify using Qiaquick columns and buffer QG

1. Pipette 1,270 µl QG in a 2 ml reaction tube.
2. Add end-repair DNA reaction mix.
3. Incubate 3 min.
4. Add 412 µl isopropanol.
5. Follow the Qiaquick protocol.
6. Elute in 34 µl (twice with 18 µl of 10 mM Tris–HCl pH 8.5).

3.3.2. A-tailing

1. Set up a 37°C water bath.
2. Set up for one reaction:

34 µl	End-repaired DNA
8.8 µl	NEB2 reaction buffer (10×)
17.6 µl	1 mM dATP
7.3 µl	Klenow Fragment 3′→5′ exo- (5 U/µl)
20.3 µl	H_2O
88 µl	*Total volume*

Incubate for 30 min in a 37°C water bath.

Purify using Qiaquick MinElute columns and buffer QG.

1. Add 352 µl QG.
2. Incubate for 3 min.
3. Add 114 µl of isopropanol.
4. Follow the Qiaquick protocol.
5. Elute in 10 µl (twice with 6 µl of 10 mM Tris–HCl pH 8.5).

3.3.3. Adapter Ligation

1. Set up a 20°C water bath.
2. Add a tenfold molar excess of adapter-oligomix to the 10 μl A-tailed DNA fragments. For 5 μg of DNA with a mean fragment size of 200 bp, add 29 μl of adapter oligo mix.
3. Mix gently by pipetting and incubate for 5 min at RT.
4. Set up a ligase master mix, per reaction:

49 μl	2× Quick ligase buffer
10 μl	Quick T4 DNA ligase
59 μl	*Total volume*

5. Add 59 μl of ligase master mix to the DNA-adapter-mix and mix gently by pipetting up and down.
6. Incubate for 15 min in a 20°C water bath.

   Purify using Qiaquick columns and buffer QG:

1. Add 392 μl QG.
2. Incubate for 3 min.
3. Add 127.4 μl of isopropanol.
4. Follow the Qiaquick protocol.
5. Elute in 30 μl (2× 16 μl 10 mM Tris–HCl pH 8.5).
6. Quantify the concentration using a Nanodrop.

3.4. Methylated DNA Immunoprecipitation

3.4.1. Blocking and Coupling the Antibody to the Magnetic Beads

Use 4°C cold solutions.

1. Pipette 700 μl 0.5% BSA/1× PBS in a 1.5 ml microcentrifuge tube. Prepare one tube per MeDIP-reaction.
2. Vortex the magnetic beads very well.
3. Add 40 μl of the magnetic beads to the tube with 0.5% BSA/1× PBS and mix by inverting the tube gently several times (see Note 7).
4. Collect the magnetic beads using a magnetic rack. After the beads are trapped at the side, invert the magnetic rack to clean the lid of the tube. Aspirate and discard the supernatant.
5. Remove the magnet from the magnetic stand (or take the tube out of the magnetic stand).
6. Resuspend the magnetic beads in 700 μl of 0.5% BSA/1× PBS by inverting the tube several times.
7. Repeat steps 4–6.
8. Repeat steps 4–5 one more time.
9. Resuspend magnetic beads in 250 μl of 1× IP-buffer by inverting the tube several times.
10. Add 10 μl of 5mC-antibody (10 μg, see Note 8).

11. Incubate overnight with over head rotating at 4°C.
12. The next day collect the magnetic beads using a magnetic rack. After the beads are trapped, invert the magnetic rack to clean the lid. Discard supernatant.
13. Resuspend the beads in 1 ml 0.5% BSA/1× PBS by inverting the tube several times.
14. Collect the magnetic beads using a magnetic rack, invert the magnetic rack to clean the lid. Discard supernatant.
15. Repeat steps 13 and 14 one more time.
16. Resuspend the beads in 1 ml 1× IP-buffer by inverting the tubes several times.
17. Collect the magnetic beads using a magnetic rack, invert the magnetic rack to clean the lid. Discard supernatant.
18. Resuspend the beads in 170 μl 1× IP-buffer and place on ice until adding the DNA.

3.4.2. Methylated DNA Immunoprecipitation

1. Dilute 4.2–4.5 μg of the library to 133 ng/μl in 10 mM Tris–HCl pH 8.5.
2. Denature library in thin-walled PCR-tubes for 1 min at 95°C in a PCR machine.
3. Immediately place on ice-water and let sit for 3–5 min (see Note 9).
4. Pipette 30 μl library and 30 μl ice cold 2× IP to the magnetic beads in 170 μl of 1× IP-buffer from step 18.
5. Incubate for 4 h at 4°C with over head rotation.
6. Trap the magnetic beads in a magnetic stand and invert the magnetic rack to clean the lid. Discard supernatant.
7. Add 1 ml of ice cold 1× IP-buffer and resuspend the beads by inverting the tubes several times.
8. Repeat the washing (steps 6 and 7) two more times.
9. After the last washing step, centrifuge for 3 min at 960 ×*g* at 4°C. Remove any residual liquid. Be careful not to disturb the beads.
10. Add 210 μl of SDS elution buffer at RT.
11. Incubate for 15 min at 65°C with briefly vortexing every 2 min. Briefly spin the contents of the tube down.
12. Trap the magnetic beads in a magnetic stand. Pipette the supernatant in a new 1.5 ml tube. *Keep* the supernatant! It contains the immunoprecipitated DNA.
13. Take the tubes containing the beads out of the magnetic stand. Resuspend the beads in 200 μl TE by vortexing. Shortly spin

the contents of the tube down and collect the beads with the magnetic rack.

14. Combine the supernatant with the first eluate.
15. Add 4 μl of proteinase K (20 mg/ml).
16. Incubate for 2 h at 55°C.

3.4.3. Phenol–$CHCl_3$ Extraction

1. Centrifuge a 2 ml PhaseLock tube for 1 min at 160,000 × *g* at RT.
2. Pipette the sample (400 μl) to the PhaseLock tube, add 400 μl phenol:chloroform:isoamyl alcohol, mix vigorously by shaking the tube (do not vortex).
3. Centrifuge 5 min, at 160,000 × *g*, RT. The organic phase (phenol) and the aqueous (upper) phase (DNA) are separated by a solid phase.
4. Repeat extraction 2× with 400 μl $CHCl_3$.
5. Pipette the upper phase (aqueous phase containing the DNA) into a 1.5 ml tube containing 16 μl of 5 M NaCl and 1 μl of linear polyacrylamide, mix, add 800 μl 100% EtOH and precipitate overnight at −20°C.
6. Spin for 30 min, 20,000 × *g* at 4°C.
7. Aspirate the supernatant.
8. Add 800 μl 80% EtOH, vortex, spin 5 min at 4°C, 20,000 × *g*.
9. Aspirate supernatant, briefly spin again and aspirate any residual liquid, air dry pellet and resuspend in 20 μl 10 mM Tris–HCl pH 8.5.
10. Optional: quantitate the DNA using the Quant-it ssDNA kit. Since the amount of immunoprecipitated DNA is low, quantitation using a Nanodrop does not work reliable in our hands. Usually, we obtain 100–200 ng of immunoprecipitated library from 4 μg of library (2.5–5%).
11. Snap freeze the sample in liquid nitrogen and store at −20°C. Avoid repeated freeze-thaw cycles.

3.5. Quantitative PCR as Quality Controls

Enrichment of methylated DNA is monitored after MeDIP and after library amplification by standard qPCR using control primers of known methylated and unmethylated genomic regions (Table 1) using SYBR-green dye (Fig. 3 shows an example). We routinely use 0.5 μl of the MeDIP sample in a 10 μl PCR-reaction and perform the reaction. The methylation status of the control regions may differ between cell types and different control regions may be suitable for different cell types due to mosaic methylation patterns.

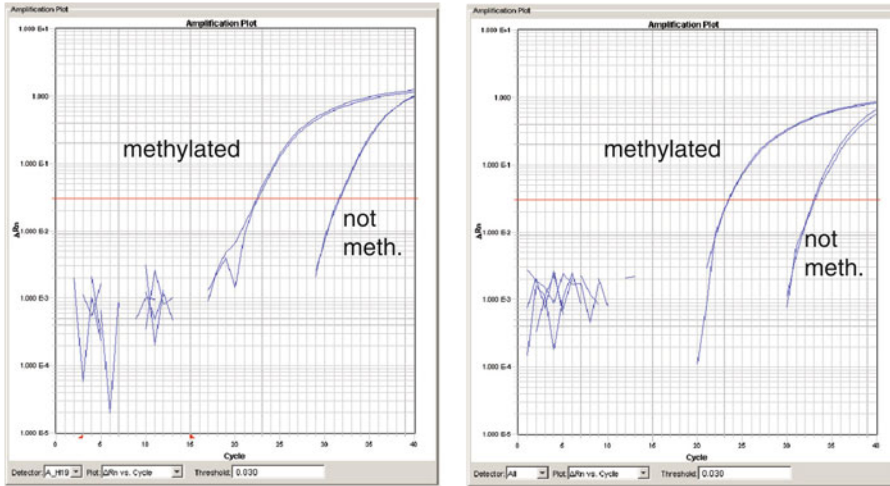

Fig. 3. Enrichment of methylated DNA before (**a**) and after library amplification (**b**). Enrichment of the methylated DNA of the same sample was assessed by qPCR using the methylated and unmethylated human genomic control regions listed in Table 1 after MeDIP and before library amplification (**a**) and after library amplification and size selection (**b**). Measurements were carried out in duplicate. qPCR amplification plots of an MeDIP from a human ES-cell-DNA sample published in ref. 11 is shown.

3.6. Amplification of the Library and Size Selection

The immunoprecipitated DNA is subjected to amplification and size-selection prior to sequencing. If desired, input DNA may also be amplified and size-selected (see Note 10).

1. Set up for one reaction:

4 μl	MeDIP-sample (1/5th of the MeDIP-sample or 40 ng of input library)
15 μl	High-Fidelity Phusion polymerase
0.6 μl	Primer 1.1 (20 μM, Illumina)
0.6 μl	Primer 2.1 (20 μM, Illumina)
9.8 μl	H_2O
30 μl	*Total volume*

For PCR cycling conditions, use a manual hot-start:

1. 98°C for 30 s.
2. 98°C for 10 s.
3. 65°C for 30 s.
4. 72°C for 30 s.

For the amplification, six PCR cycles (repetitions of steps 2–4) are used, thereby keeping the risk of introducing amplification bias low.

Size selection is performed using a 2% BioRAD/1× TAE gel. We run the gel for 2 h at 100 V and use a 50 bp Marker (NEB). If a better resolution is required, 25 bp DNA ladders may be used.

The gel chamber is cleaned thoroughly using detergent and water. The gel is loaded as follows: marker, empty well, sample, empty well, marker, empty well, sample, empty well, marker, and so on to avoid cross-contamination of the samples and to ensure a precise size-selection. We excise a 150–400 bp fragment to use for sequencing. Interestingly, the size range of an amplified MeDIP-library begins at about 200 bp while the size range of an amplified input library starts at 150 bp, indicating a selection for larger fragment sizes by the methylated immunoprecipitation (see Note 5). If desired, the excised gel slices may be frozen in liquid nitrogen and stored at –20°C. Purify the DNA using the Qiaquick Gel extraction kit and elute in 30 μl (twice with 16 μl of 10 mM Tris–HCl pH 8.5). Quantify the DNA using a Quant-it dsHSDNA kit. Typically, we obtain 240–300 ng of amplified library. Snap freeze the library and store at –20°C until sequencing. Avoid freeze-thaw cycles.

4. Notes

1. Use low-binding DNA tubes for the library preparation and the MeDIP to avoid sticking of the DNA to the tube walls. For the immunoprecipitation and the subsequent washing steps, the use of low-DNA-binding tubes is particularly important in order to reduce nonspecific binding of the DNA to the walls of the tube, which may increase the background signal.
2. Orange G (SIGMA) instead of bromophenol blue is preferred as tracking dye in the gel electrophoresis since Orange G migrates at about 50 bp and therefore does not interfere with the visualisation of the fragmented DNA.
3. RNAse digest of the DNA is important since the 5 mC-antibody will also detect 5mC in the context of RNA.
4. Shearing conditions need to be adapted to each device. We recommend not to change the shearing device within one experimental series. Also the plastic ware used (thickness of the walls) may influence shearing conditions. Cooling of the DNA during sonication is important since AT-rich regions are less stable to degradation and will be depleted from the sample.
5. Sheared DNAs within one experiment should be of equal size. MeDIP-seq is an enrichment-based method and as such it is dependent on the number of methylated CpGs within one DNA fragment. Longer fragments contain a higher number of CpGs and this will influence the enrichment.
6. Snap freezing of the DNA samples in liquid nitrogen is the best way to preserve the integrity of the sample.
7. We routinely use 4 μg of DNA library. If less DNA is used, the amount of antibody and beads needs to be adapted.

8. Avoid freeze-thaw cycles of the antibody and of your samples.
9. Ice water has a better thermal conduction compared to ice alone. Immediate cooling is important to prevent renaturation of the DNA.
10. In our point of view, sequencing of the input DNA is not generally necessary, although it serves as a control to identify a potential PCR-amplification bias. However, as an enrichment-based method, MeDIP is affected by copy number variations and especially for cancer samples, this is an important issue. In this case, sequencing of the input samples at a low coverage may be useful to identify regions of copy number variations.

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Chapter 20

Stable Isotope Labelling with Amino Acids in Cell Culture for Human Embryonic Stem Cell Proteomic Analysis

Linda Harkness, Tatyana A. Prokhorova, Moustapha Kassem, and Blagoy Blagoev

Abstract

The identification and quantitative measurements of proteins in human embryonic stem cells (hESC) is a fast growing interdisciplinary area with an enormous impact on understanding the biology of hESC and the mechanism controlling self-renewal and differentiation. Using a quantitative mass spectroscopic method of stable isotope labelling with amino acids during cell culture (SILAC), we are able to analyse differential expression of proteins from different cellular compartments and to identify intracellular signalling pathways involved in self-renewal and differentiation. In this chapter, we provide a detailed method for creating SILAC media suitable for use in hESC experiments, additionally we describe methods for the isolation of membrane fractions and cytosolic and nuclear/membrane fractions.

Key words: Human embryonic stem cells, Stable isotope labelling, Proteomics, Mass spectroscopy

1. Introduction

Mass spectrometry (MS) is a powerful, quantitative, tool for the identification of proteins by determining the mass of charged particles, and has been employed to describe the biological nature of a number of cells, including adult and embryonic stem cells (1–4). In MS, a proteolytically cleaved cellular suspension is fragmented by a high energy electron beam, accelerated through a magnetic field in a vacuum, and sorted using the positive charge on a mass-to-charge ratio (*m*/*e*), thus giving a value equivalent to the molecular weight of the fragment. Only those fragments with a proper mass-to-charge ratio will follow the path of the analyser and collide with the collector, where the *m*/*e* will be amplified and analysed. The rest (low and high momentum ions) will collide with the walls of the analysing tube and be deflected; analysis then involves the

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reassembly of the fragments on the collector to recreate the original molecule.

Mass spectroscopic analysis of human embryonic stem cells (hESCs) can be investigated in several respects. Extracellular proteomes, i.e. proteins secreted into the culture media can be evaluated to identify paracrine and autocrine growth factors secreted by both hESC and feeder layers to assist in understanding the complex microenvironment of hESC (5). Additionally, isolation of membrane (3, 6, 7), nuclear (8), or cytosolic fractions (6) of hESC or whole cell lysates (9, 10) or analysis and comparison of hESC undergoing differentiation (such as osteogenesis (11); cardiomyocytes (12); neuroectoderm (13); and haematopoiesis (14)); specific signalling pathways (tyrosine signalling (15)); or protein phosphorylation (16, 17) have all been reported thus adding to the increasing proteomic-based knowledge of hESC. Data sets acquired through these means are large (in the thousands of identifications) and can be utilised to study protein interaction and signalling networks involved in the maintenance of self-renewal or differentiation. Additionally, there has been a growth in protein sequence databases to assist in the identification and statistical validation of identified proteins (18).

Quantification of differential expression of proteins during self-renewal or differentiation is an important aspect of current proteomic studies. The development of a quantitative proteomic method based on stable isotope labelling with amino acids in cell culture (SILAC) has provided a sensitive method to create a quantitative proteomic profile of hESC. SILAC labelling involves the incorporation of heavy or light forms of amino acids into newly synthesised proteins within a given cell population (4, 19–21). During cellular replication, and over a number of divisions, naturally occurring amino acids are replaced by the light or heavy amino acids labelled with nonradioactive isotopes, thus two or three cell populations, identical except for their isotope incorporation, can be mixed in equal ratios and analysed in one experiment (22). This strategy allows the analysis of complex biological occurrences, such as post-translational modifications, phosphoproteins and secreted proteins and, additionally, facilitates comparison of signalling dynamics that determine cell fate during self-renewal or differentiation. In this chapter, we provide a detailed protocol for SILAC labelling of hESC and preparation of samples for MS analysis. Previous chapters have dealt with routine procedures for creation of conditioned media and culturing and passaging of hESC on Matrigel. Labelling media for hESC can be prepared by direct MEF conditioning of hESC media where KO-DMEM is replaced by SILAC DMEM supplemented by corresponding light or heavy Arg and lysine, as knockout serum replacement does not contain free arginine or lysine (23) and the amount of free amino acids

released into the media by unlabeled MEFs during conditioning is negligible and does not affect labelling (TA Prokhorova and B Blagoev, unpublished data).

2. Materials

2.1. SILAC Media for Conditioning (100 ml)

1. DMEM without Arg and Lys (Invitrogen, custom made as 31855 but without Arg and Lys) 80 ml.
2. Knockout serum replacement (Invitrogen, cat no: 10828010) 15 ml.
3. 20% Human serum albumin (CSL Behring, Germany vnr: 10 96 97) 2.5 ml.
4. Glutamax (Invitrogen, cat. no.: 35050038) 1 ml.
5. Nonessential amino acids (Invitrogen, cat. no.: 11140035) 1 ml.
6. 2-Mercaptoethanol (Sigma-Aldrich, cat. no.: M6250) 0.1 μM (see Note 1).
7. Human Basic FGF (Invitrogen, cat. no.: PHG0021) made in 0.1% HSA in PBS^{2-} (see Note 2).
8. Amino acids: normal "light" amino acids: L-lysine (Lys0) and L-arginine (Arg0) hydrochloride (Sigma Chemicals, Copenhagen, Denmark).
9. Stable isotope-labelled "heavy" amino acids: L-arginine-$^{13}C_6$ hydrochloride (Arg6) (Cambridge Isotope Labs, Andover, MA, cat. no.: CLM-2265) 84 mg/ml.
10. Stable isotope-labelled "heavy" amino acids: L-arginine-$^{13}C_6$, $^{15}N_4$ hydrochloride (Arg10) (Sigma-Isotec, St. Louis, MO, cat. no.: 608033) 84 mg/ml.
11. Stable isotope-labelled "heavy" amino acids: L-lysine-4,4,5,5-d_4 hydrochloride (Lys4) (Sigma-Isotec; cat. no.: 616192) 146 mg/ml.
12. Stable isotope-labelled "heavy" amino acids: L-lysine-$^{13}C_6$, $^{15}N_2$ hydrochloride (Lys8) (Sigma-Isotec, cat. no.: 608041) 146 mg/ml.
13. Proline (Sigma-Aldrich, cat. no.: P5607) 500 mg/ml.
14. Glucose 4.5 g/L.

2.2. Materials for Passaging

1. Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (PBS^{2-}; Invitrogen cat. no.: 14190094).
2. Hanks-based cell dissociation buffer (Invitrogen, 13150016).

2.3. Materials for Isolation of Membrane, Cytosolic, and Nuclear Fractions

1. PhosSTOP phosphatase inhibitor (Roche Applied Science, cat. no.: 04906845001) make according to manufacturer's instructions.
2. Complete Protease Inhibitor (Roche Applied Science, cat. no.: 04693116001) make according to manufacturer's instructions.
3. Complete Protease Inhibitor EDTA-free (Roche Applied Science, cat. no.: 04693132001) make according to manufacturer's instructions.
4. 1 ml Dounce tissue grinder (Wheaton, cat. no.: 357538).
5. Polycarbonate type 1 tubes (Hitachi Koki, cat. no.: S300535A).
6. Sequencing Grade Modified Trypsin (Promega, cat. no.: V5111).
7. Endoproteinase Lys-C (Roche Applied Science, cat. no.: 11 420 429 001).
8. Empore Disc C18 (3 M Filtration Products, cat. no.: 2215).

3. Methods

3.1. Conditioned SILAC Media

1. All cell culture steps should be carried out using aseptic techniques and inside a tissue culture hood.
2. Precoat flasks or plates with 0.2% gelatin in water for 30 min at RT.
3. Plate inactivated feeders (inactivated either through gamma irradiation (30 Gy mouse embryonic fibroblasts; or 50 Gy neonatal human dermal fibroblasts, see Note 3), or by addition of a final concentration of 10 μg/ml mitomycin C for 2–3 h (see Chapter 4 from Harkness and Kassem for a detailed protocol) at a concentration of 83,333 cells/cm^2.
4. With the exception of the hbFGF, add all the components of the media to be conditioned to a 0.2 μM Nalgene filter unit and filter sterilise. Store at 4°C until needed, adding the hbFGF just prior to use.
5. The day after plating, aspirate off the media and add the correct amount of media to be conditioned (125,000 cells/ml media to be conditioned). Incubate at 37°C, 5% CO_2 for 24 h.
6. Collect and change media every 24 h for 7 days.
7. Media can be filtered and used immediately or stored at −20°C until needed.
8. hESC should be grown on Matrigel in SILAC media using routine laboratory procedures (see Note 4) and passaging

techniques for at least five passages to ensure complete incorporation of the SILAC amino acids (see Note 5).

9. Addition of heavy or light amino acids to the media should be added after conditioning but prior to usage, media can be stored at 4°C following addition of amino acids. Cells should be used from the same passage number for both heavy and light labelling experiments to ensure that they are comparable. Figure 1 demonstrates the method for SILAC labelling of cells with a flow chart and shows the shift in peak identification between unlabelled and labelled cells.

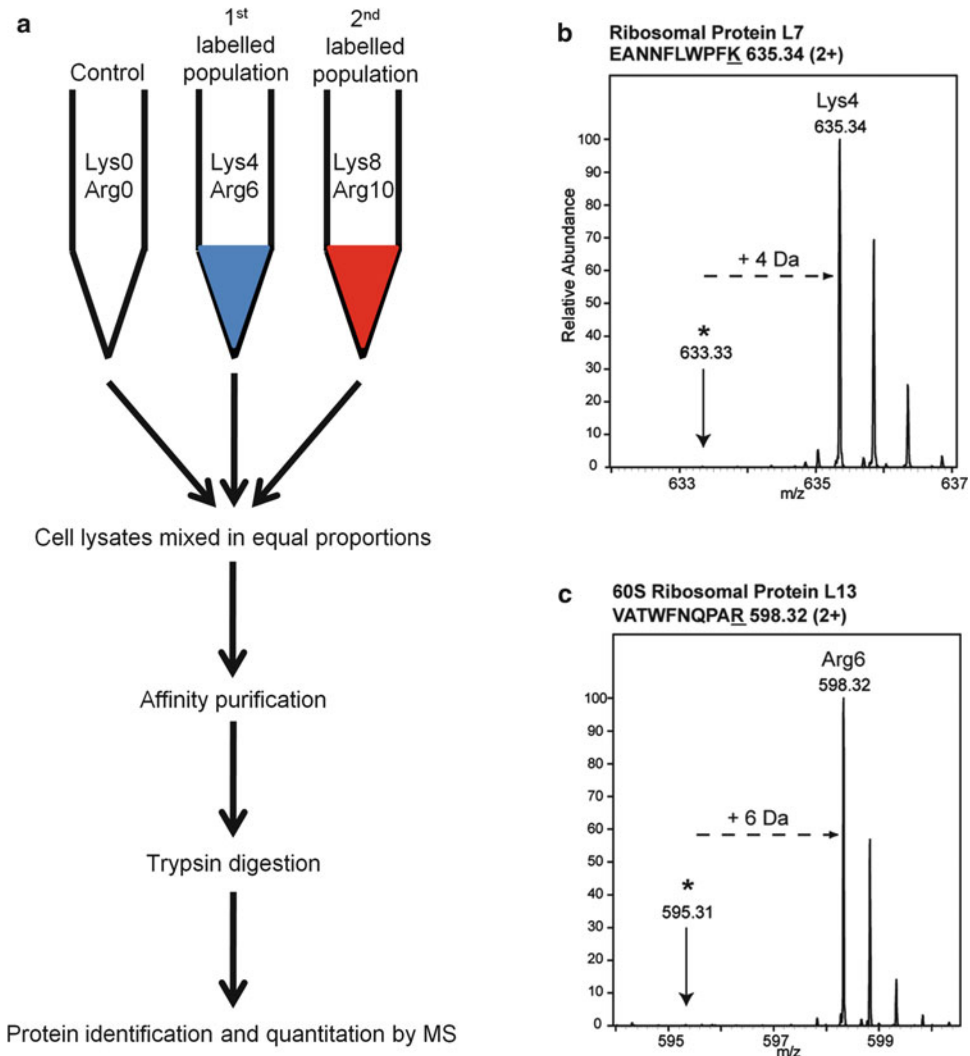

Fig. 1. SILAC labelling of hESC. (**a**) Three populations of cells are grown in media containing either Lys0/Arg0, Lys4/Arg6, and Lys8/Arg10. Cell lysates are mixed in equal proportions and subjected to optional fractionation, affinity purification, and gel electrophoreses, before being digested with trypsin and analysed by mass spectrometry. (**b**, **c**) Graphs demonstrating complete SILAC labelling of hESC. A shift in the mass spectra from corresponding unlabeled precursors (identified by *asterisk*) is shown in (**b**) the SILAC-labelled lysine-containing peptide by 4 Da (**c**) and the SILAC-labelled arginine-containing peptide by 6 Da.

3.2. Sample Preparation for Membrane Fraction (see Note 6)

1. Aspirate the media from cells and rinse cells in PBS^{2-}. Incubate the cells in pre-warmed Cell Dissociation buffer at 37° for 10 min and pipette vigorously in order to dissociate the cells to a single cell suspension. Centrifuge at 350 × *g* for 5 min; aspirate the supernatant.
2. Resuspend the cells in 1 ml sucrose buffer (255 mM sucrose, 20 mM HEPES, pH 7.4, and 1 mM EDTA) freshly supplemented with complete protease inhibitor cocktail.
3. Transfer to a prechilled Dounce homogenizer, and disrupt by 35 strokes with a tight pestle (see Note 7).
4. If desired, using a microscope, visually evaluate the cellular debris to confirm that nuclei are intact and the debris sizes even.
5. Transfer the membrane homogenate to a microcentrifuge tube and centrifuge at 20,800 × *g* for 10 min at 4°C to separate mitochondria and nuclei.
6. Transfer the supernatants to a sucrose buffer pre-washed PC1 tube, place in an S150AT-0097 (No. 7) rotor, and centrifuge in a Sorvall RC M150 GX ultracentrifuge at 245,000 × *g* at 4°C for 2 h (see Note 8).
7. Wash the membrane pellet by resuspending it in 1 ml of ice-cold 100 mM Na_2CO_3 (supplemented with EDTA-free protease inhibitors cocktail) and incubate on ice for 30 min with careful, occasional vortexing.
8. Prewash a 1PC tube (Hitachi Koki) with 100 mM Na_2CO_3, and transfer the membrane sample into it.
9. Harvest the plasma membranes by centrifugation at 245,000 × *g*, 4°C, for 30 min in an S150AT-0097 (No. 7) rotor (Sorvall).
10. After centrifugation and without resuspending it, wash the membrane pellet once with 500 mM Na_2CO_3; remove the supernatant. Repeat with the wash with 50 mM Na_2CO_3.
11. Estimate the protein concentration using the Bradford Assay.
12. Resuspend in three volumes of 6 M urea/2 M thiourea (pH 8.0).
13. Incubate for 30 min at RT, and then centrifuge for 10 min at 9,300 × *g* (Eppendorf 5415R) to pellet any insoluble material.
14. Add 1 μl of dithiothreitol (final concentration 1 mM) in water for every 50 μg of sample. Incubate for 30 min at RT.
15. Alkylate the sample with 5.5 mM iodoacetamide for 45 min in the dark, before adding 1 μg of endoproteinase Lys-C.
16. Digest the proteins for 4 h at RT then dilute in four volumes 10 mM Tris, pH 8.0.
17. Add 1 μg modified trypsin (sequencing grade) and digest overnight at RT.

18. Add trifluoroacetic acid (mass spec grade) to get a final concentration of 0.1% to quench the trypsin activity.
19. C18 StageTip: using a blunt tipped needle punch out a piece of C18 disc and transfer to a pipette tip.
20. Using a 1 ml plastic syringe, condition and then equilibrate C18-StageTips by pressing 10 μl methanol (mass spec grade) through at 50 μl/min, followed by 30 μl 0.5% acetic acid at 50 μl/min.
21. Load an appropriate volume of sample onto the StageTip and press through at 20 μl/min, and wash with 10 μl buffer 0.5% acetic acid at 50 μl/min.
22. Elute the sample with 10 μl 80% acetonitrile + 0.5% acetic acid at a rate of 10 μl/min, dry under a vacuum at 45°C to a volume of 1 μl, and dilute 1:8 with 80% acetonitrile + 0.5% acetic acid.
23. The sample is then ready to be loaded onto the analytical column of an MS.

3.3. Sample Preparation for Cytosolic and Nuclear/Membrane Fractions

1. Trypsinise the cells and wash 2× in ice-cold PBS. Aspirate dry. At this stage, pellets can be snap-frozen in liquid nitrogen and kept at −80 until needed.
2. Resuspend pellets of SILAC-labelled hESC in ice-cold modified RIPA (1% NP-40/0.1% deoxycholate/150 mM NaCl/1 mM EDTA/50 mM Tris pH 7.5) freshly supplemented with one complete protease inhibitor cocktail tablet and, if desired, two PhosSTOP phosphatase inhibitor tablets (both from Roche Diagnostics) per 50 ml.
3. Incubate on ice for 10 min and centrifuge the cell lysate at 16,000 × *g* for 10 min at 4°C. This divides the sample into a soluble fraction containing predominantly cytosolic proteins and an insoluble nuclear/membrane protein enriched fraction. These fractions can now be used for either in-gel digestion (see above 3.2 point 6 onwards) or in-solution digestion as described previously (24).

The SILAC method of labelling cells described above has shown that it can completely label hESC over a period of five passages, additionally; it has shown that this procedure does not interrupt the self renewal process necessary for the maintenance of hESC in their pluripotent state (4). Using differentiation of hESC in a non-lineage directed manner, we have been able to quantitatively compare membrane profiles of cells in their pluripotent state and cells undergoing differentiation and identify novel proteins differentially regulated between self-renewal and differentiation (25). These results demonstrate the power that proteomic platforms, such as SILAC labelling, have in identifying new and valuable markers which will assist in many aspects of research into the maintenance of self-renewal and enhancing lineage-specific differentiation.

4. Notes

1. 2-Mercaptoethanol was aliquoted and stored, neat, in 10 μl amounts at −20°C. Prior to use, it was diluted 1:10 in PBS^{2-} before adding 7 μl to 100 ml SILAC media for conditioning. It is considered a toxin by contact and inhalation and, additionally, has an unpleasant smell, so should be used within a hood and with care.
2. The concentration of hbFGF should be used according to routine methods established in the laboratory. hbFGF can be diluted in either HSA or BSA (concentrations between 0.1 and 0.2%) and should be sterile filtered, aliquoted, and stored at −20°C until needed.
3. Inactivation of feeders has been described in previous chapters (see Chapter 4, Harkness and Kassem). The amount needed to inactivate both MEFs and HDFn cells has been calculated and tested. If other feeders are considered, testing for optimal levels of gamma irradiation needs to be carried out.
4. Procedures for culture of hESC, before and during stable isotope incorporation, should be routinly established procedures used in the lab. However, culture needs to be on a matrix (such as Matrigel) to prevent interference from feeder layers during the identification of peptides.
5. Recent, unpublished data (Prokhorova and Blageov) have shown that dialysis of the medium post-conditioning is unnecessary as the amount of amino acids incorporated into the conditioned media during conditioning is minimal. The heavy amount of amino acids added is at an optimal concentration for full incorporation within five passages of hESC.
6. All buffers or solvents should be made or diluted in MilliQ water unless otherwise stated within the text.
7. A Dounce homogenizer is a glass tube with a tight fitting pestle. The number of strokes stated in the protocol has been calculated to completely disrupt hESC.
8. Post-centrifugation the supernatant contains the cytosolic fraction and the pellet contains the plasma membranes, endoplasmic reticulum, and Golgi.

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