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Substrates and supplements for hESCs: a critical review

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Abstract
Background Different laboratories around the world have succeeded in establishing human embryonic stem cell (hESC) lines. However, culture conditions vary considerably among the protocols used and the vast majority of the lines at some stage of their creation have been in contact with an animal derived component. One of the main problems to be overcome for the generation of a clinical-grade hESC line is the choice of a substrate and medium that allows derivation and culture, where animal derived components are kept to a minimum or completely excluded.
Materials and methods The following review describes past and more recent achievements in the creation and culturing of hESC. It describes protocols, giving special attention to the matrices and supplements used for derivation, mantainance and cryostorage, considering whether they included defined, undefined and/or animal-derived components in their formulations.
Conclusion This information shall be useful for the creation and choice of new substrates and supplements for future research in the field of hESC for therapeutic purposes.

Keywords Embryonic stem cells · hESC · Substrate · Supplements

Capsule This is a comprehensive review on the past and present methods employed for the creation of new embryonic stem cell lines giving special emphasis to the substrate and supplements used on their generation.

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Introduction

Human embryonic stem cells (hESC) were reported as permanent lines by Thomson and colleagues in 1998 [69]. Since these first derivation attempts, many hESC lines have been established around the world.

Different protocols for the creation of human embryonic stem cells lines for clinical use are being tested, with different degrees of success in terms of derivation, colony maintenance and genetic stability. Non-human animal derived and undefined components, particularly serum, are commonly used by different research groups as part of their derivation or culture protocols. On the other hand, there are studies aiming to completely eliminate non-human animal-derived and/or undefined components from their protocols. From now, the term "animal" will refers to non-human animals (also called *xeno* components).

The use of animal and/or undefined supplements may introduce undesired pathogens into the cells, thus creating a serious problem for human health. However, it is also possible that the exclusion of these components represents an alternative with high technical risks [10].

The present literature review aims to analize the protocols employed for the generation of hESC lines, giving special attention to the substrates and supplements used for their culture and maintenance. The study classifies the conditions described in the steps of derivation and culture of new hESC lines, considering whether they include an animal-derived component or not. In addition, we graded the protocols according to whether they included defined or undefined supplements.

The creation of new hESC lines

Removal of zona pellucida and ICM isolation

Fresh or cryopreserved surplus blastocysts, obtained from consenting donor parents are potentially the best source of

cells to establish new embryonic stem cell lines. Except when working with blastocysts that hatch spontaneously in culture, zona pellucida removal is a necessary step to expose the embryonic cells to the substrate. To this end, pronase was the first agent used to eliminate the zona pellucida [69] and it is still used by some laboratories around the world [70]. Subsequently, acid Tyrode's solution, became extensively used for the removal of the zona pellucida, because it avoids the contact of the embryo with an animal-derived product [33,60,67]. Mechanical manipulation using laser beams was proven to be an effective alternative for zona pellucida removal [38], but it requires a sophisticated set up, not easily available in a standard cell culture laboratory. For inner cell mass (ICM) isolation from the trophectoderm, immunosurgery using non-human antibodies and also non-human complement is widely used [55]. One feasible alternative to avoid the use of *xeno*-components is to manipulate the embryos with a pair of flexible metal [67] or insuline needles [58] to open the zona pellucida and isolate the ICM as much as possible from the trophectodermal cells. However, manual dissection demands good expertise and manipulation skills.

Substrates for hESC lines culture

A wide variety of natural and synthetic materials have been tested to create substrates or matrices that interact with embryonic stem cells. Biological materials typically contain sites for cellular adhesion, but there is a large variability of components in these substrates depending on their source and the method used for their isolation. Synthetic biomaterials should have a defined chemical composition and the ability to control mechanical properties, degradation rate and shape. However, the majority of the synthetic substrates tested so far have the disadvantage of their inherent lack of bioactivity, such as sites for cell adhesion [75].

Feeder cells

Mouse embryonic fibroblasts (MEF) were used on the first successful reports of human embryonic stem cells growing continuously and in an undifferentiated state. The hESC cultured under such conditions are exposed to several non-human proteins, which are immunogenic to humans. In replacing non-human feeder cells for culture, human fetal or skin fibroblasts, human adult fallopian tube epithelial cells, human placental fibroblasts, human endometrial cells, human adult marrow stromal cells, foreskin cells or fetal lung fibroblasts were all successfully used (see Table 1). Also, there are reports of good cell proliferation and maintenance of the undifferentiated state of hESC in human fibroblasts and a total animal-free culture system [20,46,54].

Furthermore, autogenic feeders derived from embryonic bodies created from hESCs, are a promising alternative. Culture of hESCs directly on autogenic feeders and conditioned medium obtained from these feeders has shown that hESCs maintain their pluripotent identity. These hESCs grown on autogenic feeders expressed the Oct4 marker at a similar fashion to hESC cultured in the presence of MEF and serum replacement [15].

Undefined extracellular matrices as substrates

The first feeder-free systems for existing hESC were cultures on Matrigel™. This is a complex mixture of several undefined and unknown components from mouse sarcoma, a tumor rich in extracellular matrix (ECM) proteins such as: laminin, collagen type IV, heparan sulfate, proteoglycan, entactin, and nidogen 1 [60,65]. Alternative undefined extracellular matrices have also been successfully tested, including those from mouse embryonic fibroblasts, human fibroblasts and human serum matrix [27,32,47].

Defined matrices as substrates

Fibronectin, laminin, and collagen can support feeder free hESC growth under defined conditions. However, efficacy varies among laboratories and some of these proteins may have disparities among lots [43]. Important to mention, in most instances, single protein matrices are used in the presence of mouse fibroblast conditioned medium and/or serum supplements. However, a combination of human collagen IV, vitronectin, laminin and fibronectin was successfully employed in a defined animal-free system to maintain hESCs growing in an undifferentiated state [44].

Polymers, proteins and peptides

More recent studies have focused on the use of polymers and copolymers, proteins and/or peptides, coating two-dimensional (2D) surfaces or as 3D scaffolds for the proliferation of hESCs. Scaffolds represent an ideal substrate for hESC culture, as they can be engineered with the desirable macro- and micro-architecture, for example, pore size and porosity [51]. Porous polymer scaffolds contain a network of channels and interconnected pores that facilitates mass transport of nutrients and metabolites leading to the formation of cellular associations, providing physical cues for cell orientation and spreading. It has already been demonstrated that hESCs differentiated within 3D bioengineered scaffolds are capable of generating complex tissue structures containing cells derived from all three somatic germ lineages [37,39].

Different extracellular matrix (ECM) proteins and signaling molecules combined in hundreds of culture conditions

Table 1 Reported data on the conditions of derivation and maintenance of hESC, focusing on the non-human components present or not in substrates and/or as supplements

Substrates for maintenance	Feeder-cell medium components	hESC medium supplements	Derivation/ Maintenance	References
MEF	SR	FBS/KOSR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[1]
MEF	FBS – KOSR	hrbFGF, b-mercaptoehanol	Dwac/Mwac	[66]
MEF	KOSR	MEF conditioned medium, bFGF	Mwac	[9]
MEF	FCS	hrbFGF, HA	Dwac/Mwac	[24]
MEF	SR	SR, bFGF, glutamine, NEAA, b-mercaptoehanol	Mwac	[4]
MEF	KOSR	KOSR, bFGF, insulin-transferrin-selenium, glutamine, NEAA, b-mercaptoehanol	Mwac	[18]
MEF	SR	SR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[79]
MEF	–	SR, glutamine, NEAA, 2-mercaptoethanal	Dwac/Mwac	[45]
MEF	–	KOSR, FBS, bFGF, LIF	Dwac/Mwac	[28]
MEF	FBS	FBS, LIF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[52]
MEF	–	KOSR, glutamine, NEAA, FGF	Dwac/Mwac	[11]
MEF	FBS	KOSR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[13]
Human foreskin fibroblasts	FBS/HS/SR	SR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[2]
Human foreskin fibroblasts	FCS	FCS, LIF, L glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[25]
Human foreskin fibroblasts	FCS	SR, bFGF, L glutamine, NEAA, b-mercaptoehanol, insulin-transferrin-selenium	Dwac/Mwac	[29]
Human foreskin fibroblasts	HS	HS, hrbFGF, glutamine, NEAA, b-mercaptoehanol	Dfac/Mfac	[20]
Human foreskin fibroblasts	FCS	KOSR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[67]
Human foreskin fibroblasts	–	KOSR, rhbFGF, glutamine, NEAA, b-mercaptoehanol	Mwac	[53]
Human foreskin fibroblasts	FBS	KOSR, glutamine, NEAA, rhbFGF	Dwac/Mwac	[17]
Human foreskin fibroblast	HS	Animal free medium: human insulin, transferrin, HSA, bFGF	Mfac	[46]
Human foreskin fibroblast	FCS	KOSR, bFGF, FCS, LIF	Dwac/Mwac	[26]
Human foreskin fibroblast		RegES xeno-free medium: HSA, bFGF, insulin, transferrin, selenium, activinA, thiamine,retinol	Mfac	[54]
Human dermal fibroblasts/Laminin	SR	HDF conditioned medium, SR, bFGF	Dwac/Mwac	[21]
Human dermal fibroblasts/Laminin	–	Serum free medium with human sourced and recombinant proteins, bFGF	Dfac/Mwac	[21]
Human placental fibroblast	KOSR	KOSR, bFGF	Dwac/Mwac	[22]
Adult skin fibroblasts	HS, FCS	FCS/KOSR, bFGF insulin-transferrin-selenium, glutamine, NEAA, b-mercaptoehanol	Mwac	[56]
Human fetal muscle fibroblasts	HS, FCS	FCS/KOSR, bFGF insulin-transferrin-selenium, glutamine, NEAA, b-mercaptoehanol	Mwac	[56]
Human fetal skin Fibroblasts	HS, FCS	FCS/KOSR, bFGF insulin-transferrin-selenium, glutamine, NEAA, b-mercaptoehanol	Mwac	[56]
hESC- derived fibroblasts	FCS	SR, bFGF, glutamine, NEAA, b-mercaptoehanol	Mwac	[64]
Human fetal lung fibroblast	SR	SR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[79]
Human embryonic fibroblasts	KOSR	KOSR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[42]
Transgenic human fetal fibroblast	FBS	KOSR, bFGF, insulin-transferrin selenium, glutamine, NEAA, b-mercaptoehanol	Mwac	[59]
Fetal lung fibroblasts	FBS	KOSR; bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[14]
Human embryonic fibroblasts	KOSR	KOSR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[80]
Human fetal fibroblast	FBS	KOSR, bFGF, glutamine, NEAA, b-mercaptoehanol	Dwac/Mwac	[34]
Matrigel	–	hESC- derived fibroblasts cond. medium, FCS	Mwac	[64]
Matrigel		MEF cond. medium, bFGF	Mwac	[9]
Matrigel	–	Bone morphogenetic proteins (BMP) antagonist “noggin”, glutamine, NEAA, KOSR, bFGF, b-mercaptoehanol	Mwac	[76]
Matrigel	–	Uncond. medium, KOSR, bFGF, glutamine, NEAA, 2-mercaptoehanol	Mwac	[40]
Matrigel	–	Defined medium, BSA, bFGF, glutamine, NEAA, 2-mercaptoehanol	Mwac	[77]
Matrigel	–		Mwac	[23]

Table 1 (continued)

Substrates for maintenance	Feeder-cell medium components	hESC medium supplements	Derivation/ Maintenance	References
		mTeSR1 medium: glutathione, NEAA, b-mercaptoehanol, insulin, transferrin, selenium, thiamine, bFGF,LiCl, gamma amino butyric acid, pipecolic acid, TGFb, HAS		
Matrigel	–	Mesenchymal stem cell- or Human foreskin fibroblast-conditioned medium, KOSR; bFGF, glutamine, NEAA, b-mercaptoehanol	Mwac	[48]
Matrigel	–	FBS, rLIF, glutamine, NEAA, b-mercaptoehanol, MEF cond. medium	Dwac/Mwac	[52]
ECM from MEFs	–	SR, plasmanate, LIF, bFGF, NEAA, glutamine, b-mercaptoehanol	Dwac/Mwac	[32]
ECM from human foreskin fibroblasts	–	ROCK inhibitor Y-27632, animal-free rhbFGF	Mfac	[47]
ECM from human foreskin fibroblasts	–	TeSR2 xeno-free medium, rhbFGF	Dfac/Mfac	[27]
Human collagen IV, vitronectin, laminin and fibronectin	–	TeSR1animal-free medium: insulin, transferrin, selenium, thiamine, bFGF,LiCl, gamma amino butyric acid, pipecolicacid, TGFb, HAS	Dwac/Mfac	[44]
Human collagen IV, vitronectin, laminin and fibronectin	–	SBX defined medium: synthetic lipid, iron carriers, insulin, bFGF, TGFb	Mwac	[50]
Human serum matrix		hES-derived fibroblasts–conditioned media, SR, bFGF, insulin-transferrin-selenium, glutamine, NEAA, b-mercaptoehanol	Mwac	[65]
Fibronectin	–	MEF cond. medium, bFGF	Mwac	[9]
Fibronectin	–	HESCO medium (Wnt3a, bFGF, insulin, transferrin, April-BAFF, cholesterol, albumin), KOSR	Mwac	[43]
Laminin	–	MEF cond. medium, FCS, NANOG over-expression	Mwac	[18]
Gelatin	–	MEF cond. medium, FCS, NANOG over-expression	Mwac	[18]

A proliferation-inducing ligand — B cell-activating factor belonging to TNF (April-BAFF); Basic fibroblast growth factor (bFGF); bovine serum albumin (BSA); bovine-sourced serum replacement medium (bSRM); chemically defined medium (CDM); conditioned medium (CM); derivation free of animal components.(Dfac); derivation with animal component (Dwac); extracellular matrix (ECM); fetal bovine serum (FBS); fetal calf serum (FCS); homeodomain transcription factor (NANOG); human dermal fibroblast (HDF); human embryonic fibroblasts (HEFs); human recombinant fibroblast growth factor (hrbFGF); human serum (HS); human serum albumin (HSA); hyaluronic acid (HA); knockout serum replacement (KOSR); leukemia inhibitor factor (LIF); maintenance free of animal components (Mfac); maintenance with animal component (Mwac); mesenchymal stem cells (MSC); mouse embryonic fibroblast (MEF); nonessential amino acids (NEAA); serum-free contained only human sourced and recombinant proteins (SFM); serum replacement (SR); transforming growth factor (TGFb)

were analyzed in an extensive work, which used an array-based technology to screen for the maintenance and proliferation of hESC [7]. This study showed that a successful defined culture system required a combination of collagen I, collagen IV, fibronectin, and laminin for hESC growth and proliferation. However, the use of purified or recombinant proteins is expensive and this fact limits their use for large scale hESC production.

Compared to isolated ECM proteins, polymer biomaterials are inexpensive, easily manufactured and represent a reliable alternative for in vitro human pluripotent stem cells (hPSC) expansion [8]. Porous polymer membranes as substrates for hESCs have advantages over conventional cultures, in that diffusible factors exchange between hESCs and feeder cells occurs without direct cell-to-cell contact. Lee and colleagues [36] found that the polyethylene terephthalate (PET) membranes of a defined pore densitie (and hardness) can provide a favorable environment, that allows good adherence, renewal and survival of undifferentiated hESCs. Also, when this membrane is used together with conditioned medium, in the absence of feeders cells, hESCs were cultured successfully for long periods. Reduced apoptosis, reduced spontaneous differentiation and enhanced homogeneity of undifferentiated cells appeared to be distinct features of the hESCs cultured on the PET membrane. Another synthetic polymer, poly (methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) showed good support for the long-term attachment and maintenance of hESC under feeder-free conditions [8]. Finally, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH), was found to sustain long-term growth of hESCs in several culture media, including commercially available defined media, without inducing differentiation [74]. The search for the ideal "stem-cell-friendly" polymer(s) that support growth and self-renewal of the hESC lines, free from exogenous adhesion molecules, is only beginning to be explored.

Individual ECM proteins have also been tested as substrates for hES cell self-renewal [9,18,43]. However, each polypeptide can connect a variety of cell-surface receptors, making it difficult to identify the key interaction molecule or active site that results in successful cell proliferation. Recently, a fusion protein consisting of the E-cadherin extracellular domain and the IgG Fc domain, called a recombinant hE-cad-Fc, was used to coat culture dishes for hESC proliferation. This substrate showed good cell expansion and maintenance of hESC in a pluripotent state, under highly defined culture conditions [49].

One major study screened over 500 unique surfaces based on 18 bioactive peptides to identify the peptide surfaces that sustained established pluripotent stem cell lines self-renewal [31]. Results revealed that surfaces presenting heparin-binding peptides (HBPs) derived from vitronectin, when coupled with the Rho kinase inhibitor Y-27632 (ROCK inhibitor), effectively supported hES cell adhesion and propagation, even when used with a defined medium. Also, cell adhesion and propagation were successfully observed, when defined HBPs were incorporated on streptavidin-coated surfaces for hESC and induced pluripotent stem cells (iPSC) cultures. The surfaces displaying HBPs promoted cell adhesion and spreading by interacting with hES cell-surface glycosaminoglycans [31]. This study further corroborates recent data suggesting that some peptides containing the core integrin-binding motif, (arginine–glycine–aspartic acid, RGD), support undifferentiated hESC growth in serum-free medium [19]. Synthetic peptide interfaces seem to represent the most promising systems for cell adhesion and proliferation. Unfortunately, the cost associated with these substrates is significant, potentially limiting their use in the large scale manufacturing industry [30]. More recently, initial attachment, survival and cloning of hESCs was successfully achieved using a vitronectin variant relatively easy to overexpress and purify (VTN-NC) together with a ROCK inhibitor or blebbistatin, in a simplified versión of mTESR™1 defined medium [16]. In a similar way [78], the successful propagation of hESCs for more than 20 passages was achieved on tissue culture-treated polystyrene plates, coated with vitronectin obtained from human plasma-purified. Interesting to point out that this study revealed a threshold surface density of vitronectin of 250 ng/cm^2 for hESCs attachment, proliferation, and differentiation. Also, on surface densities above this threshold, cell attachment and proliferation assays demonstrated that vitronectin substrate properties are equally viable.

Hydrogel scaffolds are water-swollen polymers that may be fabricated from natural ECM components or synthetic materials. Recent data showed successful culture of hESC lines for over 20 passages in chemically-defined media, on hydrogel interfaces of aminopropylmethacrylamide (APMAAm). However, more careful observations indicated that BSA, an animal derived protein source, in the mTESR™1 medium present in the experiments was the key component responsible for hESC adhesion on APMAAm hydrogel interfaces [30].

Using a different approach, Lim and colleagues [41] developed multifunctional hydrogel-based matrices that can simultaneously provide structural support, as well as electrical and mechanical cues to the embedded cells. Electrical and mechanical cues are important during embryogenesis, differentiation, and tissue repair. The anionic hydrogel developed by the authors is capable of bending in both directions in an electric field, by manipulating its crosslink density providing cells the mechanical signaling they need to proliferate. Bioreactors are devices that provide control over multiple molecular and physical regulatory signals within sophisticated 3D culture environments [12]. The incorporation of biophysical cues by integrating biomaterials that provide structural support to the cells cultured in bioreactors is another promising strategy to be explored for the efficient proliferation and maintenance of hESC in a pluripotent state [41].

Much work has been done and significant successes have been achieved for the culture of undifferentiated hESC lines and maintenance of their pluripotent status under defined conditions. However, it remains to be proved whether the reported defined culture conditions will be broadly repeatable and applicable not only for the maintenance of existing hESC lines, but also for derivation of new colonies, a key step in producing clinical grade hESC lines. As can be observed on Table 1, derivation has been performed on feeder free systems using ECM proteins as matrices [32,44], also on human neonatal foreskin fibroblasts [27], and on Matrigel™ [52]. Recently, vitronectin-coated surfaces were successfully employed to derive hESC and iPSC cells [16]. As another possibility, fibronectin alone was used as substrate for derivation of bovine partenogeneticaly generated ESC using a commercially available defined medium (Stem Pro®) [58]. Alternatively, a culture system was created that supports derivation of the hESC lines as floating clusters in suspension [63]. However this suspension culture system did not allow clonal expansion, which is a major limitation for its widespread use.

Supplements for the maintenance of hESC lines

Undefined supplements

Serum represents a complex mixture of undefined elements that has extensively been used in hESC culture as protein source. Fetal calf serum (FCS, FBS) was the first supplement used in cultures due to its strong hESC growth-promoting capacity followed by cheaper alternatives as new-born, calf, adult, donor calf or donor adult cattle serum

[10]. Also, as variants, serum replacement (SR), knockout serum replacement (KoSR) and synthetic serum supplement (SSS) have been used. One limitation for the intensive use of the sera is that batches may vary considerably in their capacity to maintain hESC in an undifferentiated state in culture [60]. However, as pointed out by Brindley and colleagues [10], for clinical therapies the human or animal derived serum must be obtained under suitable International Standards Organization-(ISO) -grade environments, that guarantee its safety for human use. In addition, three countries only, USA, Australia and New Zealand provide more than 90 % of the serum used in the commercial manufacturing of therapeutics [10]. This situation strongly limits the worldwide use of serum in experimental and clinical trials and drives scientists to investigate alternative supplements that comply with clinical standards for therapeutic applications.

Bovine serum albumin (BSA) and human serum albumin (HSA) have been given key-roles in tissue culture systems, from lipid carrier to physical protection against sheer forces. However, the work by Chen and colleagues [16] demonstrated that the removal of BSA from the medium TeSR used to derive human ESCs, leads to cyto-toxicity by β-mercaptoethanol (BME). On the other hand, in the absence of BME, BSA is no longer necessary for human ESC or iPSC culture. The simplified TeSR medium supported undifferentiated proliferation of both human ESCs and iPSCs at rates similar to those achieved, when the original mTeSR medium was employed. Important to point out, the cell lines grown in this simplified medium expressed pluripotency markers and normal karyotypes for over 25 passages and for more than 3 months.

Defined supplements

One major progress towards developing a defined system for hESC propagation came from the ability to grow hESC under serum-free conditions. For this purpose, different combinations of growth factors like basic fibroblast growth factor (bFGF), transforming growth factor (TGF), activin A, Nodal, Noggin, leukemia inhibitor factor (LIF), insulin growth factor (IGF), and glutamine and ß-mercaptoehanol, as an antioxidant, were added to the culture medium (see Table 1).

Basic fibroblast growth factor appears to play a key role in sustaining hESC self-renewal [71], and it is routinely included in most reported media formulations for hESC derivation and proliferation. High concentrations of bFGF in the medium have successfully allowed the long term maintenance of a few existing hESC lines in the absence of fibroblast feeder layers or fibroblast-conditioned medium [40]. Basic fibroblast growth factor is usually supplied as a recombinant protein derived from bacterial source, apart from one supplier (Millipore©) that produces a basic, human animal-free recombinant bFGF, which makes it expensive and limits its widespread use [47].

Bone morphogenetic protein (BMP), another growth factor commonly added to hESc cultures, acts in combination with other growth factors to induce differentiation rather than to maintain pluripotency [68,72]. Suppression of BMP appears to contribute for hESC growth in culture. Noggin, a BMP antagonist can synergize with bFGF and sustaining undifferentiated hESC proliferation in the absence of feeder cells and conditioned medium [76].

Inhibition of Activin/Nodal signalling on hESC cultured in the absence of feeders cells, using a chemically defined medium, systematically resulted in the loss of pluripotency markers including Oct-4 [72]. The combination of activin, nodal and TGF-β have been shown to be necessary components of the culture medium for the maintenance of pluripotent hESCs [5]. When using mouse embryonic fibroblasts-conditioned medium (MEF-CM; [6]), TGF-β and insulin growth factor II (IGF-II) seem to synergise with FGF pathway to maintain feeder-free culture homeostasis for hESCs. More interesting, hESCs cultured in conditioned medium from human mesenchymal stem cells (MSC-CM), without IGF-II are capable of maintaining their pluripotency and culture homeostasis [48].

A thorough analysis of published hESC derivation protocols, in which the cell lines have maintained pluripotency over long term culture conditions (over 100 passages), indicated that the vast majority of the protocols included LIF in the medium, though the role of LIF is not yet clear [28].

Inhibitors of the FGF/MEK/Erk and GSK3β pathways, when added to the culture system can modulate signaling pathways involved in the pluripotency of human blastomeres. Embryo culture in the presence of these inhibitors demonstrated a high rate of OCT3/4-and NANOG-positive cells in their ICMs. NANOG is, together with OCT3/4, is one of the most important pluripotency markers for subsequent stem cell derivation purposes. However, the manipulation of cell fate during development did not improve the capacity of these embryos to derive hESCs [73]. In addition the only hESC line created from a treated blastocyst presented a low survival rate and self-renewal capacity after passaging. Despite this new information on cell lineage segregation, there is still an enormous lack of knowledge on pathways involved in the process of derivation and creation of a new hESC line and the factors affecting them. Advances on these topics may be greatly benefited from studies performed on fully-defined culture conditions.

hESCs culture conditions and genetic stability

Just as important as their safety in terms of pathogens transmission and control of their differentiation potential,

the chromosomal and genetic stability of hESC in culture is crucial for their future clinical application. Once established, hESC lines are expected to be chromosomally stable, even after cryopreservation for subsequent culture and passages. Human ESCs may demonstrate chromosomal instability in culture, often marked by translocations and aneuploidies, in addition to other subchromosomal changes or genetic alterations that may have adverse effects and compromise hESCs populations' stability [3,62]. Small genetic alterations, also potentially deleterious for the homeostasis of the cell line request higher resolution technologies, such as the single-nucleotide polymorphism (SNP) analysis. One study employing such technology demonstrated that hESC lines have a higher frequency of regions of duplication in their genome and these aberrations were not related to the number of passages the cells have been submitted [35].

The growing possibilities of developing cellular therapies based on hESC demand cells with defined quality characteristics and safe to use in patients. The above observations highlight the need for optimization of derivation and culture conditions that should also promote genetic stability of the pluripotent stem cells populations [57]. The necessity of frequent genomic monitoring in hESC both, in the pluripotent state and when the cells are subjected to differentiation procedures is paramount to assure phenotypic stability and clinical safety [35].

Reported data

In Table 1, we present a comprehensive updated literature survey on the conditions of derivation and maintenance of hESC, giving special attention to the presence or not, of non-human components in substrates and serum as supplements. For this purpose, data was collated on a chart taking into consideration only reports, in which the steps of derivation, colony formation and proliferation were detailed described. Data are presented in an updated fashion, similar to the two thorough reviews previously reported on this subject [60,61].

Concluding remarks and future perspective

The review shows that fibroblast co-culture, conditioned media from fibroblasts, or media containing cytokines in combination with different matrices, nanomaterials, or in suspension cultures have all been tested and shown their advantages and disadvantages on the creation and proliferation of new hESC lines. At the current state of the art, it may be fair to consider that no single protocol has been proven to be optimal for derivation, proliferation and large-scale production of hESC lines for clinical use [36].

A close observation on the different protocols suggest that several factors act together or in isolation leading to the presence of one or more animal derived or undefined product in the creation of the existing hESC lines. The addition of these components was originally paramount for the successful establishment and maintenance of hES cell lines. One of the main focus of the current and future research in the field of hESC for therapeutic purposes shall gain insight into protocols, in which non-human animal products are excluded or created under strict ISO –grade conditions. In this regard, we believe present research moves forward in two different directions; in one way, with the humanized substrates, fibroblasts or matrices of human origin. In the other perspective, chemically defined substrates that contain the essential cell-interaction sites necessary for cell survival and proliferation may represent one major advance towards the creation of clinical grade hESC lines. In any instance, there shall be a strict and periodic assessment on whether the newly created hESC lines are of the highest quality to be stored and clinically used. Cells must be capable of undergoing multi-layer differentiation, never lose their proliferative capacity and express specific cell surface markers such as SSEA-4, SSEA-3 and transcription factors such as TRA-1–60, TRA-1–81, Oct-4, Sox-2 and Nanog. Last but not least, new human embryonic stem cell lines must maintain their genetic stability and keep high telomerase and alkaline phosphatase enzyme activities along passages and after cryopreservation.

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