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Embryonic Stem Cells: Isolation, Characterization and Culture

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Abstract Embryonic stem cells are pluripotent cells isolated from the mammalian blastocyst. Traditionally, these cells have been derived and cultured with mouse embryonic fibroblast (MEF) supportive layers, which allow their continuous growth in an undifferentiated state. However, for any future industrial or clinical application hESCs should be cultured in reproducible, defined, and xeno-free culture system, where exposure to animal pathogens is prevented. From their derivation in 1998 the methods for culturing hESCs were significantly improved. This chapter wills discuss hESC characterization and the basic methods for their derivation and maintenance.

Keywords Blastocyst, Embryoid body, Embryonic stem cell, Immunosurgery, Teratoma

Contents

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1 Introduction

Embryonic stem cells (ESCs) constitute a unique type of stem cells derived from the inner cell mass (ICM) of the mammalian blastocyst. ESCs differ from their adult counterpart by their distinctive potential to differentiate into every cell type of the adult body. Several items of evidence were presented for ESCs pluripotency: (1) when transferred into suspension culture in vitro, ESCs form cell aggregates known as embryoid bodies (EBs), with regions differentiate into embryonically-distinct cell types [17, 30]; (2) injection of ESCs into the hind limb of severe combined immunodeficient (SCID) mice induces the formation of teratomas which may include tissues representative for all three germ layers [70, 66]; (3) mouse ESCs were shown to contribute to chimeras and particularly to the germ cell line [7]; and finally, (4) several murine ESC lines were demonstrated to form entire viable fetuses [46]. Since the first derivation of ESCs in 1981 from mouse blastocysts [21, 40], mouse ESCs were induced to differentiate in vitro into haematopoietic stem cell-like cells [47, 31], neural precursors [8, 9], cardiomyocytes [33], endothelial cells [26, 71] and insulin-secreting cells [55, 38]. Thus the ability of these cells to differentiate into representative cell types of the three embryonic germ layers was proven.

Since their initial derivation from mice [21, 40], ESC lines or ESC-like lines have been derived from other rodents [18, 22, 23], domestic animal species [49, 53, 43], and from three non-human primates [64, 65, 56, 42]. However, only mouse ESCs demonstrate the entire set of features typical of ESCs, rendering them the most potent research model amongst other existing ESC lines.

The first step toward isolating human ESCs (hESCs) was achieved by Bongso and colleagues who described for the first time the ability to isolate ICM cells from human blastocysts and to culture them with inactivated mouse embryonic fibroblasts (MEFs) for two passages while expressing alkaline phosphate activity and demonstrating ESC-like morphology [10]. In 1998, the first hESC lines were derived by Thomson and colleagues [66]. Accumulating knowledge shows that hESCs meet most of the criteria described for mouse ESCs.

The exceptional differentiation potential of ESCs underlines them as one of the best models to study early human development, lineage commitment and differentiation processes; hopefully, in future they could also be used for cell-based therapy. Recently, a new source for pluripotent cells was proposed by Yamanaka et al., who succeeded in reprogramming mouse somatic cells and, later on, human somatic cells, to ESC-like cells [62, 63]. As their report states, an overexpression of four transcription factors, c-Myc, Oct4, Flf4 and Sox2, caused by retroviral infection, was sufficient to reprogram somatic cells [62, 41, 75]. These induced pluripotent stem (iPS) cells expressed typical ESC markers, formed the same colony morphology and were able to differentiate into representative tissues of the three embryonic germ layers both in vitro and in vivo. Later on it was shown that reprogramming of somatic cells could be obtained, albeit with lower efficiency, when oncogene C-Myc was replaced and Oct4, Sox2, Nanog and Lin28 were used [76, 45].

iPS cells were already derived from embryonic fibroblasts [62, 41], hESC-derived fibroblasts [59], fetal fibroblasts [59, 76], foreskin fibroblasts [59, 76], adult skin [25,37] and adult liver and stomach cells [4]. Future studies will reveal which culture and differentiation protocols developed for hESCs will suit these cells as well.

2 Methods for Isolating Escs

2.1 Source for Embryos

For the derivation of hESC lines, human embryos from in vitro fertilization (IVF) programs and embryos produced for research purposes [34] were used. These include surplus, apparently normal, embryos [2, 14, 57, 66], or low-grade or abnormally fertilized oocytes that were disqualified for clinical uses [35, 61, 77]. Some of the embryos are genetically-abnormal embryos after pre-implantation genetic diagnosis (PGD) that would otherwise have been discarded [68, 39]. In these studies, hESC lines harboring specific genetic diseases were derived, demonstrating all hESC characteristics.

Alongside the traditional sources of embryos for the isolation of ESC lines, other optional sources were also suggested; parthenogenetic embryos resulting from activated oocytes, or single blastomers isolated from developing embryos using similar methods to those used for PGD (allowing using the donor embryo for reproductive purposes). Vrana and colleagues demonstrated that an activated oocyte of a non-human primate can be used successfully for the derivation of ESC lines that exhibit all ESC features [67], though the extent of their differentiability is unknown. Mouse ESC lines were successfully derived from a single blastomer [13], using a technique in which a single blastomer is mixed with an already-established cell line, expansion of the newly derived line takes place and isolation is carried out by a selective tag. Both techniques have not yet been applied to human embryos.

Due to the progress in assisted reproductive medicine techniques, more embryos are currently available for hESC line derivation. It is estimated that over 500 hESC lines are obtainable for research worldwide [60]. This number indicates that the derivation of these lines is a reproducible procedure. The use of embryos for research, however, raised ethical concerns that were addressed by the publication of specific guidelines for the use of embryos for hESC studies [15].

2.2 Extraction of ICM

hESC lines are derived using the techniques developed in the 1970s for embryonal carcinoma (EC) cell lines and in the 1980s for mouse ESC line derivation.

Two principle methods can be used to isolate ICM cells from the blastocyst, namely immunosurgical and mechanical isolation.

Immunosurgical isolation is a simple method developed by Solter and Knowles [54], which aims to remove selectively the trophoectoderm layer of the blastocyst, leaving an isolated and intact ICM. A potential drawback of this method is the exposure of the embryo to anti-human whole serum antibodies, which normally attach to any human cell.However, penetration of the antibodies into the blastocyst is prevented due to cell–cell connections within the outer layer of the trophoblast, thus leaving the ICM cells unharmed. This is followed by incubation with guinea pig complement-containing medium which lysises all antibody-marked cells. The intact ICM is further rinsed and cultured with mitotically inactivated MEFs or an alternative feeder-layer that is known to support hESCs culture.

Alternatively, ICM cells can be isolated by selective and mechanical removal of the trophoectoderm layer under a stereoscope. After the embryo is released from the zona pellucida, the trophoblast layer is gently removed using 27 G needles or pulled Pasteur pipettes. Similarly to using the immunosurgery method, the isolated ICM cells should be further expanded using a suitable supportive layer.

2.3 Plating Intact Embryos Whole

ESCs lines can be derived simply by plating a whole zona-free embryo with mitotically inactivated MEFs or another suitable feeder-layer. The exposed embryo attaches to the feeder layer which, in return, permits the continuous growth of the ICM with the surrounding trophoblasts as monolayer. When the ICM reaches sufficient size it is selectively removed using mechanical methods and further propagated. Although simple, this method bears the risk of ICM differentiation, and the success rates tend to be lower as compares to the initial selective removal of the ICM.

2.4 Esc Characterization

Because of their uniqueness, much effort was invested in characterizing ESC cells. The first to be derived, i.e., mouse ESCs, are the most characterized ESCs, and therefore their list of features is used as a golden standard for other types of ESCs. The complete list of features is listed in Table 1.

When cultured in suitable conditions, ESCs are capable of prolonged undifferentiated proliferation. During culture, the cells create uniform colonies exhibiting high nucleus-to-cytoplasm ratio, two or more nucleoli, and typical spaces between the cells.

ESCs exhibit and maintain normal diploid karyotype even after prolonged culture [1]. Incidences of karyotypic instability are uncommon [1, 20, 19, 14], suggesting that those observed represent random changes which often occur in cell culture.

Table 1 List of ESC characteristics

Derived from the ICM of pre-implantation embryo, at the blastocyst stage
Capable of prolonged undifferentiated proliferation in culture
Exhibit and maintain normal diploid karyotype
Pluripotent
Able to integrate into all fetal tissues during embryonic development following injection into the blastocyst, including the germ layer (For obvious ethical reasons, the ability to examine how hESCs integrate into fetal tissues during embryonic development is restricted)
Clonogenic, i.e., each single ESC possesses all other features
Express high levels of *OCT 4* and Nanog, transcription factors known to be involved in the process of ESCs self maintenance
Can be induced to differentiate after continuous culture in an undifferentiated state
Remain in the S phase of the cell cycle for the majority of their lifespan
Do not show X chromosome inactivation

ESCs had been shown to be pluripotent, both in vitro and in vivo by EB formation [17, 30] and teratoma formation [70, 66], respectively.

ESCs express surface markers specific to the undifferentiation stage. While mouse ESCs strongly express surface marker stage-specific embryonic antigen-1 (SSEA-1), and do not express SSEA3, SSEA4, tumor recognition antigen-60 (TRA-1-60) and TRA-1-81, non-human primate ESCs and hESCs strongly express SSEA-4, TRA-1-60, and TRA-1-81, weakly express SSEA-3 and do not express SSEA1 at all [66, 57]. ESCs also express some specific genes, the most recognized is Oct 4, a transcription factor known to be involved in the process of ESC self maintenance [48]. Another transcription factor, Nanog, was recognized as having a role in the cells' renewal and is often used to define undifferentiated ESCs [11, 44]. Additional genes were found to be strongly expressed in hESCs and mESCs and were collected into a set of markers that identify undifferentiated ESCs [6].

Mouse ESCs remain in the S phase of the cell cycle for the majority of their lifespan; HESCs, like mouse ESCs, do not exhibit X inactivation. While maintained at the undifferentiated stage, both X chromosomes are active and, upon differentiation, one chromosome undergoes inactivation [16]. Recently, additional support to this finding was reported; however, it was also found that some hESC lines vary in their X-inactivation status [27, 24, 51, 52]. This may be indicative of a different and later source for some of the lines rather than the ICM, such as the epiblast stage.

As with other cell lines, single human ES cells possess all other features of the tested line, and their clonallity was demonstrated [1].

3 Methods for Hesc Culture

3.1 Defined Culture System

Any future exploitation of hESCs for clinical and industrial purposes will require a reproducible, well-defined, and animal-free culture system for their routine culture.

The traditional culture and isolation methods for hESCs, however, include inactivated MEFs as feeder layers and medium supplemented with high percentage of fetal bovine serum (FBS) [66]. The feeder layer plays a dual role of supporting ESC proliferation and preventing their spontaneous differentiation. In order to prevent any exposure of the cells to animal photogenes, hESCs must be cultured with medium supplemented with serum replacement, with no animal product, and the MEFs should be replaced by human feeder or with a cellular matrix, such as fibronectin, or laminin. A few steps toward meeting these requirements have already been achieved.

The simplest alternative to the culture method based on the use of MEF and FBS is the use of human supportive layer and medium supplemented with either human serum or serum replacement.Several cell types were found suitable to support undifferentiated hESCs, including human fetal-derived fibroblasts [58], foreskin fibroblasts [3, 28], and adult marrow cells [12]. Human fetal-derived fibroblasts and foreskin fibroblasts were also found to support the isolation of new hESC lines in animal-free or serum-free conditions [58, 28, 29].

Although these culture systems move us closer to the desired goal of animal-free conditions, they cannot be regarded as well-defined. The need to culture the feeder lines themselves, which will limit the large-scale culture of hESCs, the differences between batches of feeder-layer cells and the use of human serum rule this system out as defined. The ideal culture method would therefore be a combination of an animal-free matrix and both serum and animal-free medium. In 2001, Xu and colleagues made a significant advance in this respect: their newly culture method relied on Matrigel, laminin or fibronectin as matrix and 100% MEF-conditioned medium, supplemented with serum replacement [72].When cultured in these conditions, hESCs can be stably maintained for over a year and still exhibit their ESC characteristics. However, this method still holds the disadvantages of exposure to animal pathogens through the MEF-conditioned medium or Matrigel matrix, possible variations between batches of MEFs used for the production of the conditioned medium and the needs for simultaneous culture of both the feeders and the hESCs.

Indeed, the same group proposed an improvement to this culture system, where the MEF-conditioned medium was removed by supplementing the medium with 40 ng mL^{-1} basic fibroblast growth factor (bFGF) and 75 ng mL^{-1} Flt-3 ligand [73].

Extensive work has been carried out to improve further the feeder-layer free culture system of hESCs. As a result, several agents were reported to support undifferentiated hESC cultures in feeder layer- free conditions. Amongst them the combination of TGF_{b1} and bFGF [78], activin [5], high concentration of Noggin [74], high concentration of bFGF [69, 74], Bio [50], and a blend of five factors used in defined culture media [36]. It is therefore reasonable to assume that more than one pathway is involved in maintaining hESC potency. Further study is required in order to clarify the mechanism underlying these factors' involvement in hESC self-maintenance.

The majority of the existing hESC lines were derived with feeder layers [66, 57, 2, 14].The first report of a feeder layer-free derivation of a hESC line was reported by Klimanskaya and colleagues, in which MEF-produced matrix and

medium supplemented with a high dose of bFGF (16 ng mL^{-1}), LIF, serum replacement and plasmanate were used [32]. In this study, six new hESC lines were successfully derived, exhibiting ESC features after prolonged culture of over 30 passages. This pioneering work proves the feasibility of a supportive feeder layer-less derivation of hESCs, although the culture system includes some non-defined materials. A recent publication by Ludwig and colleagues reported the derivation of two new hESC lines using a defined serum- and animal-free medium, and feeder layer-free culture conditions [36].The matrix consisted of a mixture of human collagen, fibronectin and laminin, and the medium was supplemented with five growth factors, including TGF_{b1} and bFGF. The newly derived cells sustained most hESC features after several months of continuous culture. Thus, for the first time, defined, animal-, serum- and feeder-free culture conditions for hESCs are presented. However, the two new hESC lines were reported to harbor karyotype abnormalities; one 47, XXY after 4 months of continuous culture and the second exhibited trisomy 12 after 7 months of continuous culture. It is unknown whether the embryos were originally defected or whether these events of karyotype abnormalities occurred during prolonged culture.

3.2 Suspended Culture System

Culture of hESCs requires meticulous care which includes daily medium change, routine passaging every 4–6 days, and occasionally mechanical removal of differentiated colonies from the culture. Although hESCs can be cultured in these conditions in large quantities, the use of hESCs for therapy and for industrial applications requires a scalable and controlled culture system for both differentiated and undifferentiated hESCs. To this end we recently developed a novel suspension culture system for undifferentiated hESCs. The new three dimensional (3D) culture system is based on medium supplemented with 15% serum replacement, cytokines and bFGF. Four cell lines, H9.2, I3, I4 and I6, were cultured in suspension in Petri dishes where they spontaneously formed spheroid clumps. Cells cultured in this system for over a year, maintained all ESC features, including expression of specific markers, stable karyotype, and the developmental potential to differentiate into representative tissues of the three embryonic germ layers in vitro and in vivo. The calculated cell doubling time was 35.2 ± 1.3 h, similarly to a previous report on hESCs in 2D cultures [1]. Correspondingly, the cultures were split every 5–7 days – the same splitting interval of cells cultured with MEFs.

One month after being transferred into a stirred dynamic culture using either shaking Erlenmeyer's or spinner flasks, the spheroid clumps formed by the cells remained similar to those observed within cells cultured statically using Petri dishes. hESCs cultured for 3 months in the dynamic system maintained stable karyotype, were strongly positive for undifferentiation markers, and remained pluripotent. During 10 days of culture in the dynamic culture cell number increased 25-fold. Thus the novel culture system reported here makes it possible to expand

undifferentiated hESCs in suspension cultures which will facilitates the large-scale culture of hESCs needed in the clinic and industry.

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