

# Pluripotent stem cells and mitochondrial dysfunction

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## Abstract

Embryonic stem cells (ESCs) are derived from the inner-cell-mass (ICM) of blastocysts. ESC and induced pluripotent stem cell (iPSC) lines are immortal, meaning that they have unlimited proliferation potential. They are also pluripotent; that is, they can differentiate into lineages derived from all 3 major germ layers of the embryo. Consequently, these cells have been widely investigated in the development of regenerative medicine therapies. Human ESCs have been regarded as important research tools for the investigation into early development of the human embryo.

Mitochondria are the powerhouses capable of providing the majority of energy within the cell and performing important metabolic functions such as the Krebs cycle. The well-known endosymbiotic theory [1] has suggested that the mitochondrion was originally derived from a prokaryotic cell that invaded a larger, nucleated host cell. Mitochondria indeed contain their own mitochondrial DNA (mtDNA) in a circular form, similar to the bacterial genome. Mitochondrial genomes encode several essential genes of the eukaryotic respiratory machinery. However, most of the components of the respiratory machinery and factors controlling mitochondrial biogenesis are encoded in the nucleus. The cooperation and communication between mitochondria and nuclei are conducted by retrograde signals, such as energy supply and redox signaling and this currently poorly-understood communication is essential for balancing energy production and demand in the cell. Targeting mitochondria metabolism for inherited disease by using pluripotent stem cells is still a major therapeutic direction for cell therapy.

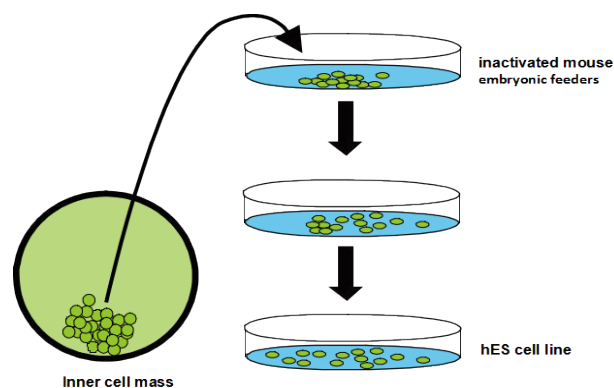
## Human pluripotent stem cells

Stem cells are endowed with a unique capacity for self-renewal [2]. In mammalian development, fertilized oocytes are able to differentiate into all types of cells and are thus classified as totipotent. ‘Totipotent’ is derived from the Latin totus, which means ‘entire’. A totipotent stem cell has the ability to generate all cell types found in the embryo and in extra-embryonic tissues. Fertilized oocytes divide and progress into 8-cell embryos and then blastocysts. Blastocysts are composed of an outer layer of cells, called trophoblasts, and inner cells, which form the inner cell mass (ICM). The ICM can develop into all cell types of the adult body and is therefore called ‘pluripotent’, a term derived from the Latin plures, meaning ‘several’ or ‘many’. Pluripotent stem cells, such as mouse embryonic stem cells (mESCs), human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs), can differentiate into ectoderm, mesoderm, endoderm, and germ cells [3].

Once blastocyst-derived cells have fully differentiated into tissues or organs, stem cells that reside in various tissues and organs remain in order to generate new tissue or repair damaged tissue. These stem cells, known as multipotent stem cells or adult stem cells (ASCs), include mesenchymal stem (or stromal) cells (MSCs) and hematopoietic stem cells (HSCs). Their differentiation ability is limited compared to that of totipotent and pluripotent cells. For example, hematopoietic stem cells, which are found in bone marrow, can differentiate into erythrocytes and white blood cells (including macrophages). These types of cells are important for homeostasis because they enable the steady self-renewal of tissue.

There are different types of pluripotent stem cells: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), embryonic germ cells (EGCs), and embryonic carcinoma cells (ECCs). ESCs are isolated from the ICM of the blastocyst (Figure 1) [4], whereas iPSCs are

artificially generated by reprogramming somatic cells using a defined set of transcription factors. iPSCs are similar to hESCs in morphology, gene expression, and differentiation ability [5]. Human embryonic germ cells (hEGCs) are derived from the primordial germ cells (PGCs) and are pluripotent stem cells [6]. Embryonic carcinoma cells (ECCs)



**Figure 1.** hESC isolation and culture procedures. hESCs are derived from the inner cell mass of the blastocyst (left). For long-term culture, hESCs are grown on inactivated mouse embryonic fibroblasts used as a feeder layer [13].

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are stem cells derived from teratocarcinomas, which are tumors that arise from embryonic tissues such as those from the testis or ovary, or from cultures of explanted cells [7]. ECCs are considered the malignant counterparts of hESCs.

### Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)

Evans and Kaufman (1981) were the first to derive ESCs from an early mouse embryo [8]. In 1998, Thomson and colleagues reported the first successful derivation of hESC lines [9]; these lines are still widely used. They are capable of proliferating extensively in their undifferentiated state *in vitro* and have the ability to differentiate into all three germ layers [6,10]. The embryo-derived hESCs were established from blastocysts discarded during *in vitro* fertilization (IVF) procedures.

iPSCs are an artificially generated type of pluripotent stem cell. iPSCs are reprogrammed from adult somatic cells to acquire stem cell-like properties through the forced expression of a combination of transcription factors, such as Oct4, Sox2, Nanog, c-myc, KLF4, and Lin28. Takahashi and Yamanaka introduced four pluripotent genes—Oct4, Sox2, c-myc, and Kruppel-like family transcription factor 4 (Klf4)—that could reprogram mouse fibroblasts into mouse-induced pluripotent stem cells (miESCs) or human fibroblasts into human-induced pluripotent stem cells (hiPSCs) [11]. Thomson and colleagues used Oct4 and Sox2 in combination with Nanog and Lin-28 homolog (Lin28), instead of c-myc and Klf4, to reprogram human fibroblasts into hiPSCs [12]. These iPSCs express stem cell markers and can differentiate into three germ layers in a teratoma *in vivo*.

iPSCs have the advantage that they do not require the destruction of an embryo. Moreover, given their origin, they provide a perfect match to the cell donor (are fully isogenic) and thus would likely avoid rejection by the donor's immune system.

### Characterization of undifferentiated hESCs

Undifferentiated hESCs express high levels of cell surface antigens that can be used as stem cell-specific pluripotency markers. These antigens include: (1) glycolipids, such as the stage-specific embryonic antigens SSEA-3 and SSEA-4 [14]; (2) glycoproteins, such as TRA-1-60 and TRA-1-81 [15]; and (3) alkaline phosphatase [4,16,17]. Pluripotency markers include the transcription factors Octamer-4, POU domain, class 5, transcription factor 1 (OCT4 or POU5F1) [18-20]; sex-determining region Y-box 2 (SOX2) [21]; and Nanog homeobox (NANOG) [22,23]. These molecular markers provide a means of identifying pluripotent stem cells, and a decrease in their expression can be used to monitor the onset of differentiation. The molecular mechanisms underlying the self-renewal of hESCs have not been fully elucidated.

### Gene expression analysis of hESCs

Investigating gene expression in various stem cell lines could give important insights into how stem cells control pluripotency and differentiation. A number of studies have measured hESC gene expression to investigate related molecular mechanisms and have reported differential gene expression in different hESC lines. Rao and Stic (2004) reported a 75% similarity in the microarray profiles of two lines; in another study, 48% of the expressed genes were restricted to one or two lines [24]. However, variations in gene expression have been observed in hESC lines derived within the same laboratory

[25] and even in the same hESC lines (38%) after three passages in different media [26]. Gene expression also changes during spontaneous differentiation [27]. For example, the expression of leukemia inhibitory factor (LIF) and its receptors is low in undifferentiated hESCs, but increases during differentiation. Differential DNA methylation of pluripotency-associated promoters such as NANOG and OCT4/POU5F1 has been observed in pluripotent and differentiated cells. Understanding gene expression in hESCs will help shed light on the molecular basis of normal differentiation and the abnormal processes that underlie human developmental disorders.

Several external signals that maintain stem cell pluripotency have been characterized. External signals are also thought to play important roles in the regulation of ESC self-renewal and differentiation. For example, the pluripotency of hESCs is maintained by several signaling pathways [28];

- The Fibroblast Growth Factor (FGF) Signaling Pathway

Exogenous bFGF is an essential factor in a defined hESC culture medium used for the maintenance of undifferentiated hESCs and hiPSCs *in vitro*. Withdrawal of bFGF induces the downregulation of pluripotency markers and the differentiation of hESCs. This suggests that FGF signaling plays an important role in self-renewal and pluripotency regulation in human ES cells and iPSCs [29,30]

- The Transforming Growth Factor- $\beta$  (TGF- $\beta$ )/Activin/Nodal-SMAD2/3 Signaling Pathway

The TGF- $\beta$ /Activin/Nodal branch is highly active in undifferentiated hESCs. The pathway supports the self-renewal of undifferentiated hESCs by activating SMAD2/3 and inducing the expression of the pluripotency markers Oct4 and Nanog [31,32]. The TGF- $\beta$ /Activin/Nodal-SMAD2/3 signaling pathway is important in maintaining the self-renewal and pluripotency of hESCs [33].

- The Phosphoinositide-3-Kinase (PI3K) Signaling Pathway

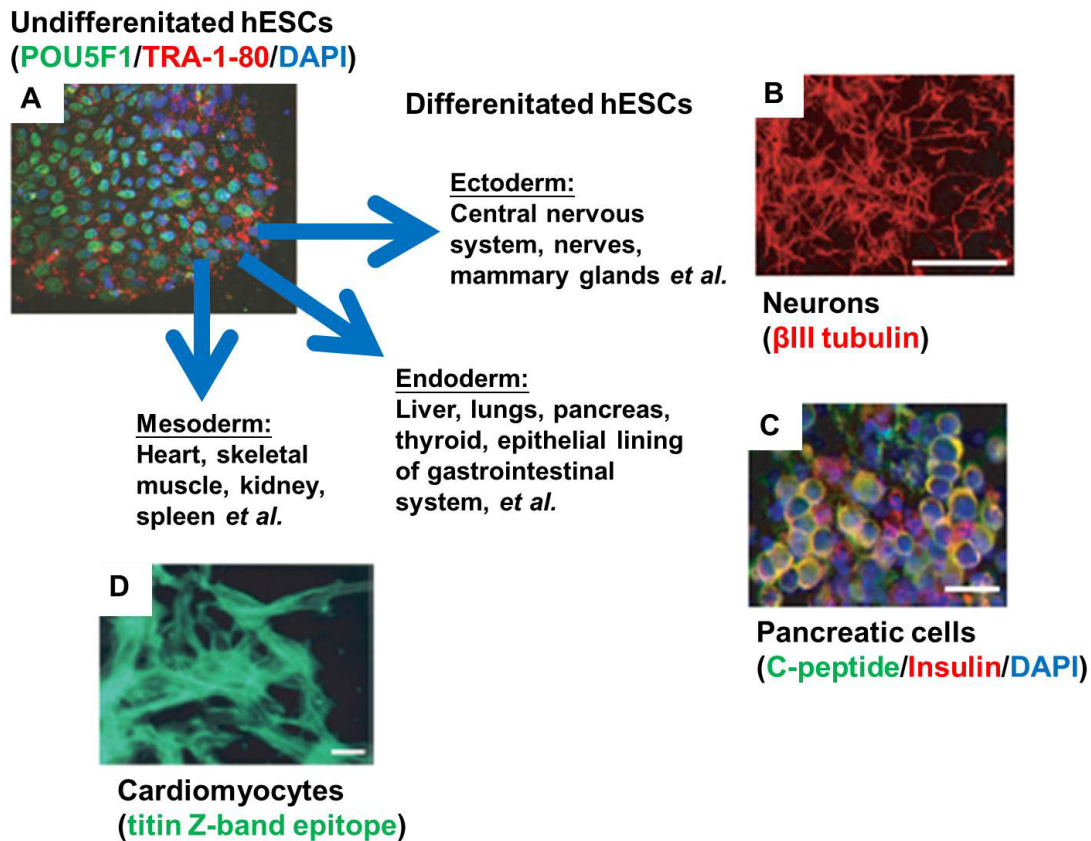
The PI3K protein is highly expressed in undifferentiated cells and is downregulated in differentiated ESCs [34,35]. Blocking the PI3K signaling pathway with the PI3K inhibitor LY294002 results in the loss of pluripotency markers and initiates cellular differentiation [36]. In addition, activation of the PI3K signaling pathway induces the PI3K-dependent phosphorylation of PKB/Akt and GSK-3 $\alpha/\beta$  proteins.

### Propagation of undifferentiated hESCs

Initially, hESCs were grown on irradiated mouse feeders or human foreskin fibroblasts [37]. However, exposure to animal-derived culture constituents is a drawback of the feeder-dependent systems [38]. Given that hESCs and hiPSCs are attractive candidates for future human cell transplantation, it is important to optimize good manufacturing practice (GMP)-compliant systems for the derivation, scale-up, and banking of cells and their corresponding quality assurance controls [39,40]. Therefore feeder-free systems are increasingly used in combination with xeno-free defined culture medium and GMP-compliant coating substrates specially designed for hESC growth [41], including laminin and fibronectin.

### Pluripotent stem cell differentiation

*In vitro* and *in vivo*, hES and iPSC cells can differentiate into cell types from the three primitive germ layers: ectoderm, mesoderm, and endoderm [4,6,16] (Figure 2). The differentiation capacity of these cells is typically tested by assessing their spontaneous differentiation in cell



**Figure 2.** Undifferentiated hES cells (A) can give rise to cell types from the three primitive germ layers: (B) neuronal cells in ectoderm-lineage differentiation, (C) pancreatic cells in endoderm-lineage differentiation, and (D) cardiomyocytes in mesoderm-lineage differentiation. This figure is adapted from Hoffman and Carpenter 2005 [13].

culture (i.e. in vitro formation of embryonic bodies or EBs). Upon the removal of growth factor (e.g. bFGF) or feeder layers and/or transfer to suspension conditions, hESCs undergo spontaneous unguided differentiation into various cells representative of the different germ layers. In two-dimensional (2D) spontaneously differentiated hESCs, the different morphologies appeared to be epithelial cells, neural cells with axons and dendrites, and cells with mesenchymal characteristics [42]. In suspension, the hESCs form multicellular aggregates of differentiated and undifferentiated cells called embryoid bodies (EBs), which resemble early post-implantation embryos and frequently progress through a series of differentiation stages [37,43,44]. Typically, EBs are allowed to grow for several days or weeks, with samples taken at intervals for analysis via flow cytometry or immunocytochemical staining. In vitro and in vivo assessments of differentiation involve determining whether the derived cells have acquired a variety of ectoderm-, mesoderm-, and endoderm-like properties (and loss of markers for pluripotency). In vivo assessment of pluripotency is performed by xenografting hESCs and iPSCs into severe combined immune-deficient (SCID) mice and observing the formation of teratomas with derivatives of all three germ layers, which indicates that the injected stem cells have the ability to differentiation along three lineages [4,16].

### Directed differentiation

To direct the differentiation of ESCs towards a particular cell type, such as a neuronal cell type or a cardiomyocyte cell type, hESCs in monolayer culture are exposed to certain growth factors or stimuli and

extracellular matrix components, either directly or indirectly through feeder cells [45].

Retinoic acid (RA) induces human embryonic stem cells to differentiate into the ectodermic lineage [46]. RA and its receptors play important roles in the development of the central nervous system by initiating the cellular differentiation of neuronal precursors [47]. Several papers have reported that RA induces neuronal differentiation in neuroblastoma cell lines (SH-SY5Y human dopaminergic neuroblastoma cells) [48,49] and human promyelocytic leukemia HL-60 cells [50]. Further, RA also induces embryonic stem cells to differentiate into neuronal cells [46,51,52], including neurons and glial cells. In order to improve the efficiency and reproducibility of neuronal differentiation, several studies have attempted to add small molecules. For example, Idelson M, *et al.* have demonstrated that nicotinamide promotes the differentiation of hESCs into neural cells and subsequently into retinal pigment epithelium (RPE) cells [53]. Moreover, Lu SJ, *et al.* have described a robust system that efficiently generates large numbers of hemangioblasts from multiple hESC lines and produces functional homogeneous RBCs with oxygen-carrying capacity on a large scale [54]. The markers of early ectoderm differentiation are paired box gene 6 (PAX6), SRY (sex determining region Y)-box 1 (SOX1), nesting, and glial fibrillary acidic protein (GFAP). Briggs JA, *et al.* have demonstrated successful neuronal differentiation via a sophisticated protocol [55].

Mesoderm differentiation has been extensively studied, particularly the families of protein growth factors that control the early stages of mesoderm formation in cardiomyocytes [56]. Hudson J, *et al.* have used small molecules to target the wingless/INT (Wnt) signaling

pathway in order to induce the differentiation of hESCs into beating cardiomyocytes [57]. Biomarkers for early mesoderm-differentiation include T-box factor Brachyury (BRY or T), the homeodomain protein MIXL1, and myosin [57]

Endoderm differentiation forms several tissues, including the liver, lung, thyroid, and foregut endoderm. The families of protein growth factors that control the early stage of endoderm differentiation into the anterior-ventral domain of the foregut endoderm are targeted by signaling through Nodal, a member of the transforming growth factor-B (TGF-B) superfamily and the SMAD signaling pathway [58]. The biomarkers of early endoderm differentiation are insulin-like growth factor 2 (IGF2) and gata binding factor (GATA4); SRY (sex determining region Y)-box 17 (SOX17) is also an indicator of the definitive foregut endoderm (Kanai-Azuma, Kanai, *et al.* 2002, Nakanishi, Kurisaki, *et al.* 2009). Little is known about the expression of mitochondrial biogenesis-related genes during hESC lineage-specific differentiation.

### Mitochondrial dysfunction

The symptoms of mitochondrial dysfunction caused by mutations or deletions in the mitochondrial genome or by mitochondrial depletion can be observed in whole animals and cellular models. Several studies have described mice with abnormal mitochondria resulting from mutations or deletions in the mitochondrial genome. To demonstrate the importance of mitochondrial morphology and function in cell-specific functions, we have established the mitochondria-depleted cells called rho-zero cells ( $\rho^0$ ), which are depleted of mtDNA, were generated in vitro through the application of different drugs, such as ethidium bromide, antibiotics [59], or the nucleoside analogue reverse transcriptase inhibitor (NARTI, an anti-HIV drug).  $\rho^0$  cells exhibit several common features: (1) they become autotrophic, relying on pyrimidine (uridine) and pyruvate supplementation for cell growth [60,61]; (2) they have a low mtDNA copy number and low expression of mitochondrial-encoded genes, but not of nuclear-encoded genes; (3) they have low mitochondrial respiratory chain complex activities, with the exception of complex II; (4) they have low ATP concentrations, respiration rates (oxygen consumption), and mitochondrial membrane potential; (5) they shift from aerobic to anaerobic metabolism if given supplemental pyruvate; and (6) they have an immature mitochondrial structure with reduced numbers of cristae membranes, circular morphology, and loss of tubular structure.

Upon a reduction in oxygen consumption, several studies have found that antioxidants can reverse the increased ROS production in  $\rho^0$  cells. In addition,  $\rho^0$  cells have decreased levels of cell proliferation, mitotic cyclin gene expression, cyclin-dependent kinase inhibitors, retinoblastoma 1 phosphorylation, and telomerase activity [62,63]. In  $\rho^0$  cells, upregulation of mitochondrial biogenesis-related genes, relative to expression in control cells, has been observed [64,65].

Interestingly, it has been suggested that  $\rho^0$  cells have increased resistance to apoptosis. However, they exhibit a normal distribution of cytochrome c within mitochondria during staurosporine-induced apoptosis (in spite of low mtDNA levels and respiratory function deficiencies). Consistently, caspase 3 activation and DNA fragmentation are not affected in  $\rho^0$  cells. However, the localization of NF- $\kappa$ B is altered (i.e. more NF- $\kappa$ B in the nucleus than in the cytoplasm), which might be related to the observed resistance to apoptosis. Moreover, in  $\rho^0$  cells, a greater amount of mass is associated with lysosome and peroxidation production [65-67]. Remarkably, the differentiation of SH-SY5Y neuroblastoma cells into neuron-like cells is not affected by defective

mitochondria, as indicated by the presence of long neurites and secretory granules, which are typical of differentiating neuroblastoma cells [68]. Therefore, targeting mitochondria metabolism or mutations in mitochondrial-related genes for inherited disease by using pluripotent stem cells is still a major therapeutic direction for cell therapy.

### Disclosure of potential conflicts of interest

The authors indicate no potential conflict of interest.

### Authorship contribution

This work was supported by authorship contribution. Wrote or contributed to the writing of the manuscript: Kao and Wolvetang.

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