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Embryonic Stem Cells: from Blastocyst to *in vitro* Differentiation

Genesia Manganelli[1], Annalisa Fico[1,2] and Stefania Filosa[1]
[1]*Istituto di Genetica e Biofisica "A. Buzzati Traverso" CNR,*
Via Pietro Castellino 111, 80131 Napoli
[2]*Developmental Biology Institute of Marseille–Luminy (IBDML)*
Campus de Luminy, Case 907, 13288, Marseille cedex 09
[1]*Italy*
[2]*France*

1. Introduction

A stem cell is a specific kind of cell that has the unique capacity to renew itself and to give rise to specialized cell type.
In terms of potentially, stem cells can be classified in three types:

- Totipotent: is the ability to form all cell types, including the extra-embryonic tissues. In mammals, the fertilized egg, zygote and the first 2, 4, 8, 16 blastomeres from the early, are examples of totipotent cells.
- Pluripotency: is the ability to differentiate into several cell types derived from any of the three germ layers (ectoderm, mesoderm, endoderm), but they are unable to produce extra-embryonic tissues. Cells from the inner cell mass of blastocyst are pluripotent.
- Multipotent: cells can form a small number of tissues that are restricted to a particular germ layer origin: e.g. blood cells or bone cells.

In according to their source, stem cells are categorized in embryonic or adult (Fig. 1):

- Embryonic stem cells (ES cells) are derived from the inner cell mass of the blastocyst (an early stage embryo) and have a high proliferative capabilities and differ from other stem cells because they have the ability to generate derivatives of all three germ layers. Embryonic stem cells have been shown to contribute to all cell lineages, including the germ line, following microinjection studies in murine embryos which give rise to chimeras (Bradley et al., 1984; Nagy et al., 1990). *In vitro*, murine ES cells can be propagated indefinitely in an undifferentiated state, under specific culture conditions they can differentiated into specific cell types.
- Adult stem cells are undifferentiated cells found among differentiated cells of a specific tissue, including bone marrow (de Haan, 2002), skin (Watt, 2001), intestinal epithelium (Potten, 1998), liver (Theise et al., 1999), retina (Tropepe et al., 2000), central nervous system (Okano, 2002), pancreas (Ramiya et al., 2000) and skeletal muscle (Seale et al., 2001). They typically can differentiate into a relatively limited number of cell types.

There is no doubt that stem cells have the potential to treat many human afflictions, including cancer, diabetes, neurodegeneration, as well as for studying basic developmental biology, and intensive screening of drug and toxic (Watt and Driskell, 2010).

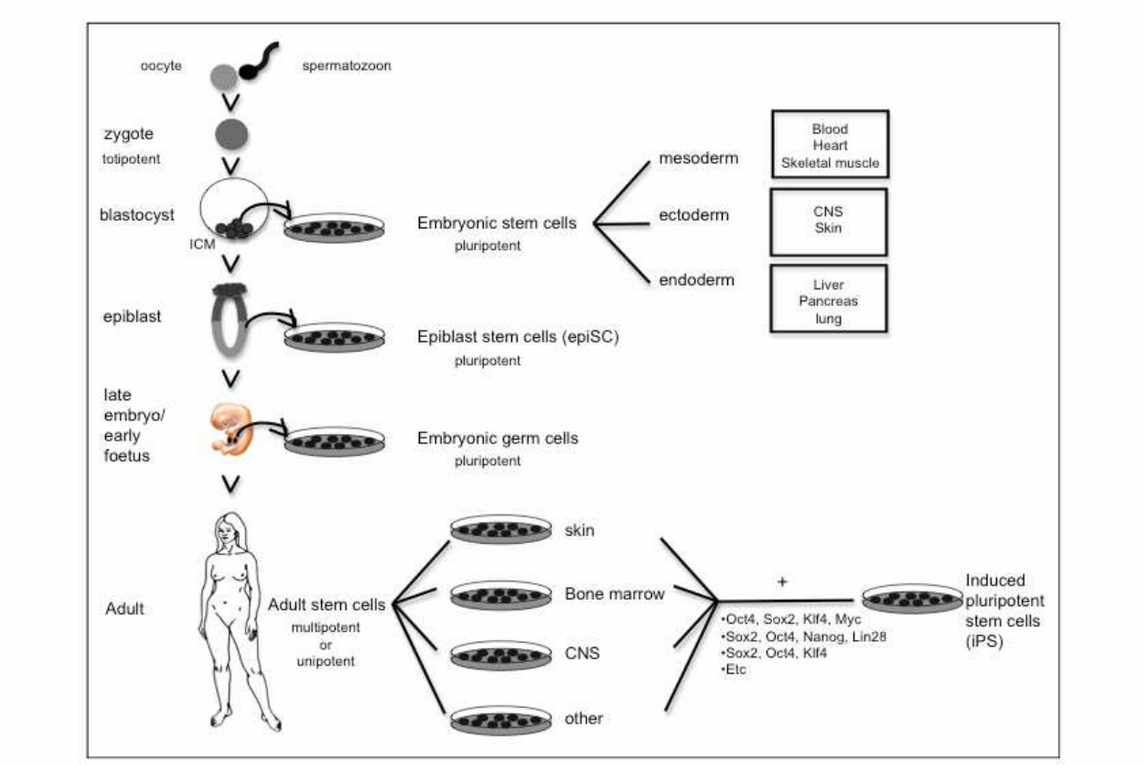

Fig. 1. Origin of embryonic and adult stem cells

2. Derivation of mouse embryonic stem cells

Embryonic stem cells are derived from the inner cell mass (ICM) of the mammalian blastocyst. The first mammalian ES cell lines were derived from mouse blastocyst in 1981 from two independent groups (Evans and Kaufman, 1981; Martin, 1981).

One distinct property of ES cells is that they remain diploid even after being cultured for many weeks. This is in contrast to other tissue culture cell lines that often do not remain diploid but spontaneously gain or lose chromosomes at high rate. A second unique property of ES cells is that they remain pluripotent and maintain the ability, like ICM cells, to form chimeras. These two properties, maintaining normal karyotype and extensive contribution in chimeras, are both necessary for ES cells to form functional germ cells in chimeras (Sedivy and Joyner, 1992) and, moreover, have made ES cells a unique tool for gene targeting and generation of genetically modified mice.

A surprising feature of mouse ES cell lines is that the majority of cell lines genetically tested are of male origin (40XY). In female (XX) ES cells, both X chromosome are active, that may result in the unsuitable propagation of ES cells (Rastan and Robertson, 1985). In either case, the XY genotype confers appreciable advantages for germ line transmission.

ES cells clonally derived from a single cell could differentiate into a variety of cell types *in vitro* and form teratocarcinomas when injected into mice (Martin, 1981). Most important, cells karyotypically normal contribute at a high frequency to a variety of tissue in chimeras, including germ cells, thus providing a practical way to introduce modifications to the mouse germline (Bradley et al., 1984).

After the first derivation of mouse ES cell lines from blastocysts, several standard protocols were developed (Robertson, 1987; Abbondanzo et al., 1993; Hogan et al., 1994; Nagy et al., 2003). The efficiency of mouse ES cell derivation is strongly influenced by genetic background. For example, ES cells can be easily derived from the inbred 129/ter-Sv strain but less efficiently from the C57BL/6 strain (Ledermann and Burki, 1991). However, mouse ES cells can be derived from some non permissive strains using modified protocols (McWhir et al., 1996; Bryja et al., 2006a; Bryja et al., 2006b). Mouse ES cells have also been derived from cleavage stage embryos and even from individual blastomeres of two- to eight-cell stage embryos (Chung et al., 2006; Wakayama et al., 2007).
ES cells or ES cell-likes have been produced in other animal models, including: medakafish from midblastulae stage (Hong et al., 1998), zebrafish from midblastulae stage (Sun et al., 1995), chickens from stage X blastoderm (Pain et al., 1996), hamsters (Doetschman et al., 1988), mink (Sukoyan et al., 1992), rabbit (Schoonjans et al., 1996), cattle (Cibelli et al., 1998; Strelchenko et al., 2004), sheep (Wells et al., 1997), and pigs (Li et al., 2003), however, only mouse and chicken ES cells are capable of colonizing the germ line.

3. Maintenance of mouse embryonic stem cells

ES cells can be stably propagated indefinitely and maintain a normal karyotype without undergoing cell senescence *in vitro* when cultured in the presence of leukemia inhibitory factor (LIF) and, depending on ES cell lines, with or without a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs). LIF, a member of the IL-6 family, is known to strongly promote self-renewal in ES cells (Smith et al., 1992). LIF binds to LIF receptor (LIFR) to dimerize with interleukin 6 signal transducer (gp130), resulting in the phosphorylation of signal transducer and activator of transcription 3 (Stat3) via Janus kinase (Jak) activation (Burdon et al., 2002). Phosphorylated Stat3 dimerizes and translocates to the nucleus to activate a variety of downstream genes. Repression of Stat3 results in differentiation (Niwa et al., 2009), whereas artificial activation of Stat3 is sufficient to maintain pluripotency without LIF in the media (Matsuda et al., 1999).

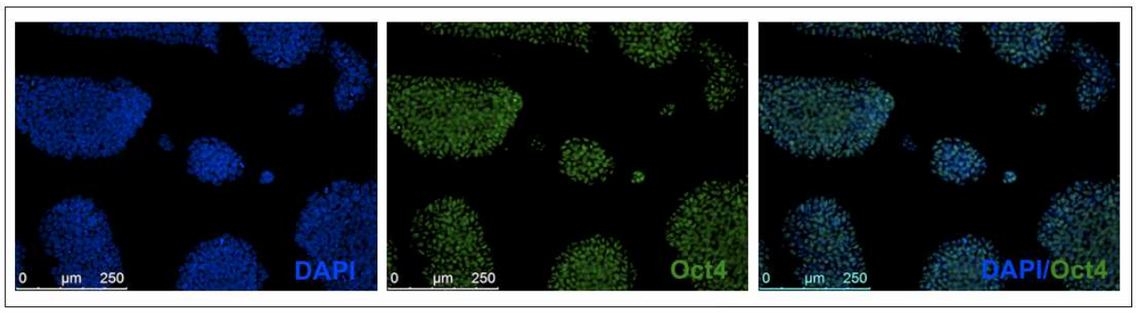

Fig. 2. Fluorescent immunostaining of undifferentiated mouse ES cells. All the undifferentiate ES cells expressed pluripotency specific marker Oct4. Immunostaining with DAPI (nuclear marker), Oct4 antibody, merge DAPI/Oct4

In combination with the LIF-Stat3 pathway, the pluripotency of ES cells is modulated by transforming growth factor β (TGFβ) superfamily members. These include Bmp and Activin, which generally play diverse roles in cellular homeostasis. In the ES cells, Bmp4 activates the MAD homolog 1 (Smad1). This upregulates the expression inhibitor of DNA-binding genes (Id), which suppress differentiation in combination with the LIF signal.

Activin/nodal signaling contributes to promote the growth of ES cells (Ying et al., 2003; Ogawa et al., 2007; Wu and Hill, 2009). Wnt signalling also contributes to the maintenance of pluripotency. In the canonical Wnt pathway, the Wnt receptor Frizzled transduces the signal to glycogen synthase kinase 3β (GSK3β) and adenomatosis polyposis coli (Apc). This enables catenin beta 1 (Ctnnb1) to traslocate into the nucleus to form the Ctnnb1/Tcf complex, which in turn activates the downstream genes (Willert and Jones, 2006). In the presence of Wnt signalling, transcription factor (Tcf3) activates the downstream genes that promote pluripotency maintenance by collaborating with the pivotal transcription factors Otc3/4 (Fig. 2), Sox2 and Nanog (Masui, 2010).

4. Derivation of human embryonic stem cells

There was a considerable delay between the derivation of mouse ES cells (1981) and the derivation of human ES cells in 1998 (Thomson et al., 1998). This delay was primarily due to species-specific ES cell differences and suboptimal human embryo culture media. In fact the first study to describe the isolation of human ICM cells was published by Bongso et al. (Bongso et al., 1994), but subsequent culture in media supplemented with LIF and serum resulted only in differentiation, not in the derivation of stable pluripotent cell lines. Human ES (hES) cells can be characterized by their immortality, expression of telomerase expression, pluripotentiality, ability to form teratomas, and maintenance of a stable karyotype and, even after prolonged undifferentiated proliferation, maintain the development potential to contribute to advanced derivatives of all three germ layers, even after clonal derivation (Amit et al., 2000). For obvious ethical reasons, experiments involving blastocyst injections and ectopic grafting in adult hosts cannot be performed in the human.

Human ES cells have been derived from morula, later blastocyst embryos (Stojkovic et al., 2004; Strelchenko et al., 2004), single blastomeres (Klimanskaya et al., 2006), and parthenogenetic embryos (Lin et al., 2007).

Previous reports suggest that the success rate in deriving hES cell lines is highly dependent on the quality of recovered blastocysts, isolation condition used and technical expertise (Pera et al., 2000; Mitalipova et al., 2003).

ES cell lines are usually derived by immunosurgery. In this process the trophoblast layer of the blastocyst is selectively removed, and the intact inner cell mass is further cultured on MEFs (Amit and Itskovitz-Eldor, 2002). Although the cloning efficiency of the hES cells was relatively poor, a several fold increase was observed when serum-free medium supplemented with basic fibroblast growth factor (βFGF) was used (Amit et al., 2000).

5. Maintenance of human embryonic stem cells

Mechanical and enzymatic transfer methods are used to maintain hES cell lines (Oh et al., 2005). The mechanical transfer method is laborious and time-consuming, although remains an efficient technique for the transfer of undifferentiated hES cells and results in similar clump sizes. The enzymatic transfer method is used when the bulk production of cells are required for various experiments and results in the more rapid growth and larger production of hES cells.

However, the cell clumps vary in size, and there is a higher probability that both differentiated and undifferentiated cell will be transferred. In the case of passaging more differentiated colonies, a combination of both methods allows mass production of hES cells by excluding differentiated colonies from passage by manual selection prior to enzyme treatment.

Another limiting factor relating to cell culture systems is that hES cells still require the presence of feeder layer. In fact, feeder-free system for hES cell culture is required if hES cell cultures are to become clinical-grade, since the use of animal feeders and/or ingredients for growth of hES cells limits the large-scale culture and medical applicability of hES cells. At present, feeder-free systems are not optimal for the derivation and growth of clinical-grade hES cell lines since the presence of animal ingredient carriers the potential risk for the cross-transfer of different infectious agents. In fact, it has been reported that hES cells embryoid body can incorporate the N-glycolylneuraminic acid (Neu5Gc) from MEFs or from conditioned medium, which resulted in an immune response (Martin et al., 2005).
The first attempt to produced feeder-free cultures of hES cells was reported by Xu et al. (Xu et al., 2001). They propaged hES cells using Matrigel, an animal based extracellular matrix (ECM) preparation, or laminin substrates in medium conditioned by MEFs. This system enabled the long term propagation of the stem cell phenotype, with strong suppression of spontaneous differentiation even at high passages (Carpenter et al., 2001).
In 2005, Prowse et al. identified 102 proteins from conditioned medium of human neonatal fibroblasts which provide invaluable information regarding the factors that may help maintain hES cells (Prowse et al., 2005).
The growth factor, ActivinA, paracrinely secreted by MEFs, is capable of supporting the growth of hES cells on laminin coated dishes for more 20 passages without the need for feeder layers (Beattie et al., 2005). Sato et al. (2004) suggest that Wnt signalling modulation can help to support the growth of hES cells cultures short-term and maintain their capacity to express some stem cell markers in the absence of a feeder cell layer (Sato et al., 2004). Another study demonstrated that noggin (BMP antagonist) combined with high βFGF concentrations in medium support the long term proliferation of undifferentiated hES cells in the absence of feeder cells and/or conditioned medium. However in this case Matrigel coated dishes were used, but this represent a problem for potential medical application of hES cells because xenogeneic pathogens can be transmitted through culture conditions (Wang et al., 2005; Xu et al., 2005).
Moreover, it has been reported that the combination of FGF2, TGFβ, LIF and a proprietary serum replacer can achieve serum-free, feeder-free maintenance of hES cells when cultured on fibronectin ECM (Amit et al., 2004).
The establishment of feeder-free system for the culture of hES cells is critical for genetic manipulation. In fact, homologous recombination could be used as a tool for the repair of specific gene defects in stem cell lines derived from patients suffering disease.

6. Comparison between human and mouse ES cells

Many of the differences between mouse and human ES cells are only beginning to be elucidated, yet it has already been demonstrated that mouse and human ES cells differ in respect to cell surface markers, with human ES cells expressing the stage specific antigens SSEA-3 and SSEA-4, the glycoproteins TRA-1-60 and TRA-1-81, and GCTM-2, none of which are detected in the mouse. In contrast, mouse ES cells express SSEA-1, which remain undetected within human ES cultures. Moreover human ES cells are insensitive to the differentiation suppressing effects of LIF pathway (Thomson et al., 1998; Reubinoff et al., 2000).
However, there remain many similarities between human and murine ES cell populations. ES cells are derived from both species using very similar protocols, and same aspects of their propagation, such as the ability of MEFs to support their growth in an undifferentiated state remain almost identical (Fig. 3). Furthermore, human and mouse ES cells possess similar

properties of spontaneous differentiation and expression of the pluripotent-associated transcription factor Oct-4.

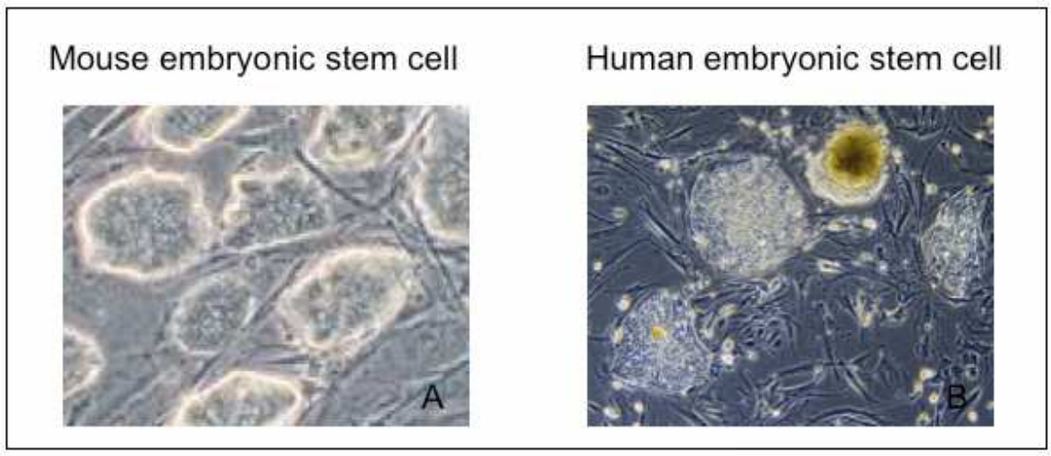

Fig. 3. Phase contrast microscopy images of mouse (A) and human (B) embryonic stem cells on mouse embryonic fibroblast

7. Differentiation of mouse embryonic stem cells *in vitro*

In the absence of feeder cells and anti-differentiating agents such as LIF, mouse ES cells spontaneously differentiate and, under appropriate conditions, generate progeny consisting of derivatives of the three embryonic germ layer: mesoderm, endoderm, and ectoderm (Keller, 1995; Smith, 2001).

Mesoderm derived lineages include the hematopoietic, vascular, and cardiac. Endoderm derivatives include pancreatic β cell and hepatocytes. Ectoderm differentiation of mouse ES cells is well established, as numerous studies have documented and characterized neuroectoderm commitment and neural differentiation.

Three general approaches are used to initiate ES cell differentiation. With the first method, the hanging drop method (Fig. 4), ES cells are allowed to aggregate and form three dimensional colonies known as embryoid bodies (EBs) (Doetschman et al., 1985; Keller, 1995). In the second method, ES cells are cultured directly on stromal cells, and differentiation takes place in contact with these cells (Nakano et al., 1994). The third protocol involves differentiating ES cells in a monolayer on extracellular matrix proteins (Nishikawa et al., 1998) or in presence of specific differentiation medium (Takahashi et al., 2003; Fico et al., 2008).

7.1 Cardiac differentiation

The development of the cardiac lineage in ES cell differentiation cultures is easily detected by the appearance of areas of contracting cells that display characteristics of cardiomyocytes. Development of the cardiomyocyte lineage progresses through distinct stages that are similar to development of the lineage *in vivo*. An ordered pattern of expression of cardiac genes is observed in the differentiation cultures, with expression of the transcription factors *gata-4* and *nkx2.5* that are required for lineage development preceding the expression of genes such as *atrial natriuretic protein* (*ANP*), *myosin light chain* (*MLC*)-*2v*, α-

myosin heavy chain (α-*MHC*), β-*myosin heavy chain* (β-*MHC*), and *connexin 43* that are indicative of distinct maturation stages within the developing organ *in vivo* (Hescheler et al., 1997; Boheler et al., 2002). Several different studies have begun to investigate the mechanisms regulating the development of the cardiac lineage in ES cell differentiation cultures. It has been demonstrated that the EGF-CFC factor Cripto, known to be essential for development *in vivo* (Ding et al., 1998; Xu et al., 1999), plays a pivotal role in differentiation of ES cells to the cardiac lineage, in fact, *Cripto-/-* ES cells display a deficiency in generating cardiomyocytes (Parisi et al., 2003). Notch signaling also plays a role in cardiac development from ES cells (Schroeder et al., 2003), in fact ES cells lacking a downstream signalling molecule of all Notch (Jk) generate more cardiac cells than wild type ES cells (Keller, 2005). However, in this case, inhibition of the pathway appears to be important for cardiac differentiation. Other factors, including BMP2 and FGF2 (Kawai et al., 2004) as well as nitric oxide (Kanno et al., 2004) and ascorbic acid (Takahashi et al., 2003), have been shown to promote or improve cardiomyocyte differentiation in ES cell cultures.

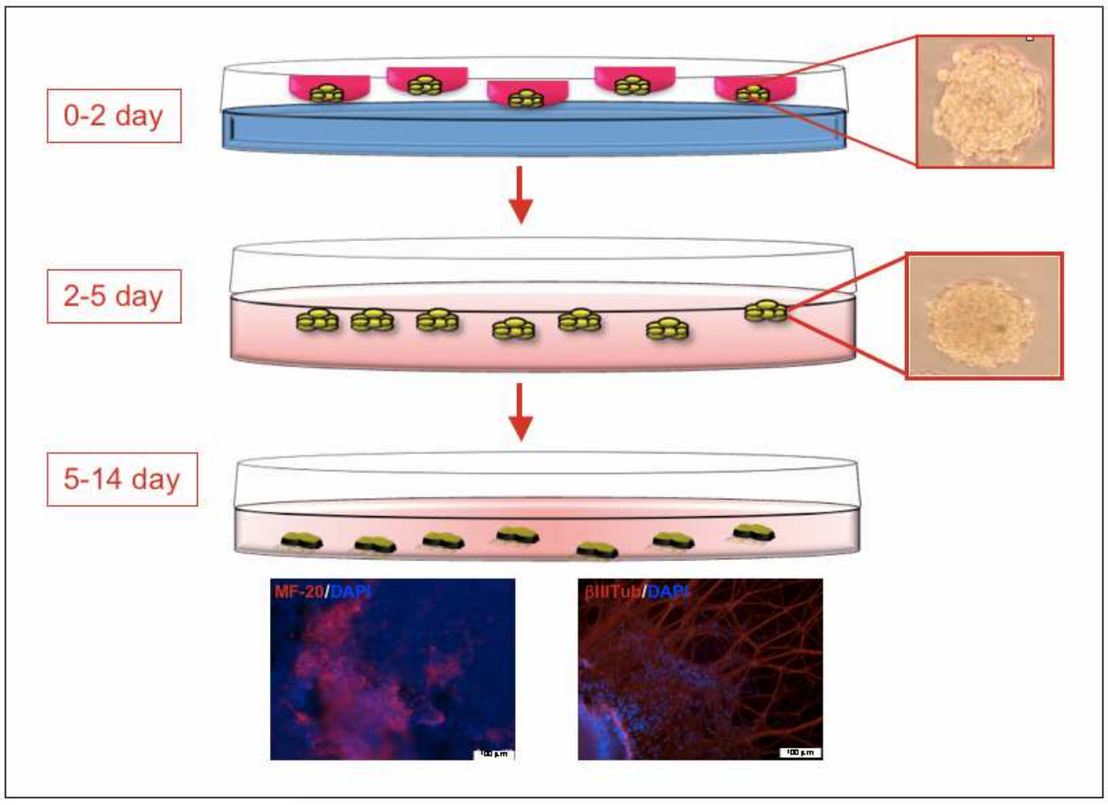

Fig. 4. Schematic rappresentation of method used to form embryoid bodies. This method is generally used to induce ES cells differentiation into cardiomyocytes or, adding retinoic acid, into neurons. MF-20 specific marker of cardiac cells, bIIITubulin (bIIITub) specific neural marker, DAPI nuclear marker

7.2 Primitive and definitive hematopoiesis

ES cells undergo hematopoietic differentiation in optimized culture conditions following serum induction (Keller, 1995). Gene expression and progenitor cell analysis revealed that

the differentiation program in these cultures closely parallels that in the early embryo, progressing through a primitive streak stage, to mesoderm, and subsequently to a yolk sac-like hematopoietic program. Detailed analysis of these early stages led to the identification of the hemangioblast, a progenitor that displays hematopoietic and vascular potential (Choi et al., 1998). After the hemangioblast appears, primitive erythroid progenitors develop in ES cells cultures, establishing the primitive erythropoiesis phase of hematopoiesis. In addition to primitive erythrocytes, other progenitors including those of the macrophage, definitive erythroid, megakaryocyte, and mast cell lineages develop in the differentiation cultures with a kinetic pattern similar to that observed in the yolk sac (Murry and Keller, 2008).
However, despite extensive efforts, to induce the formation of transplantable hematopoietic stem cells (HSCs) the development of HSCs from ESCs remains a challenge, which may reflect the complexities of embryonic hematopoietic development where different hematopoietic programs are generated at different times from different embryonic sites (Murry and Keller, 2008).

7.3 Endoderm differentiation

The generation of endoderm derivatives, in particular pancreatic β-cells and hepatocytes, has become the focus of many investigators in the field of ES cell biology. The interest in the efficient and reproducible development of these cell types derives from their clinical potential for the treatment of Type I diabetes and liver disease, respectively (Keller, 2005). Several genes used as markers of definitive endoderm (*Foxa2*, *Gata4*, and *Sox17*) (Arceci et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993; Laverriere et al., 1994; Kanai-Azuma et al., 2002), early liver (a-*fetoprotein* and *albumin*) (Dziadek and Adamson, 1978; Meehan et al., 1984; Sellem et al., 1984), and early pancreas (*Pdx1* and *insulin*) (McGrath and Palis, 1997) development are also expressed by visceral endoderm, a population of extraembryonic endoderm. Given the overlapping expression patterns, it can be difficult to distinguish definitive and extraembryonic endoderm in the ES cell differentiation cultures. Another problem encountered in endoderm differentiation from ES cells is the lack of specific inducers of this lineage.
It has been investigated the potential of ES cells to differentiate into endoderm derivatives and developed two different protocols that promote the generation of these cell types (Kubo et al., 2004). The first is a restricted exposure of the EBs to serum followed by a period of serum-free culture, and the second is induction with Activin A in the absence of serum. Endoderm development was quantified based on the proportion of cells that expressed Foxa2, a transcription factor found in the earliest stages of definitive endoderm development (Monaghan et al., 1993; Sasaki and Hogan, 1993). All of the Foxa2+ cells that developed in these cultures also expressed the primitive streak marker *brachyury*, a gene that is not expressed in visceral endoderm. This observation strongly suggests that the Foxa2+ cells represented definitive endoderm. Based on the number of Foxa2+ cells, the Activin A protocol was found to be the most efficient as >50% of the total population in these cultures expressed this protein, in fact, low level of Activin A promote a mesoderm fate, and high levels of Activin A induced the formation of endoderm cells (Green et al., 1992; Hudson et al., 1997).
In 2009 Borowiak et al. identified two potent small molecules, IDE1 and IDE2, that can direct mouse ES cell differentiation such that 70%–80% of cells are endoderm cells. This efficiency of induction compares favorably with published protocols employing TGF-β family members, e.g., Activin A or Nodal, which produce about 45% endoderm. The application of small molecules to differentiate mouse and human ES cells into endoderm represents a step

toward achieving a reproducible and efficient production of desired ES cell derivatives (Borowiak et al., 2009).

7.4 Neural differentiation

Several different protocols have evolved to promote neuroectoderm differentiation. The various approaches include (1) treatment of serum-stimulated EBs with retinoic acid (Bain et al., 1995), (2) sequential culture of EBs in serum followed by serum-free medium (Okabe et al., 1996), (3) differentiation of ES cells as a monolayer in serum-free medium (Tropepe et al., 2001; Ying et al., 2003; Fico et al., 2008), and (4) differentiation of ES cells directly on stromal cells in the absence of serum (Kawasaki et al., 2000; Barberi et al., 2003). As with the mesoderm and endoderm lineages, development of the ectoderm lineages in the ES differentiation cultures appears to recapitulate their development in the early embryo (Barberi et al., 2003). *In vitro* it is possible to form the three major neural cell types: neurons, astrocytes and oligodendrocytes.

The protocols for differentiation to specific types of neurons have included the sequential combination of regulators that are known to play a role in the establishment of these lineages in the early embryo. For instance, midbrain dopaminergic neurons have been generated in the EB system by overexpression in the cells of the transcription factor nuclear-receptor-related factor1 (Nurr1), and the addition to the cultures of sonic hedgehog (SHH) and FGF8 (Kim et al., 2002). Nurr1, SHH, and FGF8 are required for the development of this class of neurons in the early embryo (Ye et al., 1998; Simon et al., 2003). Other studies have demonstrated the development of cholinergic, serotonergic, and GABAergic neurons in addition to dopaminergic neurons, when differentiated on MS5 stromal cells in the presence of different combinations of cytokines (Barberi et al., 2003). Using the coculture approach together with the appropriate signaling molecules and selection steps, cells that display many of the characteristics of motor neurons has been successfully generated (Wichterle et al., 2002).

When cultured at low density in serum-free medium in the presence of LIF, ES cells generate a population that has been called primitive neural stem cells (Tropepe et al., 2001). These cells have been characterized by their ability to generate neurosphere-like colonies composed of cells that express the neural precursor cell marker, nestin (Lendahl et al., 1990). When cultured on a matrigel substrate in the presence of low amounts of serum, cells within these colonies generated neurons, astrocytes, and oligodendrocytes. In 2008, Fico et al. established a one-step protocol that allowed differenziation of mouse ES cells into a highly enriched population of neuronal cells, simply by culturing them on gelatin-coated dishes in a chemically defined serum-free medium. This differentiation method is able to generate a wide range of neural subtypes and glial cells from mouse ES cells (Fico et al., 2008).

The ability to generate different types of neurons from ES cells has dramatically raised the interest in repair of nervous system disorders by cell replacement therapy.

8. Human embryonic stem cells differentiation

Human embryonic stem cells are characterized by their ability to proliferate in the undifferentiated state in culture for a prolonged period, and by their capacity to differentiate into derivatives of all three germ layers. A variety of studies have described *in vitro* spontaneous and directed differentiation of hES cells into different lineages: cardiomyocytes (Kehat et al., 2001; Xu et al., 2002), neurons and glia (Carpenter et al., 2001; Reubinoff et al.,

2001), endothelial cells (Levenberg et al., 2002), hematopoietic precursors (Kaufman et al., 2001), trophoblast, and hepatocyte-like cells (Rambhatla et al., 2003). The most common method used for *in vitro* differentiation is to remove the hES cells from the feeder layer and culture in suspension in absence of MEFs. Following culturing in suspension, hES cells aggregate into EBs (Itskovitz-Eldor et al., 2000). The aggregation process itself triggers initial cell differentiation. It is thought that the EBs consist of derivatives of all three germ layers, which interact and cross-induce each other, resulting in complex differentiation into the various lineages. This process is considered to recapitulate early embryonic development from the blastocyst stage to the egg-cylinder stage.

8.1 Cardiac differentiation

In order to generate a cardiomyocyte-differentiating system from the hES cells, small clumps of 3–20 cells were grown in suspension for 8 days (Amit et al., 2000). The EBs were then plated on gelatin-coated culture dishes and observed microscopically for the appearance of spontaneous contraction. Rhythmically contracting areas appeared at 6 to 12 days after plating. Cells isolated from the beating areas expressed cardiac-specific structural genes, such as cardiac troponin I and brachyury (T), atrial natriuretic peptide (ANP), atrial and ventricular myosin light chains (MLCs). Immunostaining studies demonstrated the presence of the cardiac-specific sarcomeric proteins myosin heavy chain, α-actinin, desmin, and cardiac troponin I, as well as ANP (Kehat et al., 2001).

Cardiomyocyte differentiation can be enhanced in the mouse ES cell system following the addition of differentiation factors including, dimethyl sulfoxide (DMSO), retinoic acid (RA), and small molecoles. Addition of the demethylating agent 5-aza-2′-deoxycytidine to EB cultures has also been shown to be effective for mouse ES cell and human ES cell differentiation into cadiomyocytes. In contrast, RA in hES cells did not induce a higher proportion of cardiomyocytes *in vitro* (Schuldiner et al., 2000). An alternative method for deriving cardiomyocytes has been achieved following the coculture of pluripotent hES cell lines with END-2 cells (visceral-endoderm-like cell lines) (Mummery et al., 2003).

8.2 Hematopoietic differentiation

Several studies have documented hematopoietic development of hES cells using different induction schemes (Murry and Keller, 2008). As observed in the mouse system, the predominant population generated during the first 7–10 days of hES cell differentiation is primitive erythroid progenitors, indicating that the equivalent of yolk-sac hematopoiesis develops first in these cultures (Zambidis et al., 2005; Kennedy et al., 2007). As observed with mouse ES cell and the mouse embryo, the onset of hematopoiesis in hES cell cultures is marked by development of the hemangioblast between days 2 and 4 of differentiation, prior to establishment of the primitive erythroid lineage (Kennedy et al., 2007; Lu et al., 2007; Davis et al., 2008)

8.3 Neural differentiation

In 2001, Reubinoff and Zhang highlighted the potential of hES cells to generated neural cells (Reubinoff et al., 2001; Zhang et al., 2001). Zhang et al. have combined the techniques which were initially developed for the neural differentiation of mouse ES cells and adapted these to produce human neural stem cells. This occurs via a successive stepwise approach, which consists of inducing the formation of EBs and from these generating neural rosettes, which

are proliferating structures that mimic neural tube formation. Rosettes are subsequently harvested by selective dissociation and are cultured as free-floating aggregates of neural precursors, capable of generating neurons and glia (Zhang et al., 2001).
Reubinoff demonstrated that neural differentiation was induced by overgrowth of undifferentiated ES cells. Maintaining hES cells in culture without passage or replenishing feeder cells led to spontaneous neural differentiation within a heterogeneous population of hES cell progeny. Individual clusters of presumptive neural progenitors were identified by phase contrast microscopy and manually transferred onto uncoated dishes. Following culture in defined medium supplemented with βFGF and epidermal growth factor (EGF), these cells formed aggregates highly enriched with neural precursor cells. After withdrawal of βFGF and EGF, downregulation of nestin and mash-1 is followed by upregulated expression of neuron-specific NFM, synaptophysin, Nurr1, and tyrosine hydroxylase (TH) genes. A decreased formation of nestin-positive cells is assimilated with an increased number of neuronal cells expressing neuron-specific protein. Mature neuronal cells are evidenced by the production of neurotransmitters such as dopamine, serotonin, GABA, and glutamate. These results suggest that in presence of neuronal differentiation factors, such as retinoic acid, FGF4, FGF8, or βFGF, hES-derived cells, led to the enrichment of cholinergic, serotinergic, dopaminergic and GABAergic neurons, respectively (Okabe et al., 1996; Lee et al., 2000; Rolletschek et al., 2001; Barberi et al., 2003).
Li et al. (2005) differentiated hES cells into spinal motoneurons using retinoic acid and in the presence of SHH (Li et al., 2005).

8.4 Pancreatic β -islet cells

1–3% of cells within 60–70% of human EBs produced from hES cells have been observed to stain positively for insulin (Assady et al., 2001).
A modification of Lumelskey and colleagues (2001) method resulted in the production of insulin-secreting cells derived from hES cells (Lumelsky et al., 2001). This was achieved following an additional step of culture including, a lowering of the glucose concentration in the medium, removal of βFGF and addition of nicotinamide. Dissociating the cells and growing them in suspension resulted in the formation of clusters, which secreted higher levels of insulin than their *in vivo* counterparts and could be maintained *in vitro*. These cells expressed pancreatic genes and following immunofluorescence and in situ hybridization studies, it was confirmed that a high percentage of insulin-expressing cells were located within these cell clusters (Segev et al., 2004).

9. A new age for ES cells: induced pluripotent stem cells

Takahashi and Yamanaka recently achieved a significant breakthrough in reprogramming somatic cells back to an ES like state (Takahashi and Yamanaka, 2006). They successfully reprogrammed mouse embryonic fibroblasts and adult fibroblasts to pluripotent ES-like cells after viral-mediated transduction of the four transcription factors Oct4, Sox2, c-myc and Klf4 followed by selection for activation of the Oct4 target gene Fbx15. Cells that had activated Fbx15 were designated with a coined expression "induced pluripotent stem" (iPS) cells. These cells were shown to be pluripotent by their ability to form teratomas although they were unable to generate live chimeras. In subsequent experiments when activation of the endogenous Oct4 or Nanog genes was used as a more stringent selection criterion for

pluripotency, the resulting Oct4-iPS or Nanog-iPS cells, in contrast to Fbx15-iPS cells, were fully reprogrammed to a pluripotent ES cell state by molecular and biological criteria (Maherali et al., 2007; Wernig et al., 2007). Shortly after the reprogramming of mouse cells had been achieved the generation of iPS cells from human fibroblasts was reported (Takahashi et al., 2007; Yu et al., 2007).

While genetic experiments have established that Oct4 and Sox2 are essential for pluripotency (Chambers and Smith, 2004), the role of the two oncogenes, c-myc and Klf4, in reprogramming is less clear. Some of these oncogenes may, in fact, be dispensable for reprogramming as both mouse and human iPS cells have been obtained in the absence of c-myc transduction, although with low efficiency (Nakagawa et al., 2008; Wernig et al., 2008).

One of the promises of patient-specific ES cells is the potential for customized therapy of diseases. Previous studies have shown that disease-specific ES cells produced by nuclear cloning in combination with gene correction can be used to correct an immunologic disorder in a proof-of-principle experiment in mice (Rideout et al., 2002). In a similar approach, by using a humanized sickle cell anemia mouse model, it has been shown that mice can be rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPS cells (Hanna et al., 2007). Finally, it has been shown that iPS cells can be efficiently differentiated into neural precursor cells giving rise to neuronal and glial cell types in culture. Neural precursors derived from iPS cell were able to improve behaviour in a rat model of Parkinson's disease upon transplantation into the adult brain demonstrating the therapeutic potential of directly reprogrammed fibroblasts for neuronal cell replacement in an animal model (Wernig et al., 2008; Jaenisch, 2009).

10. Conclusion

Embryonic stem cells represent a powerful tool for future regenerative medicine due to their capacity of self-renewal and pluripotency. Studies in animal models have shown that transplantation of fetal stem cell, ES cells, or pluripotent stem cell derivatives can successfully treat many chronic diseases, such as Parkinson's disease, diabetes, traumatic spinal cord injury, Purkinje cell degeneration, Duchenne's muscular dystrophy, liver or heart failure, and osteogenesis imperfecta (Zhang et al., 1996; Horwitz et al., 1999; McDonald et al., 1999; Kobayashi et al., 2000; Li et al., 2000; Soria et al., 2000; Kim et al., 2002).

Almost every day there are reports in the media of new stem cell therapies. There is no doubt that stem cells have the potential to treat many human afflictions, including ageing, cancer, diabetes, blindness and neurodegeneration. In January 2009, the US Food and Drug Administration approved the first clinical trial involving human ES cells, just over 10 years after they were first isolated. In this trial, the safety of ES cell-derived oligodendrocytes in repair of spinal cord injury will be evaluated. Nevertheless, one of the attractions of transplanting iPS cells is that the patient's own cells can be used, obviating the need for immunosuppression (Watt and Driskell, 2010).

Adult tissue stem cells, ES cells and iPS cells can all be used to screen for compounds that stimulate selfrenewal or promote specific differentiation programmes. Finding drugs that selectively target cancer stem cells offers the potential to develop cancer treatments that are not only more effective, but also cause less collateral damage to the patient's normal tissues than drugs currently in use (Watt and Driskell, 2010).

11. References

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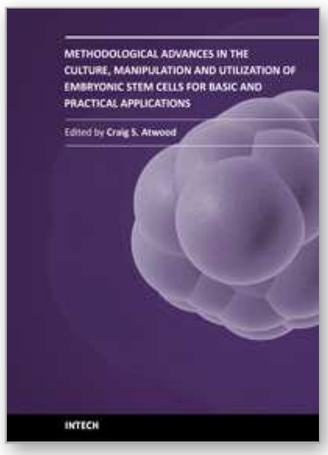

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