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ESC Cardiac Differentiation and Applications

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1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases are the leading cause of mortality worldwide. In the United States, there are more than three millions patients with a predicted increase to over six million by the year 2030. Unfortunately, current therapeutic methods are not effective and two-thirds of the patients die within five years of diagnosis (Kessler & Byrne, 1999). Thus it is important to discover new drugs which are effective. Application of high-throughput screening in the pharmaceutical industry can enhance procedures for new drug production, from target identification to preclinical compound evaluation. Reducing the lag phase between identification of a new component and production of new drugs requires cell-based methods for testing the efficacy and safety of new compounds. Application of stem cells in drug discovery has afforded new opportunities to better understand the action mechanisms of new targets, increase the safety to control their activity, reduce the amount of *in vivo* testing and evaluate components for different human genetic backgrounds. On the other hand, recently a biomedical approach called regenerative medicine have been developed. In this method a new pool of functional cells will be used in order to enhance the performance of damaged tissue. The ideal cell source for regenerative medicine should have the capacity to restore the organ's function, induce native repair and eliminate the risk of rejection by the host. In recent years, human embryonic stem cells (hES cells) with the ability of self-renewal and differentiation have been considered as ideal therapeutic cell candidates. Although tremendous developments in this field have been achieved, there are still large gaps between recent investigations and successful, safe application of these cells in humans.
In this chapter, a brief definition of ESCs followed by methods for directing their differentiation into a cardiac lineage will be presented followed by a discussion on the best strategies to purify cardiomyocytes. We will also provide a future glimpse into applications of hES cells in drug discovery and regenerative medicine.

2. Human embryonic stem cells

hES cells (Thomson et al., 1998; Reubinoff et al., 2000; Baharvand et al., 2004) are pluripotent stem cell lines which have the ability to differentiate into all three primary germ layers that

arise during development (ectoderm, mesoderm and endoderm) even after prolonged culture (Thomson & Marshall, 1998). The origin of these cells is the inner cell mass (ICM) of blastocysts. Blastocysts consist of an outer layer, the trophoectoderm. The ICM of blastocysts are cells within the trophoectoderm which give rise to all cells in the embryo. The trophoectoderm layer has a supporting role for embryonal tissues. In order to derive hES cells the trophoectoderm should be removed, and isolated ICM cells cultured on a feeder layer of mouse embryonic fibroblasts. After cells grow and form colonies they can be selected, passaged and expanded. For humans, ICM generated from IVF-produced embryos not used in the clinic and donated by individuals can be used to form hES cell lines.

hES cells have the ability for self-renewal, a continual high level of telomerase activity, and express Oct4 and Nanog which are two transcription factors that define ESC identity. They maintain a normal diploid karyotype and express undifferentiated markers such as stage specific embryonic antigen 3 (SSEA), SSEA4, Tra-1-60 and Tra-1-81 (Thomson et al., 1998; Amit et al., 2000; Reubinoff et al., 2000).

The pluripotency of these cells has been shown by injection into immunosuppressed mice. In mice, undifferentiated hES cells grew and formed teratoma which was comprised of all three embryonic layers. Pluripotency was further confirmed by the formation of three-dimensional embryoid bodies *in vitro* (Itskovitz-Eldor et al., 2000).

At first, cell populations needed to grow as compact colonies of undifferentiated cells on mouse embryonic fibroblast (MEF) (Thomson et al., 1998), human fetal fibroblast and adult epithelial cells (Richards et al., 2002) or foreskin cells as feeder layers to stay pluripotent, but later experiments have shown that hES cells can stay in an undifferentiated state if cultured on extracellular matrix such as matrigel or laminin, and in medium conditioned by MEF (Xu et al., 2001). Further investigations revealed molecules which are necessary for maintenance of hES cells in undifferentiated state *in vitro*. Basic fibroblast growth factor (bFGF) is one such protein that has been described to inhibit hES cell differentiation in the absence of feeder layers (Xu et al., 2001). Currently, they can be isolated and maintained in chemically defined medium (Ludwig et al., 2006; Yao et al., 2006). Presently, there are more than 400 hES cell lines but only 179 of these have been characterized in detail (Guhr et al., 2006). In an international study (ISCI), 59 hES cell lines were examined for the expression of several markers. Data from this experiment indicated that different cell lines show similar expression patterns despite different genetic backgrounds and derivation techniques (Adewumi et al., 2007). hES cells are attractive sources for studying cardiac differentiation because they can be isolated and survive in culture, expand greatly on feeder layer or feeder-free culture and differentiate into functional cardiomyocytes (Baharvand et al., 2005; Farokhpour et al., 2009; Kehat et al., 2001; Xu et al., 2002; Mummery et al., 2003) and form contractile clusters.

The development of hES cell technology and the ability to culture them *in vitro* has opened a new era in cell transplantation therapy, molecular genetics, drug discovery and developmental studies (Odorico et al., 2001). They also can be considered as a suitable source to determine the physiological and pharmacological properties of human cardiomyocytes and decrease the need for animals as test materials that are time-consuming and cost-prohibitive. However in order to use hES cells in cell therapy and drug screening, development of appropriate culture conditions and differentiation methods which are suitable for scale-up are needed.

3. Differentiation of hES cells to cardiac cells

3.1 Spontaneous differentiation

The most common way to initiate the differentiation of hES cells is embryoid body (EB) formation. These types of cell aggregates provide the essential signaling environment critical for cardiac differentiation. Development of the heart in vertebrates can be divided into four steps. The first step is the formation of an organizing center which secretes signals for endoderm and mesoderm induction. This step leads to activation of canonical wnt/β-catenin members such as wnt3a which then causes accumulation of β-catenin in the nucleus. β-catenin plays a key role in expression of the gene Nodal which induces and controles expression of mesoendodermal markers. In the second step Nodal, a member of the TGF-β family, causes mesoderm induction. Activation of the nodal signaling pathway will then lead to the expression of mesodermal genes, such as brachury (*bra*), in a concentration and duration-dependent manner. In the third step, after formation of the mesoderm, the wnt/β-catenin signal must be inhibited in order to continue with heart formation. Thus, several canonical wnt/β-catenin antagonists will be expressed to induce the precardiac mesoderm, such as DKK1. On the other hand, it has been shown that activation of the noncanonical Wnt signaling pathway stimulates expression of early cardiac markers such as Nkx2.5. The last step is differentiation of cardiac progenitors into beating cardiomyocytes and BMP signaling is critical for end stage gene expression.

The first report of differentiating hES cells into cardiomyocytes was in 2001. In brief, as hES cells cannot survive in single cells, they were dissociated into small clumps of 3-20 cells by collagenase and cultured in low attachment dishes for 7-10 days in suspension in the absence of self-renewal signals provided by MEF feeder layers or basic fibroblast growth factor (bFGF), then plated on gelatin coated plates for further differentiation. As the EBs mature, cells give rise to early embryonic lineages. In this method, four days after plating, 8.1% of the EBs contained beating clusters and showed a beat rate of 30-130 beats per minute. Approximately 30% of the cells in these clusters were actual cardiomyocytes and shown to express cardiac-specific structural genes, such as cardiac troponin I and T, atrial and ventricular myosin light chains (MLCs), atrial natriuretic peptide (ANP) in addition to cardiac transcription factors such as GATA4, Nkx2.5 and MEF2C (Kehat et al., 2001). Investigations indicate that these beating clusters contain cardiac cells which have been shown by several functional assays (extracellular and intracellular electrophysiological recordings, calcium imaging and pharmacological studies). They are structurally and functionally identical with nascent embryonic myocardium (Kehat et al., 2001; Boheler et al., 2002; Xu et al., 2002; He et al., 2003; Mummery et al., 2003; Passier & Mummery, 2005). The patterns of cardiac gene expression in differentiating hES cell derived cardiomyocytes is similar to embryonic cardiogenesis (Xu et al., 2006). During the early stages of differentiation, the expression of pluripotency markers such as Oct3/4, Cripto and telomerase reverse transcriptase (TERT) decrease while the mesoendoderm markers, such as Brachury, gradually increase. Subsequently, early cardiac transcription factors such as Nkx2.5, MEF2C, GATA4 and Tbx5 will be expressed. Later, the expression of cardiac structural proteins such as cardiac α-myosin heavy chain, cardiac β-myosin chain and atrial natriuretic factor appear (Beqqali et al., 2006; Synnergren et al., 2008; Synnergren et al., 2008). The similarity in the timeline of gene expression during cardiac differentiation and cardiac development indicate that data from developmental studies can be used to increase the efficiency of differentiation.

In the year 2002, other embryonic cell lines were differentiated into cardiomyocytes. On the 8th day of differentiation 25% of the EBs could beat while this number increased to 70% by the 20th day (Xu et al., 2002). A third group also reported the derivation of 10-25% beating cardiac cells from hES cells after 30 days (He et al., 2003). The reasons for the differences observed in numbers of contractile clusters are not clear, but it is assumed that the efficiency of different protocols which are applied to direct hES cells into cardiomyocytes depends on the properties of individual cell lines as well as propagation methods prior to differentiation. On the other hand, as individual EBs may contain different cardiac cells, higher beating EBs can not accurately reflect higher efficiency.

To study the events those occur during differentiation toward cardiac cells, whole genome approaches were applied. Initial investigations reported the genetic control of human embryonic development by studying the transcriptional profile of spontaneous differentiation (Brandenberger et al., 2004; Calhoun et al., 2004; Miura et al., 2004).These studies, by comparing undifferentiated hES cells and EBs, have provided important information about signals required for the self-renewal of hES cells. For example, they have indicated that FGF, WNT, NODAL and LIF pathways play important roles in maintaining pluripotency (Brandenberger et al., 2004).The first large-scale microarray has been performed 2, 10 and 30 days after EB formation (Dvash et al., 2004) and identified genes that are expressed during the early and late stages. Although these findings are valuable to understand transcriptional pathway which are critical in embryonic cardiac development, protocols to direct hES cells into cardiomyocytes with higher efficiency than spontaneous differentiation is required.

However because of heterogeneity, requirements for serum and other such limitations, spontaneous differentiation is not a proper method and much effort has been devoted to enhance the efficiency of cardiac differentiation. Thus, several techniques have been introduced to improve the efficiency of cardiomyogenic differentiation *in vitro*. One of the most common reasons for heterogeneity is that individual EBs differ in size and morphology. The first attempt to solve this issue was the application of "hanging drop" method. This method was routinely used for EB formation of mouse ES cells (Yoon et al., 2006). However this method was not successful when translated for hES cells. An alternative method was the forced-aggregation or the "spin-EB". In this method, to control the size of EBs, dissociated cells were centrifuged into V-formed ultra-low adherence 96-well plates (Ng et al., 2005; Burridge et al., 2007). Although this method provided same-size EBs, singly its application could not improve the efficiency of differentiation. Therefore several protocols have been developed to direct the differentiation toward cardiomyocytes. Strategies to induce cardiac specific differentiation rely on co-culture with different cell types, addition of different supplements to the medium and genetic manipulation of hES cells.

3.2 Directed differentiation

3.2.1 Co culture

Early efforts to find a method to enhance the differentiation of hEs cells into cardiomyocytes has led to the introduction of a new protocol by Mummery et al. (Mummery et al., 2002; Mummery et al., 2003; Passier et al., 2005). In this method, hES cells are co-cultured with mitomycin-treated mouse visceral endoderm-like cell line (END2) as a feeder layer and 12 days after co-culture, cells undergo cardiac differentiation (Mummery et al., 2003). The main advantage of this method is that hES cells are in direct contact with visceral endoderm and

their surface receptors are exposed to the autocrine and paracrine factors secreted by visceral endoderm, such as activin A and BMPs. As a result, this method mimics natural development because during development, the anterior endoderm provides signals for cells in the adjacent cardiac mesoderm and promotes differentiation into beating cardiomyocytes (Fullilove, 1970; Schultheiss et al., 1995). Furthermore, the anterior endoderm has the ability to induce cardiac differentiation of non-cardiac mesoderm (Sugi & Lough, 1994).

Whole genome microarray analysis has been performed on hES cells which were co-cultured with END2. By cluster analysis of its data, different clusters of genes corresponding to different levels of differentiation have been identified. These clusters consisted of genes which were down regulated after differentiation, such as OCT4 and NANOG, as well as genes attributed to early mesoderm formation, cardiac progenitors and fetal cardiomyocytes.

The presence of serum in differentiation medium is another factor which controls the efficiency of differentiation. Serum has negative effects on cardiac differentiation and by eliminating serum, the efficiency of co-culture with END2 increases more than 20-fold. On the other hand, the presence of serum in culture media converts the protocol into a less reproducible one because the exact component of serum is unknown and varies from one batch to another (Passier et al., 2005). The efficiency of this protocol has been further enhanced by addition of ascorbic acid. These findings have led to a near 25-fold increase in the number of beating areas per cell preparation. The phenotype of nearly 90% of cells derived with this protocol is similar to fetal ventricular cells, although atrial and pacemaker-like cells are also observed (Mummery et al., 2003). In another experiment, the differentiation efficiency has been optimized by removal of serum and insulin, and the addition of prostaglandin I2 to the culture medium (Zaffran & Frasch, 2002; Passier et al., 2005; Graichen et al., 2008; Xu et al., 2008). Co-culture has some advantages that limit the clinical application of this method. One obstacle is the risk of transmission of pathogens, especially viruses. In this situation, the differentiated cadiomyocytes would already be diseased and not suitable for transplantation, thus these cells could not be expanded for drug testing and toxicology.

Another limitation with the use of co-culture is the difficulty of separating co-cultured cell populations. Although cells can be separated by magnetic affinity cell sorting (MACS) (Siegel, 2002), this method does not have the proper purity. Fluorescence-activated cell sorting (FACS) can provide the highest degree of purity but the instrument is expensive, skill-intensive and not available to everyone (Herzenberg et al., 2002). Separation of co-cultured cell populations leads to detachment of the cells from each other and disrupts gap junctions which are necessary for electrical coupling between differentiating cardiomyocytes. These problems can be solved by using condition medium or commercially available Transwell inserts (Giovino et al., 2002).

Since all the previously mentioned protocols produce cardiomyocytes with low efficiency and past experiments with mouse embryonic stem (mES) cells have efficiently resulted to cardiomyocytes, therefore different cardiogenic reagents which have been shown to enhance the cardiac differentiation of mES cells were also tested on hES cells. Unfortunately, no significant improvement was achieved by addition of DMSO, retinoic acid (Kehat et al., 2001; Xu et al., 2002) or BMP-2 (Mummery et al., 2003; Pera et al., 2004). It is unclear whether these protocols need to be manipulated or whether these factors are not important in human cardiac differentiation. Investigators, by evaluation of various growth factors and agents, have provided a diverse array of agents capable of inducing cardiomyogenesis from ES cells.

3.2.2 Growth factors

As spontaneous differentiation is low and both undifferentiated and differentiated hES cells express receptors for different growth factors, the addition of appropriate growth factors in defined time and concentration may enhance differentiation toward cardiomyocytes (Xu et al., 2008).

Data from developmental studies indicate that members of the transforming growth factor (TGF) superfamily (BMPs and activin), the Wnt family, as well as the fibroblast growth factor family which are expressed in the ectoderm and endoderm adjacent to the heart-forming region have key roles in cardiac lineage induction (Filipczyk et al., 2007; Behfar et al., 2002). With the addition of BMP2 and SU5402, a FGF receptor inhibitor, into EB medium Tomescot et al. were successful in increasing expression of mesodermal and cardiac genes by more than three-fold (Tomescot et al., 2007). Burridge et al. indicated that by application of Activin A and FGF2 in the early stages of EB differentiation, the number of beating areas would increase five-fold (Burridge et al., 2007). Both of these experiments have proven that TGF-β superfamily members play an essential role in the cardiac differentiation of hES cells.

It has been shown that BMP signaling promotes commitment of cells into the cardiac lineage by posteriorization of the primitive streak, but it does not play a role in primitive streak induction. This induction is attributed to both the activin/Nodal and the canonical Wnt pathways (Nostro et al., 2008). Thus activin A, which is secreted from the visceral endoderm, has the ability to enhance cardiac differentiation (Yao et al., 2006; Laflamme et al., 2007; Yang et al., 2008). Based on this information, Laflamme et al. reported a differentiation protocol using RPMI that contained 2% B27 supplemented with activin A and BMP4, which obtained over 30% efficiency (Laflamme et al., 2007).

As well as BMP, members of the Wnt signaling pathway have the ability to increase cardiac differentiation efficiency by stabilizing β-catenin which activates cardiac gene expression (Lev et al., 2005). Activation of canonical Wnt signaling in the early stages of differentiation leads to mesoderm formation and enhances cardiac differentiation (Marvin et al., 2001; Schneider & Mercola, 2001; Ueno et al., 2007; Klaus & Birchmeier, 2009). With these results at hand, an improved differentiation protocol has been developed by Tran and co-workers. In this protocol, hES cells at the early stages of EB formation were treated by Wnt-signaling activator Wnt3a or BMP4, followed by reduction in the amount of serum and insulin in the medium (Tran et al., 2009). However, after mesoderm induction this signal inhibited further cardiac differentiation (Cohen et al., 2007; Qyang et al., 2007; Yang et al., 2008). In this stage, activation of non-canonical Wnt signaling such as Wnt11 activates protein kinase C and Jun N-terminal kinase signaling pathway and enhances cardiac differentiation (Terami et al., 2004).

In another stage, the specific differentiation protocol by Yang et al. succeeded in forming cardiac progenitor cells from hES cells by using a combination of activin A, BMP4,FGF2, VEGF and DKK1 in serum-free medium. They were able to identify a population of cardiovascular progenitors which had low expression of KDR and no c-kit (KDR low/c-kit neg) and observed that these cells had the ability to differentiate into cardiomyocytes with greater than 50% efficiency (Yang et al., 2008).

3.2.3 Small molecules

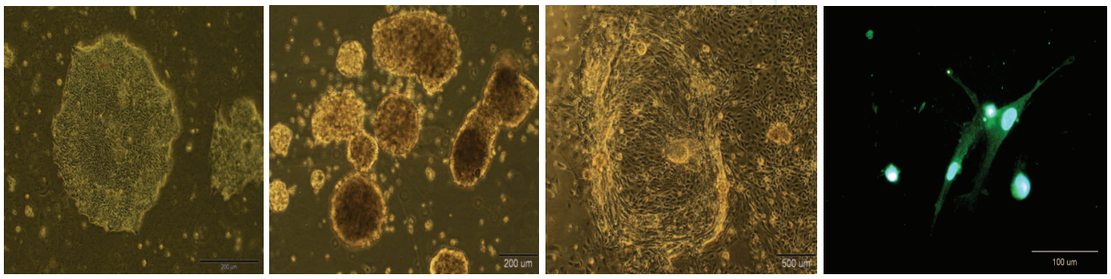
In addition to growth factors, synthetically small molecules have been described to have procardiogenic differentiation effects upon cultured ES cells by affecting a specific signaling pathway. These molecules usually have more half life in the medium and as they are

synthetic, they are more structurally and functionally defined. They are stable and inexpensive when compared with polypeptide differentiating agents. To find the best differentiating agent from a library, hES cells which express a reporter gene under the control of a cardiac specific promoter, are used. In this situation, by the addition of compounds from the library, active agents will be identified and the most appropriate one can be chosen. By using this method, Takahashi et al. have screened 880 small molecules and reported that ascorbic acid enhances the efficiency of cardiac differentiation. They noted that this property is independent from its antioxidant property, as other antioxidant agents did not have a positive effect on the efficiency of differentiation (Takahashi et al., 2003).
One factor shown to enhance differentiation into cardiomyocytes is the demethylating agent 5_deoxyazacytidine. Treatment of human EBs with 5_deoxyazacytidine increases expression of cardiac alpha-myosin heavy chain up to two-fold. (Xu et al., 2002).
5-Azacytidine and 5-aza-2′-deoxycytidine are cytosine analogues which are incorporated into DNA and form stable covalent complexes between DNA methyltransferases and DNA. They act as demethylation agents and cause expression of cardiac genes. Therefore treatment of hES cells with 5-aza can improve the efficiency of cardiac differentiation (Xu et al., 2002; Yoon et al., 2006).
Lithium chloride, by inhibition of glycogen synthase kinase 3β and Wnt3a-conditioned medium, increases differentiation efficiency (Nakamura et al., 2003). In addition, other small molecules which are inhibitors of p38 MAP kinase, such as SB203580, have the potential for mesoderm induction and experiments show that addition of such components into differentiation medium almost double the cardiogenic efficiency from 12% to 25% (Graichen et al., 2008). These data indicate that synthetically small molecules are useful agents for directing the differentiation, however experiments in this field still continue.

3.2.4 Suspension

hES cells are routinely cultured as adherent colonies, however culturing hES cells in an adherent position is labor intensive and requires a number of large incubators in order to provide enough cells for cell therapy or drug discovery. Although this kind of cell expansion supports hES cell lines in terms of growth and survival, it limits their application in large scale systems. As a result, several groups have tried to overcome this problem by propagation of hES cells in suspension and expansion of unattached hES cells in spinner flasks for short periods as reported (Lock & Tzanakakis, 2009; Nie et al., 2009; Oh et al., 2009; Krawetz et al., 2010). Recently, Steiner et al. have published a new protocol for prolonged culture of hES cells in suspension in the absence of microcarriers (Steiner et al., 2010). They cultured hES cells in neurobasal medium containing KO-SR, FGF2, activin A, Nutridoma-CS, ECM components and neurotrophic factors. Analysis indicates that cells after 10 passages in suspension culture express pluripotency markers such as SSEA-4,3, TRA-1-60, TRA-1-81 and OCT4; they also express alkaline phosphatase while showing a normal karyotype. These data indicate that cells after 10 weeks in suspension cultures are still pluripotent normal hES cells. These findings indicate that hES cells have the ability for large scale culture in bioreactors and have helped hES cell technology to move toward industrial applications.
hES cells have the ability to survive as single cells in the presence of Rho-associated kinase (ROCK) inhibitor (Watanabe et al., 2007). Thus, by treating hES cells with ROCK inhibitor at least 2 hours before dissociating them from Matrigel, our group succeeded to culture undifferentiated hES cells as spheroids in suspension. Six days after propagation in

suspension, the culture medium was changed to a differentiation medium. Differentiation was performed according to the Laflamme protocol (Laflamme et al., 2007). Briefly, cells were treated one day with 100 ng/ml activinA followed by 4 days with 10 ng/ml BMP4 in RPMI/B27 medium, then plated on gelatin-coated plates. In this protocol, beating clusters were observed 10-20 days post-plating (Fig.1). Our data have indicated that 20 days after plating more than 80% of the cells expressed Nkx2.5 when examined by flow cytometery, which is a marker of cardiovascular progenitors. Below, the gene expression profile of hES cells in different differentiation stages is presented (Fig.2). The advantage of this protocol is that it can be used in large scale culturing of cells in bioreactors with the intent to produce with high efficiency large numbers of contractile cardiac cells [unpublished data].



(a) (b) (c) (d)

Fig. 1. a) Undifferentiated adherent hES cell colony, b) spheroids c) a beating colony d) immunoflurecence staining of differentiated cells with anti-desmin antibody.

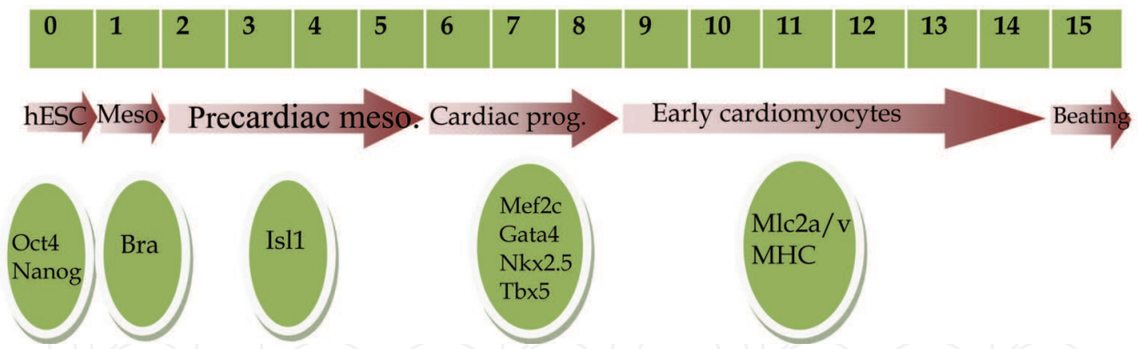


Fig. 2. Gene expression profile of hES cells during differentiation.

3.2.5 Genetic manipulations

Genetic manipulation of hES cells, whether transiently or by stable transformation, can provide a proper tool for understanding cardiac lineage specification and differentiation mechanisms, and lead to the establishment of a protocol for directing hES cells into cardiomyocytes. However, because of the poor transfection efficiency and difficulty of genetic manipulation of hES cells, reports for experiments in this field are limited (Moore et al., 2005). Experiments have demonstrated that transfection efficiency highly depends on the hES cell lines; overall, by the application of lentiviral infection a high efficiency (He et al., 2005), variable efficiency by plasmid transfection (Liew et al., 2007) or low efficiency with the use of adenoviral infection (Smith-Arica et al., 2003) can be achieved. Homologous recombination and electroporation for genetic modification of hES cells has been applied

and are the methods of choice for gene targeting in special hES cell lines (Zwaka & Thomson, 2003; Costa et al., 2007). Recently, gene transfers to 12 independent hES cell lines without the loss of stem cell markers has been reported (Braam et al., 2008).

By improving transfection efficiencies, the introduction of a reporter gene into hES cells provides a powerful tool to study gene expression profile during development (Eiges et al., 2001; Matin et al., 2004). Homologous recombination or RNA interference enables us to knockout a specific gene in order to explore its function during differentiation (Zwaka & Thomson, 2003). Overexpression of transcription factors which play important roles in differentiation might direct hES cells to the cardiac lineage (Grepin et al., 1997). As a result, Fijnvandraat et al. overexpressed TBX5 in P19C16 embryonic carcinoma cells and observed that transfected clones began to beat earlier and the number of beating areas tended to increase. Thus, this data indicated that TBX5 plays a role in increasing cardiogenesis (Fijnvandraat et al., 2003). Overexpression of other cardiac transcription factors such as Isl1 (Bu et al., 2009), Gata4 and Nkx2.5 have also been reported, and one of the best results was achieved by using a combination of two transcription factors Nkx2.5 and Tbx5 (Hiroi et al., 2001).

Transdifferentiation to cardiomyocytes is a new topic in cardiac differentiation which by the use of defined factors, different cells can be pushed to express cardiac genes. The transdifferentiation of mouse mesoderm into contractile cardiomyocytes has been reported with the introduction of Gata4, Tbx5 and Baf60c (which is specifically expressed in precardiac mesoderm) into cells (Takeuchi & Bruneau, 2009). Recently, Ieda et al. succeeded in transdifferentiating fibroblasts to cardiac cells. They have shown that with the application of just three cardiac transcription factors (Gata4, Tbx5 and Mef2c), dermal or cardiac fibroblasts can directly be converted to functional cardiomyocytes (Ieda et al., 2010). Although further investigation in this field is required, such experiments are valuable in better understanding both the developmental process and signaling pathways.

3.3 Selection

When a population of ESCs is differentiated into cardiomyocytes, yet another challenge exists with isolation and purification in order to avoid contamination by ESCs. Within EBs, a heterogeneous cell mixture exists; thus, obtaining a homogenous population of cardiomyocytes depends on a selection process. Currently, investigators have developed selection methods to further enrich *in vitro* differentiation populations. These methods fall into two main strategies: mechanical and genetic. In the mechanical strategy, contractile clusters can be isolated manually from a culture plate (Kehat et al., 2004). However, this is not a suitable technique for large-scale, high throughput isolation of cells which are required for pharmaceutical applications and, additionally, the final purity will range from 50-80%. Another mechanical separation method is the Percoll density gradient-based isolation method. Although this strategy improves the throughput of isolation in comparison with manual dissection, the degree of enrichment is insufficient for clinical or research purposes, as the highest purity of cardiomyocytes reported by this method is only 70% (Xu et al., 2002). A third strategy for enrichment of hES cell- derived cardiomyocytes is the formation of cardiac body. After differentiation, cells will be enzymatically dissociated into single cells. In this situation, cardiac cells will re-associate in suspension cultures and form cardiac bodies which contain more than 60% cardiomyocytes (Xu et al., 2006).

Another strategy for the isolation of differentiated cardiomyocytes involves sorting them on the basis of a surface marker expression. Recently, in an experiment cardiac cells were sorted by the endogenously expressed surface marker, ALCAM. However the application of this method is limited due to the lack of cardiomyocyte specific cell surface markers (Rust et al., 2009).

In genetic strategies, selective markers such as an antibiotic-resistance gene, colorimetric markers or cell-surface markers are expressed under the control of a cardiac specific gene. Therefore, when ESCs differentiate into cardiomyocytes and the promoter is activated, selective markers will be expressed and cardiomyocytes can be isolated by exposing cells to an antibiotic or FACS (fluorescence-activated cell sorting). Based on this, Klug et al. have used an antibiotic selection method to purify ES-cell-derived cardiomyocytes. In their research, a fusion gene which consisted of an alpha-cardiac myosin heavy chain promoter followed by an aminoglycoside phosphotransferase gene, an enzyme which causes resistance to the cytotoxic agent geneticin (G418). Once the transfected cells began to differentiate and the cardiac specific promoter was activated, aminoglycoside phosphotransferase was expressed and allowed them to survive under treatment of the culture system with G418. Surviving cells provided a population of cardiomyocytes with approximately 99% purity (Klug et al., 1996). In another experiment, a ventricular-specific promoter ,myosin light chain-2v was linked to enhanced green fluorescent protein (Muller et al., 2000). After differentiation of murine ES cells into cardiomyocytes, GFP protein was expressed and cardiac cells could be collected with 97% purity by FACS (Muller et al., 2000).

These data indicate that hES cells can be manipulated in order to obtain enhanced efficacy or purity during cardiac differentiation. Recent studies have proven the benefit of using transgenic approaches, which include cardiac resistance genes alone or in combination with reporter genes (Anderson et al., 2007; Xu et al., 2008; Kita-Matsuo et al., 2009). By the use of lentiviral infection, hES cells were transfected with a construct in which eGFP has been expressed under the control of a cardiac specific promoter (human myosin light chain-2V promoter) After EB formation, cardiomyocytes could be detected by GFP expression and sorted by FACS with more than 90% purity (Huber et al., 2007). By using the same method, cardiomyocytes derived from hES cells could be sorted on the basis of cardiac troponin I expression (Gallo et al., 2008). Another recent study used the same method to isolate cardiac cells based on the expression of dsRed fluorescence reporter gene under the control of MLC2v promoter (Fu et al., 2009).

All the above mentioned studies have selected matured cardiac cells, however due to poor differentiation efficiency and limited proliferative potential of cardiomyocytes, the application of these selected cells have not been fully established (Lyon & Harding, 2007). Therefore, several groups have focused on cardiac progenitors (Yamashita et al., 2005). Yang et al. reported the isolation of a cardiovascular progenitor from differentiated hES cells which were KDR low/c-kit neg in which it was observed that this population had the ability to produce more than 50% contracting cardiomyocytes (Yang et al., 2008). KDR is a marker which can be used for enrichment of early cardiac progenitors (Motoike et al., 2003; Ema et al., 2006; Kattman et al., 2006; Moretti et al., 2006; Yang et al., 2008). However KDR is not a suitable single marker for isolation of cardiac progenitors as it is expressed in different stages of differentiation. Other progenitors which have been selected were ISL1+ cells that give rise to cardiomyocytes, smooth muscle and endothelial cells. In this experiment, ISL1+ cells have been isolated and cultured on mouse embryonic fibroblasts (Bu et al., 2009). The advantage of ISL1 is that it is expressed in a special stage of differentiation and as cells start

to complete the process of differentiation, it will be rapidly down regulated. Thus, ISL1 can be considered as a proper pool of cardiac progenitors for different applications.

4. Applications

4.1 Drug discovery

Drug discovery is a long process of about 5-10 years which requires tremendous effort and money. However, all these efforts are necessary to ensure the safety and efficacy of new drugs. Statistics show that more than 40% of new drugs fail to pass phase III clinical trials, which then contribute to waste of time and money (Kessler & Byrne, 1999). Thus, it is critical to discover unacceptable targets in the early stages of development. Pharmaceutical industries need novel technologies to assist with improving the efficiency of drug discovery. High throughput technology in the fields of screening, evaluation and toxicity assays can be beneficial (Kola & Landis, 2004). High throughput screening is the automated screening of a library (more than 100 molecules) in order to find the best molecule for a specific purpose. In this case, ES cells are the proper choice for cell-based screening. In many cases the toxicity of new components will not be discovered until the final stages; in clinical trials or when tested on animals. However, using animals as experimental models are costly and surrounded with ethical and legal considerations. On the other hand, the answers from animal models cannot always be translated into humans. ES cell technology has overcome some of these problems and made the discovery process more efficient.

hES cells have the potential to differentiate into cardiac cells *in vitro*. Phenotypic analysis indicates that they are morphologically and ultrastructurally similar to adult cardiomyocytes, although their myofibrillar and sarcomeric organization are immature (Snir et al., 2003; Olson, 2004; Norstrom et al., 2006; Yoon et al., 2006). The answers from these cells may be different from the *in vivo* situation. Fortunately there are indications which prove the possibility of *in vitro* maturation of hES cell-derived cardiomyocytes (Snir et al., 2003). In this case they can approximate adult cardiomyocytes and can be used as pharmacological models. The technology to direct differentiation toward cardiac cells can reduce the heterogeneity and improve their application in screening. One of the most important advantages of cardiomyocytes derived from hES cells is that they keep their contractile ability in culture for an extended time which is beneficial for testing different components on the same cells. In this situation the answers can be compared easily.

Pharmaceutical industries have began to use hES cell-derived cardiomyocytes as models for evaluation of the biological activities of different components. Functional assays are necessary in different stages of drug discovery; from target identification to end stage detailed pharmacological analysis.

The application of hES cell-derived cardiomyocytes in drug development can be divided into two categories. The first category concerns the necessity of caridac drug discovery in cases where the heart is a diseased organ. One of the applications of hES cells in drug discovery is examining responses of cells with different genetic variations. On the other hand, cardiomyocytes can provide specific disease models (Friedrich Ben-Nun & Benvenisty, 2006). Secondly, when the safety of a new component for the heart needs to be tested, hES cells can be used in both fields of drug development.

Every new drug needs to be tested for safety. From 1991 to 2000 about 30%of the new components failed because of toxicology and clinical safety (Kola & Landis, 2004). A significant portion of the toxicity is due to cardio- and hepatotoxicity, thus it is necessary to

be sure that new components are safe for the heart before their approval for medical applications. Some of the mechanisms for cardiotoxicity are due to the formation of reactive oxygen species, apoptosis, altering proper molecular signaling or cardiac gene expression.
The unavailability of hES cells is one of the most important barriers for the application of hES cells in drug discovery due to the necessity of large scale expansion. On the other hand, after differentiation it is necessary to improve both homogeneity and cardiac cell yields. By overcoming some of the obstacles of hES cells, they can make the process of finding new drugs more efficient and easier as they reduce the need for *in vivo* experiments.

4.2 Cell therapy

Human adult cardiac muscles have low ability to regenerate (Bergmann et al., 2009) and only a limited number of species such as newts and zebrafish are able to renew their myocard, therefore loss of massive cell population as a result of infarction or other heart diseases is irreversible and often leads to heart failure (Kubo et al., 2008). Today, the treatment of choice to prevent death caused by heart failure is heart transplantation. However, because of a shortage of donor organs, the complication of rejection and difficulty of transplantation surgery, this strategy cannot be used easily. On the other hand, to prevent unwanted immune response lifelong immunosuppressive therapy is required and even after successful transplantation failures of donor organs are frequent.
Recent developments in molecular biology, stem cell culturing and tissue engineering have provided a biomedical approach called regenerative medicine. The main objective of cell therapy is to repopulate damaged tissue with a new pool of functional cells in order to perform its normal actions. Several different cells that have been considered as suitable sources are: skeletal myoblasts (Murry et al., 1996; Taylor et al., 1998; Menasche et al., 2003), bone marrow-derived hematopoietic stem cells (Orlic et al., 2001; Zafarghandi et al., 2010) and mesenchymal stem cells (Min et al., 2002; Shake et al., 2002; Toma et al., 2002; Mangi et al., 2003). All these cell types have serious problems and for this reason, have gained limited application in cardiac cell therapy. For example, skeletal myoblasts following transplantation can not express cardiac markers and beat in synchrony with the host myocardium (Reinecke et al., 2000; Leobon et al., 2003; Rubart et al., 2004). Similarly, hematopoietic stem cells and mesenchymal stem cells when injected into the heart rarely differentiate into cardiac cells (Balsam et al., 2004). Other cell sources for regeneration, therefore are necessary. hES cells with their ability to self-renew and differentiation to cardiac cells can be considered as a suitable source for cell therapy. Thus far, several experiments have shown their ability and potential for regenerating infarcted heart as they express molecular components required for electromechanical integration with host cardiomyocytes (Rust et al., 1997; Xu et al., 2002; Mummery et al., 2003; Caspi et al., 2007; Laflamme et al., 2007; Leor et al., 2007; Tomescot et al., 2007; van Laake et al., 2007).
In order to use hES cells in drug development or regeneration, some points need to be considered. First, a scalable system for culture and maintenance of these cells in an undifferentiated state is necessary. Additionally, a well-defined protocol to direct hES cells toward cardiac cells with a high efficiency, a method to isolate and further purify cariomyocytes, a system to check their functionality and finally a high throughput system to test a library of candidate components are all necessary.
The main obstacle of cell therapy is the isolation of purified cardiomyocyte populations suitable for transplant.

As a result, several groups pushed ahead with experiments to evaluate ES cell-derived cardiomyocytes transplanted into the hearts of animal models. Both Gepstein (Kehat et al., 2004) and Li's groups (Xue et al., 2005), in separate experiments, spontaneously transplanted contracting EBs from hES cells into uninjured hearts to show electrochemical integration of transplanted cells with the heart and provide strong evidence of this ability, at least in an uninfarcted heart. In the year 2005, transplantation of hES cell- derived cardiomyocytes into arrythmic rat hearts demonstrate that they can successfully engraft, proliferate and express several cardiac markers, without evidence of teratoma formation up to 4 weeks post-transplantation (Laflamme et al., 2005). Engraftment of these cells into guinea pig hearts have been tested as well and their ability for function and integration with host cardiac tissue has been proven (Xue et al., 2005). Tomescot et al. showed that treatment of hES cells with BMP2 and SU5402 (a fibroblast growth factor receptor inhibitor) followed by transplantation into infarcted rat hearts lead to improvement in the heart performance without teratoma formation (Tomescot et al., 2007). Moreover, electrophysiological analysis indicates that transplanted cells functionally integrated into host myocardium. These data confirmed past evidence that showed electromechanical and structural coupling of transplanted hES cell-derived cardiomyoblasts with the host myocardium in pigs (Kehat et al., 2004). Although these findings suggest that hES cell-derived cardiomyoblast transplantation might be feasible and safe, but before such therapy is applied in humans several concerns must be considered. Some major areas for concern are: immunogenicity, risk of post-transplant cellular misbehavior such as teratoma formation, controlling cell survival and delivery, potential for post-transplant arrhythmogenecity as well as ethical concerns of isolating hES cells from human embryos.

Immunorejection by recipients may decrease the therapeutic potential of transplanted allogeneic hES cells. Because of conflicting evidence by several investigators, it is still unclear whether hES cells are immunoprivileged or not. Drukker et al. have shown that hES cells have low natural killer cell receptor expression (Drukker et al., 2002). In another experiment Li et al. have shown that hES cells did not elicit an immune response when injected into the muscle of immunocompetent mice (Li et al., 2004). In contrast to these findings, some groups have indicated that hES cells can cause an immune response in the host. So, immunogenicity of these cells should be examined before transplantation. One approach to prevent immunorejection of transplanted cells is to minimize allo-antigenic differences between donor and recipient. This can be achieved by establishing a bank of different MHC antigens such as the banks for tissue and organ transplantation. Another approach is to make universal hES cell lines by silencing or modulation of genes associated with the immune response.

Another potential problem of ES cell therapy is the risk of teratoma formation. It is obvious that undifferentiated hES cells have the potential for tumorogenicity. Although further experiments have shown that the pure populations of cardiomyocytes do not create teratomas, there is still the risk of contamination with undifferentiated or dedifferentiated cells. Therefore, selection strategies to separate fully differentiated cardiomocytes from mixed populations are necessary. Another strategy is to genetically manipulate hES cells in order to activate apoptotic signals in undifferentiated cells at special time points after directed differentiation.

One of the most serious problems of cell transplantation is that only a few number of engrafted cells will be survive (Robey et al., 2008). To solve this problem Laflamme et al.

used a "pro-survival cocktail" to improve cell survival after transplantation. This cocktail consists of a mixture of anti-oncotic and anti-apoptotic factors which cause cell survival in the harsh infarct environment (Laflamme et al., 2007). The issue of cell delivery and survival still needs further investigations and optimizations.

Ethical concerns are another serious barrier to the application of hES cells in a clinic setting. Currently, the production of hES cell lines leads to the destruction of embryos which have the potential to live. Thus, new strategies for generating hES cell lines are needed. In the year 2006, the production of ES cells from single cell biopsies of a developing mouse embryo has been reported. If such a strategy can be translated into humans, several ethical concerns will be solved.

Recently, growing evidence suggest that there are multipotent cardiovascular progenitors (Beltrami et al., 2003; Oh et al., 2003; Laugwitz et al., 2005; Smith et al., 2007; Kattman et al., 2006; Yang et al., 2008; Bu et al., 2009).They can be isolated from the adult heart or derived from hES cells. These cells can be separated from adult myocardium on the basis of spheroid or expression of special markers such as c-kit, Isl1 (Laugwitz et al., 2005) , Sca-1 (van Vliet et al., 2008) and Abcg2 (Martin et al., 2004). By injecting c-kit+ population into an infarcted rat model, ventricular function could be improved. In this experiment, transplanted cells were positive for cardiac myosin, but morphologically smaller than cardiomyocytes, yet similar to fibroblast cells (Dawn et al., 2005). The Sca-1+ population can differentiate into cardiac cells by treatment with 5-azacytidine. These populations demonstrated proper survival and integration after transplantation. However, the use of 5-azacytidine in clinical application is not safe as it can cause widespread DNA demethylation. Although these progenitors seem to be suitable tool for cardiac regeneration, still more investigation is required in order to fully characterize them and understand their nature.

Experiments indicate that cardiac stem cells have the ability to functionally integrate with myocardium when injected to infarcted rat and mouse hearts (Bearzi et al., 2007). These populations have less risk of teratoma formation but less differentiated cells have more potential to integrate and survive.

An alternative approach is the use of induced pluripotent stem cells (iPS) which are reprogrammed adult cells. It has been shown that iPS cells derived from dermal fibroblast are able to differentiate into cardiomyocytes (Takahashi & Yamanaka, 2006; Yu et al., 2007). The other application of iPS cells is that they can form models of human disease to study molecular mechanisms involved in disease, drug screening, safety and toxicology on cells of different genetic backgrounds and investigate new treatment opportunities (Dimos et al., 2008; Park et al., 2008).

5. Conclusion

Taken together, the data that has been reviewed above indicate that hES cells are pluripotent cells which have the ability to differentiate into cardiomyocytes *in vitro*. The process of differentiatin can be both spontaneous and directed. Although experiments show that directed differentiation has more efficiency than spontaneous differentiation, however the efficiency is not satisfiable for scientists in order to apply differentiated cells in drug discovery or regenerative medicine. The main obstacles in the way of developing a proper protocol for directing hES cells toward the cardiac fate is our limited knowledge of the exact signaling pathways which control cardiac differentiation. As the best protocol is the one which mimics processes in normal development, by improving our knowledge of

developmental processes and finding growth factors which have the ability to induce cardiac differentiation and/or genes involved in natural development, therefore new, nore effective protocols can be achieved which will cause huge improvements in the fields of new drug discoveries for cardiovascular patients and cell therapy.

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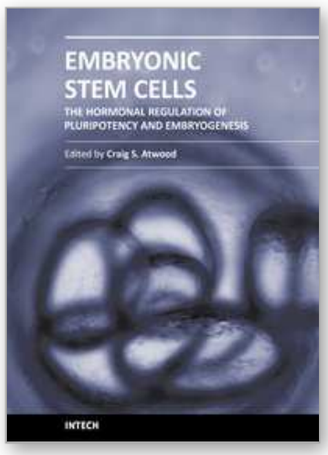

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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