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Recent Advances in Controlling Cardiacmyoctye Differentiation from Embryonic Stem Cells

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1. Introduction

As a high proliferative ability cell line, embryonic stem (ES) cells can be propagated in undifferentiated state, while maintaining their pluripotency to form various kinds of adult tissue cells (Thomson et al., 1998). Under appropriate conditions, ES cells can from embryoid bodies (EBs) and subsequent differentiation into cardiacmyoctyes (CMs) that retain the function of excitability and spontaneous contractions (Wobus et al., 1991) (Maltsev et al., 1994). These spontaneously beating cells contained in EBs have almost the same ion channels that control pacemaker function in the heart (He et al., 2003) (Yanagi et al., 2007).

Although multipotent progenitors have previously been shown to give rise to CMs, smooth muscle, and endothelial cells, the mechanism governing the generation of large numbers of differentiated progeny remains poorly understood. Current research indicates that the level of differentiation is low and that few cultured EBs will ultimately convert into functional CMs (Norstrom et al., 2006). The potential use of ES cells to replace functional loss of particular tissues may depend on efficient differentiation protocols to derive tissue-specific cells. By manipulating the culture conditions in which ES cells differentiate, it has been possible to control and restrict the differentiation pathways and thereby generate cultures enriched in lineage-specific cells in vitro. Regulation of multipotent cardiac progenitor cell expansion and subsequent differentiation into CMs, smooth muscle or endothelial cells is a fundamental aspect of basic cardiovascular biology and cardiac regenerative medicine.

More recently, direct reprogramming of adult somatic cells to become pluripotent ES-like cells (a.k.a. induced pluripotent stem cells, iPS cells) has been achieved. The cardiogenic potential of iPS cells is comparable to that of ES cells and that iPS-derived CMs (iPS-CMs) possess all fundamental functional elements of a typical cardiac cell, including spontaneous beating, hormonal regulation, cardiac ion channel expression and contractility. Therefore, iPS-CMs can be regarded as a potentially valuable source of cells for in vitro studies and cellular cardiomyoplasty (Pfannkuche et al., 2009). The availability of human ES cells and iPS cells, and their successful differentiation into genuine human cardiac cells have enabled researchers to gain novel insights into the early development of the human heart as well as to pursue the revolutionary paradigm of heart regeneration.

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2. Embryoid body formation

There are two major ways for EB from ES cells in vitro: suspension culture and hanging drop methods. In suspension culture, ES cells will differentiate into aggregates known as EBs in a manner similar to the early embryo. Suspension methods allow spontaneous aggregation of EBs but usually give rise to heterogeneous cell clusters. The heterogeneous size and shape of EBs resulting from suspension culture can influence their differentiation potential. Hanging drop methods can provide relatively uniform EB size but technically challenge by limited number of EBs for characterization. Recently, other methods such as using spinner flasks and bioreactors for stirred-suspension culture have been utilized. Enhanced growth of EBs in spinner flasks has been reported due to effective nutrient and oxygen supply. In addition to stirred-suspension culture for scalable production of EBs can provide a readily available source of CMs (Lecina et al., 2010). These strategies have been shown to be advantageous compared to conventional suspension method due to their ability to control EB-EB interactions.

Without induced differentiation, the differentiation level is low and that few cultured EBs will ultimately convert into functional CMs. Kehat et al. (Kehat et al., 2001) cultured human ES cells in suspension and plated to form aggregates termed EBs. Spontaneously contracting Cells appeared in only 8.1% of those EBs. Controlling the differentiating pathways permitting efficient generation of more mature and functional cells for basic studies or cardiac repair applications is a pressing need. As molecular mechanisms and signaling pathways leading to efficient differentiation from ES cells have not been understood and well-summarized, the potential factors that impact CM differentiation from ES cells include cell factors, drugs, hormone, culture method, and extracellular matrix.

3. Cell factors and CM differentiation

Schuldiner el at. (Schuldiner et al., 2000) examined the potential of eight growth factors including basic fibroblast growth factor (bFGF), transforming growth factor beta1 (TGF-beta1), activin-A, bone morphogenic protein 4 (BMP-4), hepatocyte growth factor (HGF), epidermal growth factor (EGF), beta nerve growth factor (βNGF), and retinoic acid to direct the differentiation of human ES-derived cells in vitro. They found that the EBs differentiate selectively into mesodermal, endodermal, or ectodermal lineages. However, these studies did not result in homogeneous differentiation of ES cells.

Many differentiating factors, including hepatocyte growth factor (HGF), transforming growth factor beta1 (TGF-β1), bone morphogenic protein (BMP)-2/4, and fibroblast growth factor (FGF) have been investigated in attempts to improve the efficiency of specific CM differentiation processes in mouse or human ES cells (Gassanov et al., 2004) (Roggia et al., 2007) (Schuldiner et al., 2000) (Ronca et al., 2009). These investigations have had some early encouraging results, but pluripotency and cellular differentiation are intricate biological processes that are coordinately regulated by a complex set of factors and epigenetic regulators, which need increasing research aiming to understand the molecular mechanisms that regulate stem cell differentiation.

Growth factor signaling is required for cellular differentiation, tissue morphogenesis, and tissue homeostasis. Wnt proteins are one important family of growth factors and its signaling is an important regulator of differentiation and morphogenesis that can also control stem cell fates. Tran et al. (Tran et al., 2009) found that mesendoderm formation and

CM differentiation were enhanced by early and transient treatment of human ES cells with Wnt3a. The induction of mesoderm and subsequent cardiac differentiation from human ES cells requires fine-tuned cross talk between activin A/BMP4 and Wnt/beta-catenin pathways (Paige et al., 2010). Cripto is one of epidermal growth factors and its signaling pathway is recently reported as essential role for cardiac myogenesis in ES cells. D'Aniello et al.(D'Aniello et al., 2009) demonstrated that APJ/apelin in the Cripto signaling pathway governs mesoderm patterning and CM differentiation via activation of mitogen-activated protein kinase/p70S6 through coupling to a Go/Gi protein. Hypoxia failed to induce differentiation of mouse ES cells into CMs in the absence of Cripto-1 expression, demonstrating that Cripto-1 is required for hypoxia to fully differentiate mouse ES cells into CMs (Bianco et al., 2009). Phosphorylation of fibroblast growth factor receptor-1 is also reported as essential process required for CM differentiation in ES cells (Ronca et al., 2009).
However, the differentiation of ES cells inevitably results in a heterogeneous mixture of cell types present in EBs. It would be more difficult to transform these into a homogenous population of CMs or to stop their differentiation at the functional CM stage. Neuregulin (NRG)-1beta/ErbB signaling regulates the ratio of nodal- to working-type cells in differentiating human ES cell-CM cultures and presumably functions similarly during early human heart development. By manipulating NRG-1beta/ErbB signaling, it will be possible to generate preparations of enriched working-type CMs (Zhu et al., 2010). Calreticulin may have a housekeeping role to play in mature CMs as well as during cardiomyogenesis. Calreticulin, an ER Ca (2+) storage protein, is a crucial regulator of cardiomyogenesis whose presence is required for controlled CM development from ES cells (Papp et al., 2009). Sik1 (salt inducible kinase 1), a serine/threonine kinase that belongs to the stress- and energy-sensing AMP-activated protein kinase family, involves in cardiac cell differentiation and/or heart development. Romito et al. (Romito et al., 2010) studied the role of sik1 in cardiac differentiation and found that sik1-mediated effects are specific for cardiomyogenesis regulating cardiomyoblast cell cycle exit toward terminal differentiation.
MicroRNAs are a newly discovered endogenous class of small noncoding RNAs that play important posttranscriptional regulatory roles by targeting messenger RNAs for cleavage or translational repression. microRNAs as important transcriptional regulators may provide a new means of manipulating stem cell fate (Sartipy et al., 2009). By modulating miR-1 and -499 expression levels, human ES cells function can be altered and differentiation directed, thereby enhancing cardiomyogenic differentiation (Sluijter et al., 2010).

4. Drugs used for the CM differentiation of embryonic stem cells

Dimethyl sulfoxide (DMSO), 5-azacytidine, retinoic Acid (RA), ascorbic acid have been investigated in attempts to improve the efficiency of CM differentiation processes in mouse or human ES cells (Paquin et al., 2002) (Yoon et al., 2006) (Drab et al., 1997) (Takahashi et al., 2003). Recently, the synergy of these drugs in inducing ES cell differentiation into matured and functional CMs is quite attractive. Chan et al. (Chan et al., 2009) have investigated Salvianolic acid B-vitamin C synergy in cardiac differentiation from ES cells and found that Salvianolic acid B (saB)- ascorbic acid synergy improves ES cell differentiation into CMs.
Recently, accumulating evidence points to reactive oxygen species (ROS) as important signaling molecules for CM differentiation from ES cells. Endogenous ROS control is important for CM formation from ES cells (Crespo et al., 2010). Nitric oxide (NO) has been shown to promote ES cell differentiation by increasing both the number and the size of

beating foci in EB outgrowths (Kanno et al., 2004). Exogenous electric fields have been implied in CM differentiation of mouse ES cells and the generation of ROS, which plays a role in CM differentiation of human ES cells, through mechanisms associated with the intracellular generation of ROS (Serena et al., 2009).

5. Hormones used for the CM differentiation from embryonic stem cells

Recent researches indicate there are some hormone receptors existing in ES cells, so hormone used as inductor to promote the CM differentiation from ES cells receives increasing attention. ES cells expressed cell surface kappa opioid receptors. The prodynorphin gene and its product, dynorphin B, have been found to promote cardiogenesis in embryonic cells by inducing the expression of GATA-4 and Nkx-2.5, two transcription factor-encoding genes essential for cardiogenesis (Ventura et al., 2003). Oxytocin receptor was discovered in undifferentiated ES cells and derived differentiated cells, which plays an important role in cardiogenesis by promoting CM differentiation. Treatment with oxytocin improves the EBs with spontaneous contraction, but has no effect on ultrastructural characteristics of CMs in any stage of development (Paquin et al., 2002) (Hatami et al., 2007). Thyroid hormone is essential for normal cardiac development and function, Lee et al. (Lee et al., 2010) showed that Thyroid (3) supplementation promotes cardiac differentiation of ES cells and enhances maturation of electrophysiological, as well as calcium homeostasis, properties of ES cell-derived CMs.

6. Co-culture differentiation

Although progress has been made towards differentiating stem cells to specific cell lineages, the efficiency is often poor and the number of cells generated is not suitable for therapeutic usage. Recent studies demonstrated that controlling the stem cell microenvironment can influence differentiation. As the differentiation of ES cells closes the development of the embryonic heart (Banach et al., 2003), factors that contribute to essential functions during early embryogenesis are expected to be involved in the formation of EBs (Behfar et al., 2002). However, the precise growth factor combinations that enhance cell differentiation in ES cells are unclear (Schuldiner et al., 2000). Consequently, ES cells are co-cultured with special cells or conditioned media to promote differentiation. For example, when ES cells are co-cultured with visceral-endoderm-like (END-2) cells, the majority (>90%) of the ES -CMs have a phenotype similar to fetal ventricular cells (Mummery et al., 2003). The efficiency of cardiogenic differentiation in ES cells can be readily enhanced by a culture medium that has been conditioned by END-2 cells (Graichen et al., 2008). Similarly, when medium conditioned by mouse embryo fibroblasts is used, the homogeneity of beating EBs can be significantly improved (Burridge et al., 2007).

The niche in which stem cells reside and differentiate is a complex physicochemical microenvironment that regulates cell function. Local microenvironments are considered to be the key inducers in directing the site-specific differentiation of ES cells. Recently, it is reported that endothelial cell play an essential role in facilitating CM differentiation from pluripotent stem cells and regulate regeneration of CMs via EphB4 signaling (Chen et al., 2010). Nevertheless, the effect and the mechanism of the cardiac microenvironment on the development of CM differentiation and EB growth have not yet been systematically studied. We developed a novel protocol to generate functional CMs from ES cells by an in vitro

indirect co-culture inducing strategy that looks promising for tissue engineering applications (fig.1). The propagation and CM differentiation of an ES cell line were studied in prolonged culture in this system (fig. 2). ES cells cultured on membranes by indirect co-culture with native cardiac cells were significantly enhanced ES cells to differentiate into CMs that possessed expression of cardiac specific genes and functions of excitability and

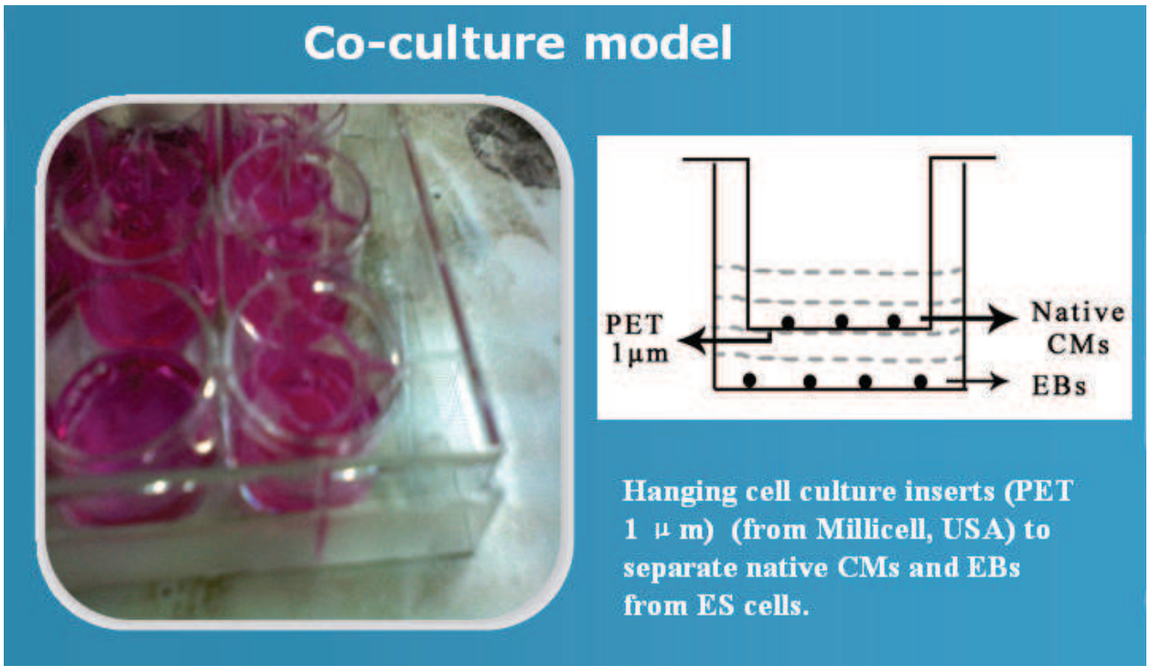

Fig. 1. The indirect co-culture system was used for CM differentiation from mouse ES cells.

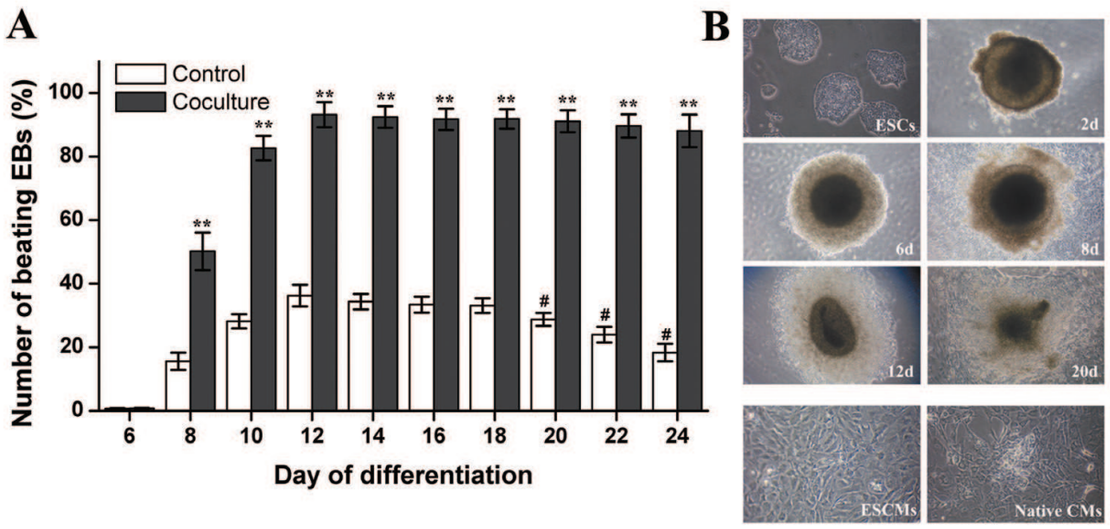

Fig. 2. The differentiation of embryonic stem cells (ES cells).
(A) The percentage of embryoid bodies (EBs) with spontaneously contracting areas in control and co-culture groups during plating culture.
(B) The in vitro differentiation process of ES cells in co-culture system.

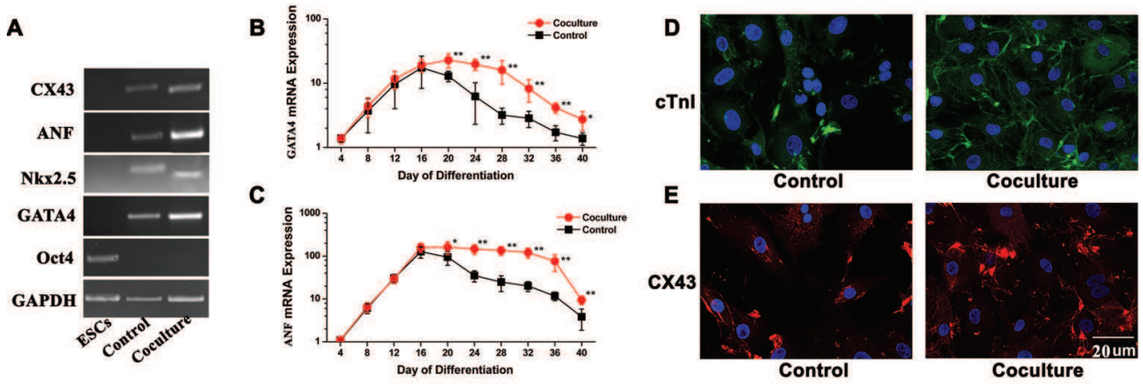

Fig. 3. The expression of cardiac markers in ES cells. (A) OCT4, GATA4, Nkx2.5, ANF and CX43 expressions were almost increased in the co-culture system. (B),(C) Real time-PCR was performed to detect mRNA expressions of GATA and ANF in prolonged time course. *: $P<0.05$, **: $P<0.01$. (D),(E) the protein expression of cardiac troponin I (cTnI) and connexin 43 (Cx43) in co-culture system.

spontaneous contractions (fig. 3). This co-culture system is a significant advance in ES cells culture methods, providing possibility to efficiently control the differentiation of ES cells and to more reliably obtain a large number of more mature and homogenous CMs for large-scale tissue engineering applications and other stem cell-based regenerative medicine (Ou et al., 2009a).

7. 2D vs. 3D differentiation

Previously the most common model in vitro for cell research has been 2D monolayer culture. Even though EB has 3D structures, the researches on the differentiation of ES cells are mainly based on 2D cultured systems. However, these 2D cultured systems don't mimic the physiological environment in vivo and may lead to low differentiated level of ES cells. Significant differences were found in the differentiation efficiency of ES cells when they were cultured in 2D and 3D environment (Liu et al., 2006).

The extracellular microenvironment for 3D differentiation plays a significant role in regulating ES cells to differentiate into special lineages, as well as in cell migration and proliferation (Philp et al., 2005) (Chen et al., 2007). The extracellular matrix is in close contact with cells and involved in their maintenance and regulation. The mechanical and chemical signals (e.g., integrin signals) elicited by the extracellular matrix regulate the activities of cytoplasmic kinases, receptors for growth factor and ion channels, impacting differentiation of stem cells. Extracellular matrix proteins independently trigger differentiation of human mesenchymal stem cells and that differentiation in this context can be guided down multiple lineages using the same Extracellular matrix protein stimulus (Santiago et al., 2009). A new scalable bioprocessing system as a scalable platform for CM production has been developed (Lecina et al., 2010). Use of a 3-D cuboidal microwell system to culture human ES cells in colonies of defined dimensions, 100-500 microm in lateral dimensions and 120 microm in depth, enabled formation of more uniform-sized EBs. The regulation of microwell-engineered EB size can be used for more efficient and reproducible formation of human ES cell-CMs (Mohr et al., 2010). In addition, proliferation of stem cell-derived CMs appears to be regulated by microtopography through tension-generation of contractility in the third-dimension (Biehl et al., 2009).

Bioreactors, recent methods to achieve CM differentiation by engineering the stem cell microenvironment, are used to control the differentiation and produce large numbers of desired cells. Based on bioreactors, it is very likely to control the differentiation of ES cells: (1) biomaterials technology, biomaterials with different growth factors or morphogenetic factors delivery systems can be utilized to mimic embryogenic process, our unpublished data demonstrates that specific factors released from biomaterials via diffusion can enhance long-term survival and differentiation of ES cells, and (2) the use of bioreactors, cultivation in rotating bioreactors can promote and maintain CMs electrophysiology and molecular properties (Bursac et al., 2003).

8. The potential applications

The availability of ES and iPS cells and their successful differentiation into genuine human cardiac cells have enabled researchers to gain novel insights into the early development of the human heart as well as to pursue the revolutionary paradigm of heart regeneration. Their potential applications for ES cells in cardiac research area include developmental biology, functional genomics, pharmacological testing, cell therapy, organ regeneration, and biological pacemaker.

8.1 Pharmacological testing

Recent withdrawals of prescription drugs from clinical use because of unexpected side effects on the heart have highlighted the need for more reliable cardiac safety pharmacology assays. Human or mouse ES cell-derived CMs serve together as a valuable model for drug safety screening and advance preclinical drug development. Assays based on human ES cell-derived CMs could complement or potentially replace some of the preclinical cardiac toxicity screening tests currently used for lead optimization and further development of new drugs (Braam et al., 2010) (Liang et al., 2010). Human ES cell-derived CMs have been used to predicate of drug-induced cardiotoxicity, e.g., to study cardioprotective effects of dexamethasone in doxorubicin cardiotoxicity (Farokhpour et al., 2009) (Braam et al., 2010).

8.2 Cell therapy

ES cells, which can be differentiated into cardiac progenitors and CMs, represent a candidate cell source for cardiac cell therapy. However, it is not known what specific cell type or condition is the most appropriate for transplantation. It is reported that transplanted mouse ES cells in the infarcted heart inhibit apoptosis, fibrosis, and hypertrophy, thereby reducing adverse remodeling, resulting in the improvement of cardiac function (Singla et al., 2007) (Singla and McDonald, 2007) (Xie et al., 2007). Several recent studies have demonstrated that human ES cell-derived CMs survive after transplantation into infarcted rodent hearts, form stable cardiac implants, and result in preserved contractile function. To investigate the ischemic environment of host myocardium affects transplanted pluripotent cells, van Laake et al. (van Laake et al., 2009) cultured mouse ES cells in medium containing ischemic myocardial interstitial fluid (iMIF) and found that the ischemic/infarcted environment is favorable to stem cell-mediated angiogenesis, but hostile to cardiac myogenesis. These findings indicated mouse ES cell-mediated improvement of cardiac function after transplantation of pluripotent cells do not reflect remuscularization. Although cell transplantation has modestly improved cardiac function, major challenges including increasing cell survival, engraftment, and functional integration with host tissue.

8.3 Cardiac muscle regeneration

Tissue-engineered cardiac muscle transplantation may have advantages over direct cell transplantation or replacement therapy for myocardial defect. The use of engineered heart tissue as a model system to accelerate development of cardiac cell therapy strategies has been demonstrated (Song et al., 2010). Human ES cell-derived CMs alone or with human endothelial cells (human umbilical vein endothelial cells) and embryonic fibroblasts (triculture constructs) were seeded onto biodegradable porous scaffolds. This tissue-engineered human vascularized cardiac muscle have been established in vitro and transplanted in vivo to form stable grafts (Lesman et al., 2010b) .

Effective engineering of viable thick complex tissue-constructs requires intense vascularization. More recently, a beating human cardiac muscle-construct containing an endothelial network was established by co-culturing human embryonic stem cell-derived-CMs, fibroblasts, and endothelial cells within biodegradable scaffolds (Lesman et al., 2010a). The success of cardiac muscle regeneration and transplantation is dependent on a combination of several factors, such as: 1) the formation of new blood vessels, 2) the release of pro-survival, pro-angiogenic and anti-inflammatory factors (paracrine effect), and 3) the functional contribution of CMs and functional integration with host tissue.

Components of the extracellular matrix are considered to be important physiological regulators and provide mechanical cues, direct differentiation and improve cell engraftment into damaged tissue. However, there are also adverse effects of scaffold materials, e.g. necrosis at the tissue core, low vascularization, and poor survival after transplantation. Scaffold-free for vascularized human cardiac muscle tissue that markedly improved viability after transplantation has been developed (Stevens et al., 2009).

8.4 Biological pacemaker

Sudden cardiac death due to abnormal heart rhythm from sick sinus syndrome kills millions of people each year, the reduction of the morbidity and mortality depends on how we can restore the generation of the cardiac impulse effectively. A conventional approach is implantation of electronic pacemakers, which are commonly used to treat cardiac impulse generation defects. However, there are many shortcomings for electronic pacemakers in clinical applications, such as the limited battery life, lack of catecholamine responsiveness, and associating with inconvenience in daily life (Rosen et al., 2004). Biological cardiac pacemaker, mimicking the sinoatrial node (SA-node), has been served as a promising way to restore the generation of the cardiac impulse for better alternative to the present routine.

ES cells can be induced to differentiate into CMs with excitability and spontaneous contraction activities (Wobus et al., 1991) (Maltsev et al., 1994), which raises the prospect of the probable therapeutic application of ES cells in cardiac pacing. Their use as biological pacemakers has also been explored (Kehat et al., 2004) (Xue et al., 2005) (Menasche, 2004 ; Yanagi et al., 2007). Kehat et al. (Kehat et al., 2004) had discussed the potential of human ES cell-derived CMs to act as a rate-responsive biological pacemaker. The transplanted human ES cell-derived CMs can functionally integrate with other quiescent ventricular cells and pace the heart of swine with complete AV block, as assessed by detailed three-dimensional electrophysiological mapping and histopathological examination. Xue et al.'s work (Xue et al., 2005) was similar with Kehat's. A functional human ES cell-derived pacemaker could be implanted in the left ventricle in vivo and successful spread of membrane depolarization was confirmed from surrounding myocardium in the site of injection.

Nevertheless, the use of ES cells in cardiac pacing is challenged by how to control the differentiation of ES cells and overcome the neoplasia, proarrhythmia or immunogenicity after transplantation. As a potential approach to solve these difficult problems, tissue engineering techniques may provide a precise control on the different cell components of multicellular aggregates and the forming construct with defined architectures and function properties (Ou et al., 2009b). For any biological pacemaker to be considered a potential medical therapy, it must functionally integrate with the heart and provide an impulse initiation enough and steady in the conducting system to ensure physiological activation of the heart. The applications of tissue engineering techniques not only provide a 3D environment for cells growth and expanding, but also provide structural support for higher order tissue organization and remodeling. The combined interactions among ES cell-derived pacemaker cells, supporting cells and matrices may result in a steady functional unit to induce rhythmic electrical and contractile activities and completely reproduce pacemaker properties.

9. The challenges in stem cell research

These investigations for ES cell differentiation have had some early encouraging results, but pluripotency and cellular differentiation are intricate biological processes that are coordinately regulated by a complex set of factors and epigenetic regulators, which need increasing research aiming to understand the molecular mechanisms that regulate stem cell differentiation. The major direction of human ES cell use is derivation of a specific differentiated progeny, which has lower proliferative potential and immune privilege, yet poses fewer risks. There is report that immaturity of the sarcoplasmic reticulum and the beta-adrenergic response can be found in CMs from ES Cells and iPS cells (Xi et al., 2010). The heterogeneity in electrical properties of the human ES cell-CMs is exist (Pekkanen-Mattila et al., 2010).

Pluripotent stem cell biology and technology is in need of further investigation and development. The development of efficient and reproducible culture systems for culturing ES cells in complete xeno-free conditions to reduce the risk of cross-transfer of pathogens without loss pluripotency is an essential pre-requisite for regenerative medicine. Synthetic, xeno-free, scalable surfaces that support the self-renewal and differentiation of ES Cells and iPS cells will be useful for both research purposes and development of cell therapies (Melkoumian et al., 2010).

Efficiently controlling the differentiation of ES Cells to produce more mature and homogenous CMs is currently the most challenging task. Thus, a future challenge will be to design strategies that eventually may allow the cells to reach a higher degree of maturation in vitro.

The recent development of reprogramming of differentiated human somatic cells to iPS cells should overcome ethics obstacles and facilitate clinical applications of stem cells. The most advantages of reprogramming is that it allows the establishment of patient- and disease-specific in vitro models of human hereditary diseases for pathophysiologic and developmental studies. However, although human ES cells and human iPS cells have been shown to share a number of similarities, there are still differences electrophysiology properties between human ES cells and human iPS cells (Jiang et al., 2010). It has been showed that foreign genes were silenced or removed after reprogramming, but those approaches have low reprogramming efficiency, and either leave residual vector sequences,

or require tedious steps. Whether reprogramming methods can be improved will depend on a better understanding on the molecular cell biology of pluripotent stem cells.

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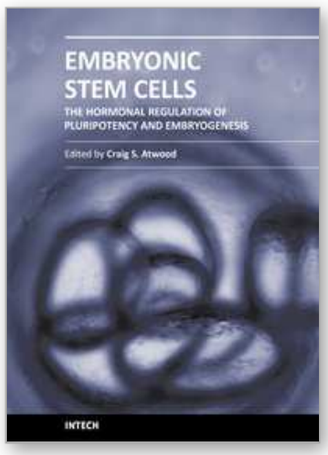

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem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

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