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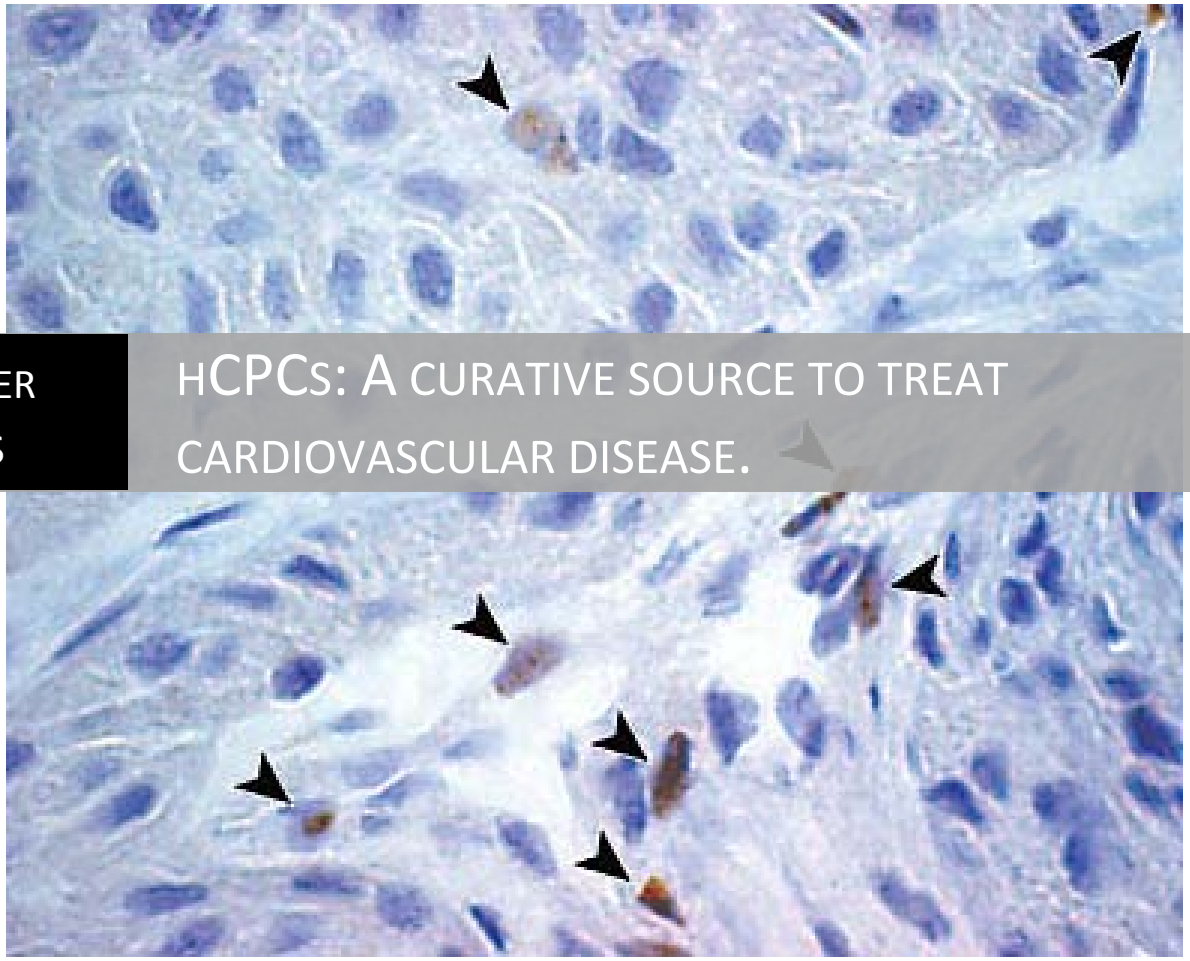
Cancer Genomics and Developmental Biology Masters



MASTER THESIS

HCPCS: A CURATIVE SOURCE TO TREAT CARDIOVASCULAR DISEASE.

Human cardiac progenitor cells (arrowheads) were found in a part of the atrial septum that is routinely discarded during surgeries to correct heart defects in neonates. (Photo credit: Peter J. Gruber, MD, PhD.)

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Contents

Abstract

The treatment of cardiovascular disease in modern society is of high importance, considering that it is a major cause of death worldwide. One of the approaches used to treat cardiovascular disease utilizes recent advancements in regenerative medicine. This involves the use of cell based therapies, which include human embryonic stem cells (hESCs) as well as other resident populations of stem cells either from the adult body (i.e. skeletal myoblasts) or human cardiac progenitor cells. In this thesis the hESC potential are reviewed, as well as the issues both practical and ethical that arise from their application. Also, the regenerative potential of other stem cell populations are investigated, including their electrophysiological potential to compare their ability to adapt in the diseased area after transplantation. Last but not least, it is shown that hCPCs posses both the properties and the electrophysiological potential to better adapt on the host tissue, and provide the best candidate for treatment of cardiovascular disease by utilizing cell-based therapies.

Introduction

Heart failure resulting from myocardial infarction is a leading cause of death in modern society [1]. After sustaining an infarction, the heart muscle whose cardiomyocytes do not have the ability to divide anymore, gradually uses fibroblasts, to form a scar tissue in the area of the incident. This increases the workload for the surviving cardiomyocytes, since the amount of pumping force required remains the same, while the effort is now spread to a lesser number of active cardiomyocytes, thus eventually leading to heart failure (Figure 1).

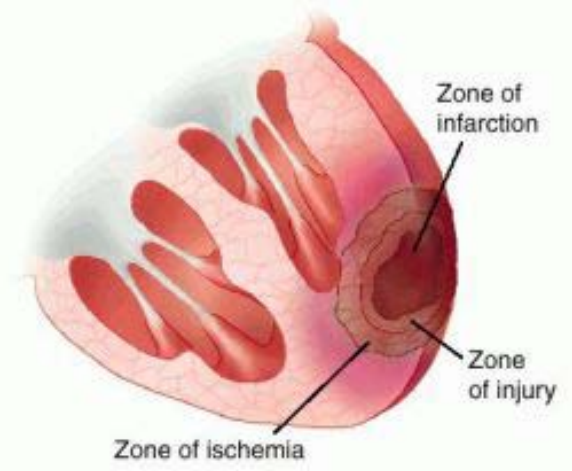

Figure 1. *Schematic representation of a heart that has undergone an ischemic event. The zone of infarction is now scar tissue. (Photo: http://img.tfd.com/dorland/thumbs/infarction_myocardial.jpg)*

There have been a number of approaches suggested [2, 3] to deal with this, including transplantation of cultured cardiomyocytes. This strategy could dramatically restore contractile activity, and prevent the progressive loss of cardiac function. To achieve such a goal, the first step would be the production of human cardiomyocytes in amounts capable of repopulating the tissue in the scar, as well as making sure that they have the capability to couple to the surrounding viable tissue without forming arrhythmic substrates [4, 5]. To be able to provide such a function, the desired cells should have a certain specific degree of maturity, which would allow them to express high levels of functional cardiac gap junction channels [6], while at the same time refrain from exhibiting autonomous pacemaker activity [7]. Such advancement would offer an extremely valuable *in vitro* tool, in the research not only of the treatment of heart failure, and direct drug innovation, but also of the core human cardiac muscle physiology and pathology. Studies embarking on stem cell lines, as a potential source for cardiomyocytes, have been ongoing for some time, the only positive results have been produced by human embryonic stem cells (hESCs), which have shown the capability to differentiate to

cardiomyocytes and have distinct structural and functional early-stage cardiomyocyte characteristics [8].

Cell-based therapy

Human embryonic stem cells (hESCs)

Modern advances in molecular biology as well as tissue engineering have created the potential for a new approach to biomedicine, regenerative medicine. Regenerative medicine attempts to utilize stem cells, to replace diseased or absent tissue, thus circumventing the shortage of organs for transplantation, as well as making sure that the transplants will not be rejected [9]. These advances have paved the way for extensive research in the stem cell physiology and development. The first embryonic stem cell isolation was performed twenty-five years ago, by utilizing immunosurgery to isolate mouse embryonic stem cells (mESCs) [10], while the first successful isolation of human embryonic stem cells (hESCs) was first accredited to Thomson et al., in 1998 [11]. Stem cells are located in the inner cell mass of the developing blastocyst of the embryo (Figure 2).

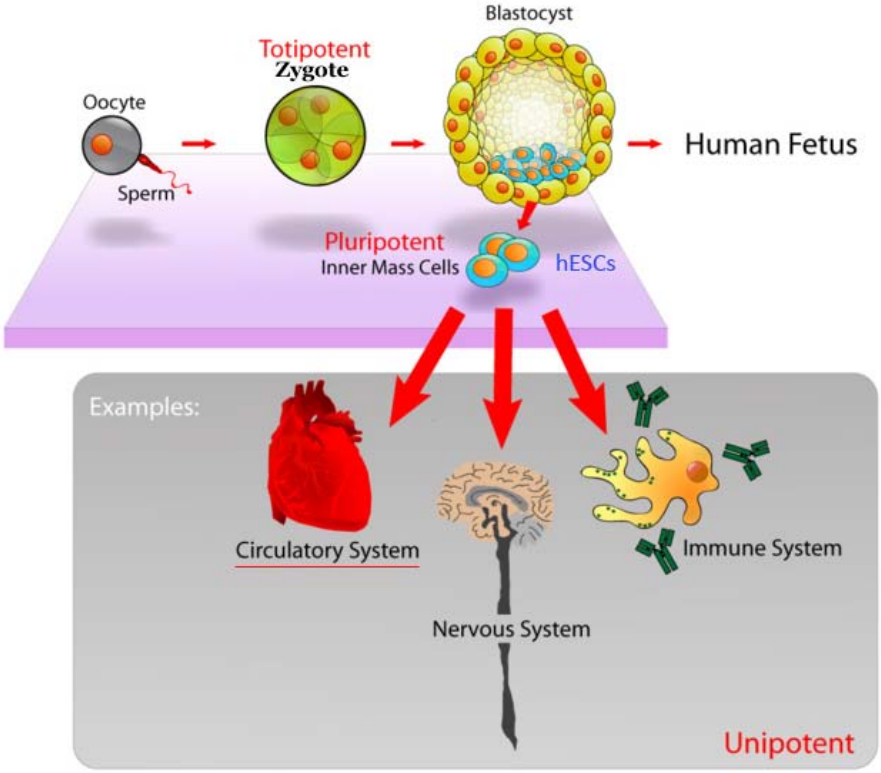

Figure 2. *Schematic representation of the origin of hESCs, and their ability to produce any kind of tissue. (image accredited to Mike Jones from Wikipedia. Additional text by LARTL)*

They have the potential to either divide and give rise to more stem cells or differentiate into all the derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm, which in turn include all the cell types in the adult body. Thus embryonic stem cells are defined as pluripotent and they maintain this pluripotency through multiple cell divisions.

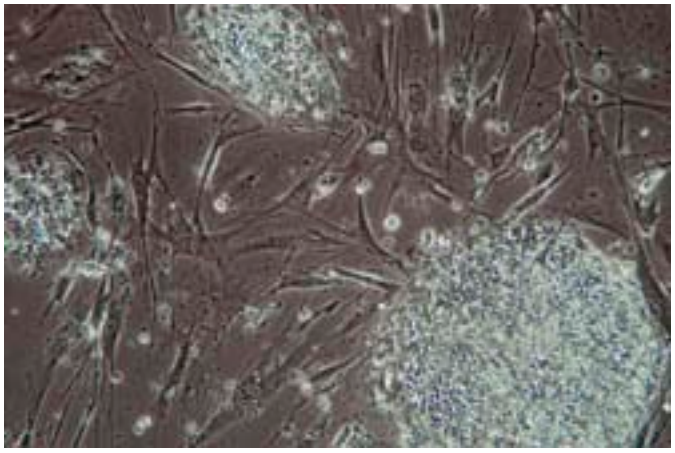

Figure 3. *Microscopic view of a colony of original human embryonic stem cell lines from the James Thomson lab at the University of Wisconsin-Madison*

Although there have been major advances in the research, as well as the techniques used to treat and maintain stem cell lines, a wide variety of issues concerning the applicability of embryonic stem cells have surfaced.

Practical issues concerning hESCs

The cardiogenic potential of hESCs has been well-established, by studies that have showed that reliable differentiation of cardiomyocytes is possible [12]. Still, the differentiation efficiency is very low, ranging from 5% to 20%, thus leading to additional purification procedures. Recent studies using mouse embryonic stem cells, have showed that engraftment of undifferentiated embryonic stem cells in the heart can lead to tumorigenesis [13]. The aim of this approach is of course the transplantation of the cardiomyocytes derivatives of these stem cells, without the undifferentiated cell population. This requires selection of the cardiomyocytes population from the differentiated mESCs before transplantation takes place [14]. Moreover there still remains the chance of rejection of the transplant due to immunological responses from the patient. Another problem that is encountered is that stem cell derived cardiomyocytes express a plethora of predominantly immature electrical phenotypes, which poses a pro-arrhytmogenic risk upon therapeutic transplantation of those *de novo* generated cardiomyocytes.

Ethical issues

Apart from the practical roadblocks hESC research has to overcome to become a valuable tool, a major issue that still remains is the moral and ethical debate [15]. Since the collection of embryonic stem cells requires the destruction of the embryo from which they are harvested, a lot of questions arise, often seen in the abortion debate as well. On one hand there are those that support, that harvesting hESCs equals to the destruction of a human life; while on the other hand others suggest that these embryos were destined to be destroyed in the first place. This of course leads to another even more delicate question, which is whether the creation of embryos solely for research purposes is in accordance to the moral and ethical standards of our society. Apart from the ethical aspect, there is also the legal one. Are there any limitations that should be imposed on this type of research, and if yes who will be the judge of that? What will be the limits of the government influence on scientific research? Regardless of the above, the fact remains, that these ethical as well as legal issues, do hinder the evolving of stem cell research as well as the cell-based therapy solutions. [16, 17]

Alternatives to hESCs

A high variety of adult stem cell types have been tested for their cardiogenic potential, including skeletal myoblasts, bone marrow cells and fat cells. Most of the above studies have managed to provide only biochemical or molecular characterization of small sets of markers, rather than evidence for actual physiological cardiac phenotype. Skeletal myoblasts reside in skeletal muscle, and are stem cells that contribute to regeneration in vivo. Transplantation of skeletal myoblasts has been shown to improve cardiac contractile function but still it was not possible to observe transdifferentiation into cardiomyocytes [18, 19]. Histological evaluation has shown that small patches of skeletal muscle exist in the cardiac wall. Still, transplantation and improved function was accompanied by various forms of arrhythmias [20, 21], most likely due to the absence of connexin43, as well as the difference in action potential waveform between skeletal and cardiac muscle.

Transplantation of various bone-marrow derived cells has taken place, and although in the beginning it showed increased potential, it was later questioned since the reliability of the methods involved, was concerned [22, 23]. Later studies have shown that transdifferentiation of bone marrow derived cells into cardiomyocytes does not take place [24]. Still, cardiac performance has been shown to be improved in a number of studies, after transplantation of BM derived cells in the infarcted heart [25]. The mechanisms behind these processes have yet

to be identified. One possible mechanism would be vasculogenesis although there are conflicting studies as far as this process is concerned [26]. Another possible mechanism would be secretion of cytokines by BM-derived cells, which would drive some level of cardiac repair, by neovascularization [27] or prevention of apoptosis in native myocytes at risk. Still, the utilization of BM-derived cells remains just a tool to decrease the mortality from myocardial infarction but not an actual cardiac repair approach.

Apart from the above, a number of other stem cell populations have been used to attempt to regenerate lost or diseased myocardium, including adipose tissue stroma cells [28], endothelial progenitor cells [29, 30], mesenchymal stem cells [31], as well as angioblasts [27]. All of the above have had limited long-term effects, while some could be differentiated at very low percentages into cardiomyocytes *in vitro*.

Progenitor cells

Recent identification of progenitor cells has opened a whole new area of research, as far as cell-based therapy is concerned. Progenitor cells have been identified in humans [32-34] as well as other species including mouse [32, 35], rat [36, 37] and dog [38]. They can only give rise to a limited number of cell types, usually deriving from the tissue they reside on [39]. Progenitor cells have been identified in the brain [40], liver [41] as well as the heart [42]. These cardiac progenitor cells (CPCs), suggest that the heart is not a terminally differentiated organ,as was always believed, but it also contains a stem cell niche [43], suggesting regenerative capacity of the heart muscle. These CPCs are defined as multipotent cells, and retain the ability to differentiate in all the cell types constituting the heart [32, 36]. There have been implications that these resident progenitor populations have the ability to induce cardiac repair, as far as minor injuries are concerned, but lack the ability to respond effectively when larger scale damage occurs [36]. They are usually identified and isolated by the use of specific surface markers. These stem cell markers are traditionally associated with bone marrow-derived or blood-derived stem cells. A variety of different markers have been used which could be used to categorize these populations. c-Kit$^+$ cardiac stem cells, have been found to express c-Kit, Sca-1, or MDR-1 genes as well as a number of cardiac-specific markers like GATA4 and a-sarcomeric actin [36]. By using rat models, to investigate the cardiogenic potential of these cells, under both wild-type as well as diseased conditions, this study has showed that c-Kit$^+$ cells express early cardiac-specific transcription factors like Nkx2.5. Their self-renewing and clonogenic ability was also established. They have been also shown to significantly improve left ventricular ejection fraction (LVEF), when delivered through a reperfusion protocol. Another cardiac progenitor cell population is characterized by the expression of the Sca-1 surface marker. Sca-1$^+$

cells have been isolated in mouse hearts and they have been shown to express GATA4, Mef2C but not Nkx2.5 [44]. They don't spontaneously differentiate *in vitro* but when stimulated with a cytosine, some of them where shown to express A-sarcomeric actin, troponin and Nkx2.5. When Sca-1^+ cells were injected intramyocardially into infracted mouse hearts, these mice showed considerably improved LVEF than the untreated ones, and the graft was able to form endothelial cells as well as cardiomyocytes *in vivo.* Isl-1^+ cells have been also described as cardiac stem cells [45]. These cells have been identified in the rat, mouse as well as human heart. These cells contribute to endothelial, smooth muscle and pacemaker lineages in the embryonic and postnatal heart, but so far no evidence have been submitted to support their existence in the human adult heart [46]. Another stem cell surface marker that has been used to identify stem cell lineage include SSEA-1^+ which has been so far identified in rats [47].

Cardiac electrophysiology

From the beginning of human life in the early embryonic stages, till the very end, the heart continuously drives our circulatory system in order to oxygenate tissues and to remove wastes. The first contractions appear in the heart tube stage, where the mammalian heart develops into a four chambered organ, maintaining two circulations. These consist of the pulmonary circulation, which transfers oxygen-depleted blood from the heart to the lungs, and returns oxygenated blood back to the heart; as well as the systemic circulation, which transfers oxygenated blood from the heart to the body. The contractile rate of the heart is determined by the sinus node (also known as the sinoatrial (SA) node, or the heart's pacemaker) (Figure 4), which is located close to the superior caval vein of the right atrium and is maintained autonomously. Still it can be subjected to outside stimuli, by means of vagal and sympathetic innervations. These innervations can determine the resultant heart rate. This translates in 60 to

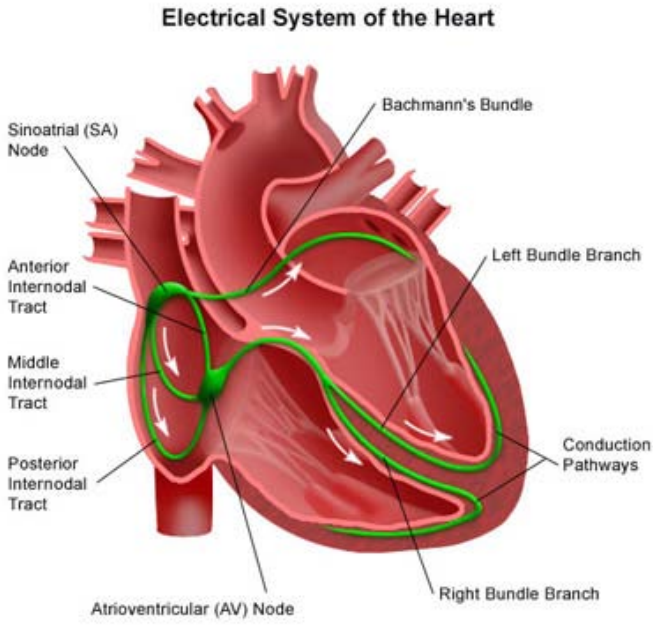

Figure 4. *Schematic representation of the electrical heart system, where the SA node can be identified. (http://images.main.uab.edu/healthsys/ei_0018.jpg)*

74 beats per minute in adults, 100 to 120 for infants and children and after tension or exertion it can vary between 55 to 200 beats per minute. Present in the SA node, are cardiomyocytes with the ability to impose their spontaneous beating rate on the surrounding atrial tissue, from where the signal is propagated towards the atrioventricular (AV) node (Figure 4), located right above the interventricular septum. After a slight delay, to allow for proper filling of the ventricles, the impulse is then transferred to another bundle of muscle fibers, the Bundle of His. From there it branches to the Purkinje system. Careful observation of the above pattern, shows that both atria contract simultaneously as well as both ventricles, with a brief delay between the contraction of the two parts.

The above sequence produces potentials that can be detected in the body surface, and thus measured. By utilizing an electrocardiogram (ECG), it is possible to assess cardiac function in a non-invasive manner. The ECG provides valuable information on the contractile function of the heart and can be used to identify a high variety of rhythm disorders. To better understand the electrophysiology of the heart though, one must first look into the components used to produce it, which are the ion channels and the gap junctions of the cardiomyocytes.

Every cell in the human body retains a membrane potential, due to the permeability of its cell membrane for special ions. This membrane potential, usually has a resting value of approximately (- 0.1 V or -100mV). Among these cells, there are excitable cells, like cardiomyocytes or neurons, which retain the ability to rapidly reverse their resting membrane potential from negative resting values to slightly positive values. This change is called an action potential, and is caused by the rapid change in the permeability of the cell to certain ions. Cardiomyocytes produce APs, after being triggered by a stimulus that can reach a specific threshold. This AP production triggers contraction of the cardiomyocyte through a process dependent on intracellular calcium handling, known as excitation-contraction coupling [48]. To further analyze the human cardiac AP, we have to divide its various parts into phases (Figure 5). To achieve this excitability, a variety of interactions and changes in the state of the various ion channels must be established. The rapid changing in the balance between the depolarizing and repolarizing current by specific ion channels controls the human cardiac action potential. The state of those ion channels is controlled by the membrane potential, which defines whether a specific voltage-gated ion channel, like the ones mentioned above, will be open or closed. It is believed that there must have been a common prokaryotic ancestor for all the voltage-gated ion channels [49].

At resting state cardiomyocytes have a stable membrane potential of -85mV. Phase 0 describes the immediate depolarization caused by the sudden increase in membrane permeability to sodium (Na^+) ions, attributed to the cardiac sodium channel encoded by the SCN5A gene [50],and at the same time, decrease in potassium (K^+) permeability. This change increases the voltage level past the 0mV (milliVolts) level to +20mV, thus making it positive. In Phase 1, the voltage begins to decline due to decrease in high Na^+ permeability, thus initiating a transient repolarization. This is followed by a steady point at 0mV where the AP reaches a plateau, which is described as Phase 2. The reason behind this small lag is the fact that the flow of inward flow of calcium ions (Ca^{2+}) is equal to the outward flow of K^+ ions via I_{Kr}. Still the Ca^{2+} permeability declines, following the decrease of the membrane potential, while on the other hand the K^+ permeability continues to increase, initiating repolarization and being described as Phase 3. Finally, the voltage declines until it reaches again -85mV (Phase 4), where it will remain at resting state, until the next AP [50, 51]. All of the above ion channels are usually described with a capital letter I and their ion subscript (I_{K1}, I_{Na}, I_{Ca}).

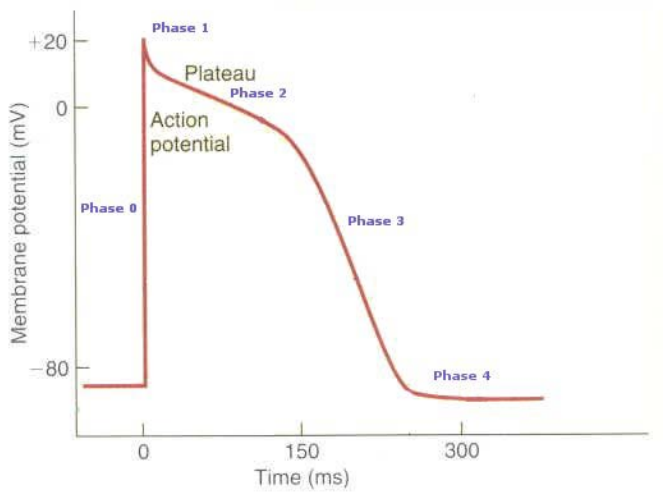

Figure 5 *Schematic representation of the Action Potential of a cardiomyocyte. Designated are also the 5 Phases (Photo: http://www.mona.uwi.edu/fpas/courses/physiology/muscles/CardiacAP&Twitch.jpg, additionally processed by Leptidis S.)*

Another factor that governs the transduction of the rhythm generated in the SA node, apart from the ion channels, are the gap junction channels. Gap junction channels couple cells together through intercellular attachment. The morphology of these channels comprises of two hexamers of connexin proteins, each one of which are contributed by each of the connected cells. The predominant gap junction isoforms expressed in the human cardiomyocytes are Connexin 40 (Cx40), Cx43 and Cx45. These isoforms show a different expression profile regarding location and function, with Cx43 being the mainly expressed isoform [50]. Cardiac disorders are often accompanied by changes in the expression of ion channels as well as gap junctions like Cx43. Last but not least, important role in the propagation of the heart impulse also plays the extracellular matrix, comprised of fibroblasts and a collagen network. During the early stages of life, this matrix remains thin, and grows in thickness with aging. It also has been observed that during aging and several forms of cardiomyopathy the damaged tissue is replaced by fibroblasts and collagen, a process known as fibrosis. Fibrosis severely reduces heart muscle's contraction ability, by creating regions of slow conduction. These regions contribute to impairing of the impulse propagation of the heart, creating one of the three ingredients that are required for rendering the heart susceptible to the occurrence of arrhythmias [52]. The currents that characterize mature cardiomyocytes are L-type calcium, sodium and rectifier potassium currents, which are important in the plateau, upstroke and repolarization phase.

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Electrophysiological potential of stem cell based therapies

As far as the success of the transplantation in a damaged heart tissue is concerned, two goals have to be achieved. First of all, cardiac performance must be maintained and increased, via the replenishment of lost or damaged cardiomyocytes. Also, the development of biological pacemakers, to replace the current electronic ones, is one of the most promising applications of transplanting stem cell derived cardiomyocytes [53]. Electronic pacemakers are unable to respond to increased adrenaline levels by increasing pacing frequency, a feature that the physiological pacemakers are envisioned to achieve, thus greatly improving the quality of life for the patients. The aforementioned goals require two different electrophysiological properties from the transplanted cardiomyocytes. As far as the replacement therapy approach is concerned, the transplanted cells should express all the necessary gap junction proteins, to produce well-coupled, quiescent cardiomyocytes. On the other hand, in the creation of a biological pacemaker, the transplanted cardiomyocytes should have the ability to beat spontaneously and maintain an electrical coupling with their neighboring cells that does not hinder their activity but can transfer the pace to the surrounding myocardium.

It has been so far shown that stem cell transplantation in the infracted area can improve myocardial performance by a variety of means. . These include functional regeneration, by differentiation of cardiac progenitor cells and maturation into myocytes; paracrine signaling to stimulate cell survival and angiogenesis; fusion with host cells, conferring survival and proliferation and mechanical stabilization [54]. Still, one major issue that remains is the ability of the graft to augment in the contraction of the cardiac muscle, which requires functional coupling of the host and grafted cells, by means of gap junctions. As far as functional integration is concerned, it has been shown that neonatal rat ventricular myocytes showed action potential propagation with cocultured human embryonic stem cell-derived cardiomyocytes [55]. However, another study, that utilized skeletal myoblasts in coculture with rat neonatal cardiomyocytes demonstrated the opposite [56], which is no surprise, considering that when forming myotubes, skeletal myoblasts lose their ability to form gap junctions and express Cx43. To further investigate, the integration potential of transplanted cells, with their surrounding tissue, investigators utilized two-photon microscopy imaging of calcium transients [57]. This study has showed that it is possible to image deeper layers of viable tissue and also documented electrical integration between the grafted tissue and the host one, which was not the case when using skeletal myoblasts [58]. Another study utilized mouse fetal cardiomyocytes tagged with green fluorescent protein (GFP), and injected them in both viable tissue as well as cryoinjured tissue. Their results showed that there was electrical integration between the graft and the healthy cardiomyocytes, while no coupling was observed between the graft and the infarcted area [59].

As far as the electrophysiological properties of the hES cells are concerned, while undifferentiated they do not express any sodium or calcium currents; instead they only express the delayed rectifier potassium current [60] as one study showed. However according to another study the same cell line did harbor I_f and Ca currents. By utilizing blocking of potassium conductance, it has also been shown that proliferation of the above cell line was severely reduced. Upon differentiation to cardiomyocytes, hESCs exhibit an immature electrical phenotype. Although they do beat spontaneously and display action potentials, the resting membrane potentials are much higher than expected, around -50mV. Also the upstroke velocity is measured to be around 7V/s, which is rather slow, indicating low expression or even absence of I_{Na}. However, studies have shown that hESC-derived cardiomyocytes do display a significant sodium current [61]. These results could be explained by the relatively high membrane potential which limits sodium channel availability. Another factor that is in accordance with the immature electrical phenotype exhibited by the hESC-derived cardiomyocytes is the absence of a functional sarcoplasmatic reticulum, as well as the fact that excitation-contraction coupling does not involve intracellular calcium stores [62]. They also have been shown to display heterogeneity in action potential waveform as well as gap junction expression, thus increasing the chances for the development of pro-arrhythmic effects.

Considering the successful integration of the transplanted tissue and the damaged area the risk of developing arrhythmias is a very important factor. In the case of skeletal myoblast transplantation, there has been reported a high percentage of ventricular arrhythmias, rendering these approaches highly risky for further clinical trials [63, 64]. As discussed above, skeletal myoblasts lack the ability to form gap junctions with the surrounding cardiomyocytes, thus acting more as an anatomic obstacle rather than assisting in the repair of the contractile function. Nevertheless, studies that induced overexpression of Cx43 in myoblasts has shown considerable improvement as far as arrhythmogenic potential is concerned [56]. Also induced overexpression of Cx43 in wild-type skeletal myoblasts, resulted in hampering of pro-arrhythmogenic potential [14, 65]. Loss of Cx43 has been shown to be directly associated with disruption of the ventricular activation pattern, as well as hampering of impulse conduction, rendering Cx43 deficient mice highly susceptible to complex ventricular arrhythmias [66, 67]. Cx43 is expressed in undifferentiated embryonic or embryocarcinoma cells [68], and hESC lines have been shown to express Cx43 as well as other connexin isoforms apart from 40.1 and 50 [69, 70]. Upon differentiation though, hESC-derived cardiomyocytes exhibit downregulation of Cx43, which is a vital factor for mature electrical phenotype [71].

Displaying a mature electrophysiological phenotype is a key factor to the successful transplantation of stem cell derived cardiomyocytes. There have been a variety of clinical as well as experimental studies which have shown that cell transplantation is often accompanied by pro-arrhythmic side-effects. Bone marrow stem cell transplantation has been shown to be

relatively safe. Although they cannot be used to provide a physiological pacemaker since they lack the ability to spontaneously beat, they can still form cx43-based gap junctions, thus enabling them to connect with the host tissue. Although no clear pro-arrhythmic effect has been observed, there are several indications that these cells can pose a threat by the formation of spiral waves [72]. Also, after injecting these cells directly to scar areas of patients with ischemic heart disease, did not alter the electrophysiological properties of the injected areas [73]. On the other hand transplantation of skeletal myoblasts results in ventricular tachycardia, as well as induction of ectopic pacemaking [55]. Skeletal myoblasts do possess the ability to be excited intrinsically but cannot form successful gap junction due to their lacking of expression of connexins, they cannot propagate the electrical impulse [74]. The pro-arrhythmic risk from the transplantation of skeletal myoblasts derives from the fact that they increase tissue heterogeneity and heterogeneity of conduction, even though they are not coupled to the host myocardium.

The use of hCPCs has been shown to have specific advantages. Administration in animal models, of CPCs that have been expanded *in* vitro substantially improves cardiac performance. However, they do not seem to possess the ability to repair a large scale infracted area, under physiological conditions. Nevertheless, they do differentiate into functional cardiomyocytes and vessels [36, 38]. Also secretion of certain factors has been shown to have potential of mobilizing resident cardiac progenitor cells to participate in cardiac repair mechanisms [75, 76]. Additionally there is a possibility that transplanted stem cells could fuse with the host cardiomyocytes, thus producing a multipotent cell capable of proliferation and integration with the damaged tissue [77]. The problem that many stem cell based therapies encounter upon transplantation is the acquisition of the adult phenotype and properties by the grafted tissue. hCPCs have been shown to be able to differentiate into an adult phenotype after transplantation at a certain distance from the infracted area, thus suggesting that the borderline of the damaged tissue could hamper the maturation of the graft. Another explanation would be that the necessity for the functional differentiation intercellular coupling, between the adult myocytes and the transplanted cells, is hampered by the destroyed cardiac tissue [78]. Recent studies have also shown that hCPCs do not need to be co-cultured to be differentiated [79], maintaining a high efficiency potential of up to 90%.

Apart from the advantages that we described earlier, hCPCs show a more mature electrophysiological phenotype, than any other source used for cardiac repair. Upon induction of differentiation, hCPCs exhibit upregulation of a-actinin, b catenin and N-cadherin. They also express Cx43 and Cx45, both required for correct impulse propagation in the myocardium. Cells isolated from human or pig endomyocardial biopsies, grow *in vitro* and they express a

variety of cardiac specific markers like MHC, as well as expressing Cx-43, while they tend to form cardiospheres, which are clusters of cells that differentiate toward the cardiac lineage. By utilizing patch-clamp recordings from single CPCs under physiological conditions, no discernable ionic currents with unstable membrane potential were observed. To assist cardiac differentiation the hCPCs were cultured on a monolayer of neonatal rat cardiomyocytes (NRCMs). By utilizing current clamp recordings it was shown that the hCPCs on the cardiosphere exhibited spontaneous action potentials with a maximum diastolic potential of -50mV. They also exhibited intracellular Ca^{+2} transients in synchrony with the neighboring NRCMs, indicating electrical coupling between them. This suggested that hCPCs maintain the ability to differentiate and display cardiac cellular electrophysiology, as well as functional coupling with the surrounding host myocardium *in vitro [54].* In a recent study by [80] it has been shown that excitation-contraction coupling on hCPCs relies on L-type calcium channels. The current density observed was in the lower range of reported densities for human atrial and ventricular cardiomyocytes. The membrane potential in hCPC-derived cardiomyocytes exhibited a rather negative value (-73mV), which demonstrates the presence of functional I_{K1} currents.

Discussion

Cardiovascular disease is increasingly becoming one of the leading causes of mortality worldwide, boosted also by the improvement in the economic conditions and life quality of previously third world nations [1]. The current treatment status of the disease can only elongate the life expectancy and improve the quality of the remaining life of the patient, but so far no cure for the disease has been developed. Myocyte loss, resulting by either myocardial ischemia or infarction, leads to extensive fibrosis and creation of scar tissue, thus affecting the contractile function of the heart muscle and potentially causing arrhythmias. One approach to treating and improving the contractile function would be to replace the damaged tissue with healthy cardiomyocytes. Since adult cardiomyocytes are terminally differentiated, there are two options remaining. One would be transplantation of exogenous cardiomyocytes or cells that retain the potential to become cardiomyocytes after transplantation, to repopulate the damaged area of the heart muscle. However, stem cell derived cardiomyocytes have been shown to express a plethora of electrical phenotypes. The vast majority exhibits spontaneous nodal-like action potential, with a high resting membrane potential, long action potential duration, low upstroke velocities as well as limited gap junction coupling with the host tissue. This poses a pro-arrhythmogenic risk upon therapeutic transplantation of de novo generated myocytes, which should be taken carefully into consideration. Another approach would be to stimulate the resident cardiac progenitor cells, to re-enter cell cycle and differentiate into healthy cardiomyocytes, thus repairing the damaged tissue.

Since the adult myocardium harbors a variety of progenitor cells that are capable of differentiation, the ability to stimulate those cells to proliferation and differentiation could lead to cardiac regeneration and functional improvement. Another approach would be to differentiate them in vitro and transplant them in an infarcted or scarred tissue. A series of studies and animal experiments have taken place in the past years concerning these two approaches. The first sign of improved functional performance *in vivo* included injection of CPCs in induced myocardial infarcts in rat models [36]. This has led to the production of a substantial band of regenerating myocardium. The CPCs have developed into differentiated cells and the cardiac function has been significantly improved. Also, by utilizing the second approach, that includes stimulation of the resident CPCs, studies have used insulin-like growth factor 1(IGF-1) and hepatocyte growth factor (HGF) to boost the repair of damaged cardiac tissue in mice and dogs respectively. This approach significantly improved the repair rate of the cardiac tissue, although it became evident that CPCs require additional factors to fully differentiate [54].

Still, there are a variety of setbacks to a successful implementation of such a technique. First of all the controlled growth and maturation of CPCs poses a significant challenge, which has yet to

be achieved. Also, as we have seen from the research done on the electrophysiological phenotype of both hESCs and hCPCs, the focus of future studies must be the functional competence of the transplanted cells and their ability to correctly integrate to the existing matrix, form direct cell surface interaction and intercellular connections with their surrounding cells. It is not sufficient any more to provide proof, by relying on the expression of a specific limited set of transcription factors or sarcomeric proteins, since these might only represent an acquired biphenotype due to a partial nuclear reprogramming [54]. A variety of techniques involving tissue engineering can be used to amplify the success rate of the differentiation potential in simple cell cultures *in vitro* [81]. As far as future approaches are concerned there have been quite a few suggested, including application of different subtypes of stem cells as well as progenitors to the infracted tissue, with the first ones preceding, to secure blood supply through angiogenesis for the subsequent cell transplantations [54].

However we are far from completely understanding the underlying relationships between the different populations of CPCs as they have been isolated in different species. The function of the aforementioned surface markers remains widely unknown as far as their correlation with self-renewal and differentiation is concerned. Advances should be made towards improving performance of the graft survivability as well as identifying cytokines that can stimulate resident CPC populations. Still there is an extremely low amount of cells that survive the delivery process and even a lower amount that actually do differentiate. We do know that these cells are capable of forming muscle and vessels but these processes seem to be incomplete or at least require further factors. We have also seen that the use of hESCs as well as stem cells derived from other tissues, like bone marrow or skeletal muscle, do provide a level of improvement but have a high pro-arrhythmogenic risk, thus rendering the hCPCs as the next best choice for this approach.

Last but not least, the future of cell-based therapy seems to be enhanced by the discovery and the amplification of techniques based on progenitor cells transplantations, and only time will tell if these resident cells in the patients' myocardium will prove to be the optimum source for one step further in the curing of cardiovascular disease.

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