Perspective

Calcium Signals Induce Liver Stem Cells to Acquire a Cardiac Phenotype

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ABSTRACT

Heart failure is a major cause of premature death and disability in the United States. Stem cell therapy has attracted great interest for the treatment of myocardial infarction and heart failure. Some tissue-specific adult-derived stem cells demonstrate plasticity in that they are multipotent, react to inductive signals provided by a new micro-environment, and acquire the phenotype of cells endogenous to the new micro-environment. The mechanism through which this phenotype is acquired is unknown. We have demonstrated that a liver-derived clonal stem cell line, WB F344, differentiates into cardiomyocytes in vivo and in vitro. Using a coculture model of neonatal heart cells and WB F344 cells, we have found that cytosolic communication between the two cell types results in calcium-induced transcription of cardiac transcription factors and appears to usher in the cardiac phenotype. Functional gap junctions and IP3 receptors appear to be required for this process. We propose that the observed low frequency of stem cell differentiation into cardiomyocytes when transplanted into the injured heart is due, in part, to their inability to establish functioning intercellular communications with healthy cardiomyocytes and receive instructive signals needed to activate a cardiac gene program.

INTRODUCTION

Heart failure is a major cause of premature death and disability in the United States. Loss of cardiomyocytes following myocardial tissue damage results in their replacement with fibrosis leading to myocardial dysfunction and heart failure. The ability of myocardial cells to regenerate is limited and insufficient to restitute the normal function of the heart.1-3 Furthermore, currently available therapies are not adequate.

Stem cell therapy has attracted great interest for the treatment of myocardial infarction and heart failure.2,4 Embryonic stem (ES) cells and adult-derived stem cells have been examined as potential sources for such cellular therapy. ES cells are totipotent and have been shown to differentiate into immature cardiomyocytes in vitro that become functional cardiomyocytes in vivo.5,6 However, ES cells form teratomas in adult tissue in vivo and being from allogeneic donors, pose a serious life-long immunologic barrier. Furthermore, the use of progenitor cells from embryonic sources, raises ethical, religious and political concerns.

Adult-derived stem cells from various tissue origins have been introduced in the heart in vivo.2,4 Animal studies and early clinical trials have suggested that transplanting adult-derived stem cells into the damaged heart may be helpful in the treatment of heart failure.7,8 Yet, double-blind randomized placebo-controlled trials in patients have been less encouraging, and the reported therapeutic benefits have been the subject of great controversy.4,9,10 The beneficial functional effects of the transplanted stem cells have been attributed to the production of cytokines and other factors by the transplanted stem cell population that minimize the damage or enhance the recovery of injured (host) cardiomyocytes in the recipient heart.11 Where the transplanted stem cells were found to have acquired a cardiomyocyte phenotype in the heart, in vivo, several mechanisms have been proposed to explain this apparent plasticity. Fusion between the stem cells and host cardiomyocytes, a rare event in the absence of selection pressure, has been proposed to explain transplanted stem cell adoption of a cardiac phenotype.12 Despite the reservations of investigators in the field, accumulated data in the literature suggest that some tissue-specific adult stem cells exhibit plasticity in that they are multipotent, react to inductive signals provided by a new micro-environment, and acquire the phenotype of cells endogenous to the new micro-environment.13-15

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PLASTICITY OF ADULT-DERIVED STEM CELLS

The plasticity of adult-derived stem cells may not necessarily reflect "transdifferentiation" from one cell type into another, but rather persistence of multipotent embryonic stem cells that are acquired during development and are residual in adult tissue. Microchimerism reported between male fetal stem cells and maternal tissues suggests that fetal stem cells can integrate, persist, and appropriately differentiate into multiple maternal adult tissues. Alternatively, once these primitive stem cells are removed from their protective niche in their tissue of origin and propagated in culture, their multipotent properties may resurface as a result of gene reprogramming. “Ordered heterogeneity” and “community effect” may be controlling the behavior of stem cells in vivo, but once dissociated and propagated in culture, these undifferentiated naïve stem cells could become susceptible to epigenetic influences that modify their gene expression and phenotype in culture. For example, the gene expression profile of two human glioma cell lines was significantly different when grown independently in vitro, yet, when grown intracerebrally under orthotopic conditions, the gene profiles of these cell lines were similar.

Cell-fate switching of adult somatic cells has recently been documented with mammalian nuclear transfer technology where in response to environmental cues these cells reprogram their gene expression and differentiate into diverse types of cells. Forced transcription factor expression in differentiated cells has been reported to regulate cell-fate switching as evidenced by the myogenic regulator Myo D inducing myogenesis in differentiated cell lines from fibroblast, nerve, liver, and pigment cells. Collectively, these findings suggest that a regulated induction of a stem cell gene profile change into that of a desired cell phenotype may provide a potential solution for enhancing stem cell therapy.

LIVER STEM CELLS

We sought to investigate signals that induce adult stem cells to differentiate into cardiomyocytes. Because of the suspicion that a mixed population of cells, used in many studies that reported differentiation of adult stem cells into multiple lineages, might confuse the interpretation of the results, we used cells from a cell line derived from a cloned single epithelial stem cell from the liver of a normal young adult male rat (WB F344 cells). Although epithelial cells of the liver are conventionally thought to be of endodermal derivation, some hepatic epithelial cells may be of mesenchymal origin. Thus, epithelial stem cells within the canals of Herring in the liver terminal biliary ductules may originate from mesenchymal precursors (reviewed in refs. 26 and 27).

The WB F344 cells are primitive poorly differentiated cells that express some of the “stemness” markers reported in other stem cell types (Table 1), and express, at low level, few of the proteins expressed in immature liver cells. Importantly, WB F344 cells express connexin 43 (Cx43), the most common isoform in ventricular cardiomyocytes.

When transplanted into the heart in vivo, WB F344 cells acquire a well differentiated cardiac phenotype and shared intercalated discs (ID) with surrounding host cardiomyocytes. These IDs contained structures that were suspected to be gap junctions, suggesting that WB F344-derived cardiomyocytes contribute to the function of the cardiac syncytium in vivo. In agreement with studies by other investigators who employed other kinds of stem cells, WB F344 cells acquire structural and functional cardiac phenotypes when cocultured with neonatal cardiomyocytes. This acquisition depends on the juxtaposition of the WB F344 cells with the neonatal cardiomyocytes in the coculture and was independent of cell fusion with the latter. We furthermore found that neither conditioned medium nor separation of the WB F344 cells from the neonatal cardiomyocytes by a porous membrane resulted in the WB F344 cells acquiring a cardiac phenotype, suggesting that a humoral factor secreted in the coculture medium from the neonatal cardiac environment did not induce differentiation.

CYTOSOLIC STEM CELL COMMUNICATION WITH NEONATAL CARDIOMYOCYTES ALLOWS WB F344 CELLS TO ACQUIRE A CARDIAC PHENOTYPE

Since the juxtaposition of the WB F344 cells with neonatal cardiomyocytes was a necessary condition for the former to differentiate and express a cardiac phenotype, we examined cytosolic communication between the two types of cells as early as 24 hrs and before the WB F344 cells modified their phenotype. Given that the half life of Cx43, which is expressed in both WB F344 cells and cardiomyocytes, is approximately 1-2hrs, we rationalized that the formation of Cx43-derived gap junctions shared among the two cell types may be the first event in a sequential process that requires intercellular communication between the cytoplasm of the stem cell and that of the cardiomyocyte. Such a conduit would allow small signaling molecules (<1000 Da) to move freely between the cytoplasm of adjacent cells. Indeed, we found by fluorescent recovery after photobleaching that a fluorescent dye diffused from juxtaposed cardiomyocytes into photobleached WB F344 cells, supporting the presence of inter-cytoplasmic communication.

This was associated with de novo calcium signals in the WB F344 cells that oscillated in synchrony with the calcium transients in adjacent neonatal cardiomyocytes. These calcium signals, seen as early as 24 hrs in coculture, consisted of calcium spikes located close to the cell membrane interfacing the stem cell and the cardiomyocyte and in the stem cell nuclear region (Fig. 1). These signals were abolished from the WB F

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<th>Table 1: Partial “stemness” transcriptional profile of WB F344 cells compared to other stem cells(1)</th>
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<tr>
<td><strong>Growth Factor</strong></td>
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<td>P53(2)</td>
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<td>C-kit(3)</td>
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<td>Notch1(3)</td>
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<td>Nestin(3)</td>
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<td>Smad(d)(3)</td>
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<td>Oct4(3)</td>
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Calcium Signals Induce a Cardiac Phenotype in Stem Cells

We and others have found that IP3Rs are localized in the perinuclear region of the WB F344 cells and that nuclear [Ca\(^{2+}\)] increases result from IP3-induced calcium release from the nuclear calcium stores via IP3R activation. Indeed, the nuclear but not the cytoplasmic calcium signals in the WB F344 cells were abolished with IP3R inhibitors. These nuclear calcium signals were associated with de novo expression in the WB F344 cells of cardiac specific transcription factors, namely Nkx2.5 and Tbox5, and Mef2c and myocardin. The expression of these transcription factors was downregulated when the calcium signals in the nuclei were abolished with the use of IP3R inhibitors in the culture medium. In combination with a priori expression of GATA4 and SRF in the WB F344 cells, we believe expression of the cardiac transcription factors, Nkx2.5 and Tbox5, and Mef2c and myocardin activate a cardiac gene expression program in the WB F344 cells. The expression of cardiac specific contractile proteins was detected 2–4 days later and the acquisition of a morphologic cardiac phenotype 3–5 days later (Fig. 2). Gene reprogramming in the WB F344 cells was evidenced by downregulation of the expression of the stem cell marker, c-kit and upregulation of the expression of the cardiac specific L-type calcium channel.

We recently used siRNA to silence the expression of Cx43 in the WB F344 cells and found a significant decrease in the expression of the cardiac transcription factor Nkx2.5 in WB F344 cells cocultured with neonatal cardiomyocytes (unpublished). We, also, found that cells from a chemically mutagenized subclone of the WB F344 cell line (WBAB1) selected for nonfunctioning gap junctions, a result of hypophosphorylation of Cx43, do not recover fluorescence when photobleached, do not significantly upregulate the expression of cardiac transcription factors, and do not acquire a cardiac phenotype when cocultured with cardiomyocytes (unpublished). These observations combine to suggest that Cx43-dependent gap junctional communication between WB-F344 cells and cocultured cardiomyocytes is required for differentiation of WB F344 cells and expression of the cardiac phenotype.

Other types of stem cells, reported to differentiate into a cardiac phenotype, including some stem cells endogenous to the heart, have been demonstrated to acquire a cardiac phenotype by virtue of...
being cocultured with neonatal cardiomyocytes but not with adult cardiomyocytes.\textsuperscript{39} Ventricular cardiomyocytes isolated from adult hearts are quiescent in culture. They do not cycle calcium nor do they support the differentiation of stem cells into a cardiac phenotype unless they are electrically paced.\textsuperscript{40} In contrast, neonatal cardiomyocytes which support cardiac differentiation of stem cells into cardiomyocytes are usually isolated from whole ventricles. In addition to cardiomyocytes, such cell populations contain pacemaker cells which undergo spontaneous and repetitive depolarizations generating action potentials that propagate and trigger excitation-contraction coupling and oscillating calcium transients in the cardiomyocytes. We propose that such oscillating calcium signals are transmitted via Cx43-derived gap junctions from the neonatal ventricular cardiomyocytes into adjacent WB F344 cells as early as 24 hrs after the two cell types are cocultured.

**CALCIUM SIGNALS AND TRANSCRIPTION**

Evidence is mounting that calcium signals trigger transcriptional responses. Calcium-driven transcription of cell specific genes has been reported in many types of cells\textsuperscript{41-44} including cardiomyocytes.\textsuperscript{45-47} Calcium sensing cascades through calmodulin\textsuperscript{48} and its down stream effectors, CamKII\textsuperscript{49} and CamKIV, calcineurin,\textsuperscript{41} protein kinase C,\textsuperscript{50} and Ras\textsuperscript{51} have been reported to sense changes in \([\text{Ca}^{2+}]_{c}\) and transduce calcium signals into specific transcriptional responses. The diverse responses in different types of cells are believed to be the result of variation in the frequency, amplitude, duration of the calcium signals and their spatio-temporal properties.\textsuperscript{52-54} In neurons, hepatocytes, and epithelial cells, cytosolic \([\text{Ca}^{2+}]_{c}\) and nuclear \([\text{Ca}^{2+}]_{n}\) oscillations appear to affect different transcriptional pathways.\textsuperscript{55-57} When \([\text{Ca}^{2+}]_{c}\) and \([\text{Ca}^{2+}]_{n}\) were independently buffered through targeting the expression of the calcium-binding protein parvalbumin to the cytosol or to the nucleus, suppression of \([\text{Ca}^{2+}]_{n}\), but not \([\text{Ca}^{2+}]_{c}\), inhibited epidermal growth factor (EGF)-induced expression of Elk-1.\textsuperscript{58} A role for IP3-dependent perinuclear calcium signaling has been implicated in cardiomyocyte “excitation-transcription” coupling and the expression of cardiac hypertrophy genes.\textsuperscript{55,56}

By virtue of these reports by others and our findings, we propose that “cardiac-like” calcium signals, oscillating in the WB F344 cell nuclei at the same frequency as those in adjacent cardiomyocytes, trigger in the WB F344 cells the expression of cardiac transcription factors, ushering in a cardiomyocyte phenotype. We find this process to be dependent on cytosolic communication between the WB F344 cells and adjacent cardiomyocytes.

We propose that a signal, possibly calcium, diffuses down its concentration gradient across shared Cx43-derived gap junctions into the WB F344 cells as the \([\text{Ca}^{2+}]_{c}\) in cardiomyocytes increases during excitation. Whereas the uniform \([\text{Ca}^{2+}]_{c}\) throughout the cardiomyocyte cytoplasm is consistent with a calcium-induced calcium release process, the decrease in the amplitude of the \([\text{Ca}^{2+}]_{c}\) signal in the WB F344 cell cytoplasm with distance away from the interface with the adjacent cardiomyocyte suggests that the calcium signal passively spreads through the WB F344 cell cytoplasm. Our investigation does not address the signaling pathway between the calcium signal at the WB F344 cell membrane and the IP3-induced calcium signal in the nucleus. Our observations do suggest that the nuclear calcium signals depended on the proximity of the WB F344 cell nucleus to the juxtaposed cardiomyocyte (Fig. 1).\textsuperscript{51}

**INSIGHTS INTO STEM CELL THERAPY**

Findings from our study may contribute to the understanding of obstacles encountered in stem cell-based therapy for the heart. We suspect that the observed low frequency of stem cell differentiation into cardiomyocytes when transplanted into the injured heart\textsuperscript{2,3} is, in part, due to (a) a low number of healthy cardiomyocytes in the area of damaged myocardium decreasing stem cell ability to establish functioning intercellular communications with healthy cardiomyocytes and (b) damaged cardiomyocytes not transmitting the required instructive signals needed to activate a cardiac gene program in the stem cells. In addition, stem cell promotion of host cardiomyocyte survival through the exogenous delivery of angiogenic\textsuperscript{59} and anti-apoptotic factors\textsuperscript{51} may not be sufficient to reestablish normal cardiac function. We propose that, similar to ES cells, successful adult-derived stem cell-based therapy may require ex vivo differentiation of the adult-derived donor stem cells before they are transplanted into the injured heart.

Stem cells derived from bone marrow, specifically human mesodermal cells (hMSCell), have been successfully differentiated ex vivo into cardiomyocytes. Bone marrow Oct3/4\textsuperscript{4} stem cells acquired a cardiomyocyte phenotype in culture through a stage-dependent paracrine mechanism consisting of PDGF-AB/PDGF R alpha signaling pathway,\textsuperscript{60} and a set of recombinant trophic factors has been used to transform a cardiopoietic population from hMSCells into a cardiac phenotype.\textsuperscript{61} Interestingly, undifferentiated hMSCells have been reported to have spontaneous \([\text{Ca}^{2+}]_{c}\) fluctuations.\textsuperscript{52,63} Unlike the fast “cardiac-like” \([\text{Ca}^{2+}]_{c}\) fluctuations we observed in the WB F344 cells cocultured with neonatal cardiomyocytes, the frequency of the spontaneous \([\text{Ca}^{2+}]_{c}\) fluctuations in undifferentiated hMSCells in culture is low (1 oscillation per 2 minutes).\textsuperscript{62} Alone, these low frequency \([\text{Ca}^{2+}]_{c}\) fluctuations may not be sufficient to induce a cardiac phenotype in the hMSCells. However, it is tempting to speculate that these \([\text{Ca}^{2+}]_{c}\) fluctuations in association with growth factor or cytokine signaling, which often lead to phosphoinositide turnover and possibly a greater increase in intracellular \([\text{Ca}^{2+}]_{c}\), may initiate commitment and early differentiation into a cardiac phenotype. Similar spontaneous intracellular \([\text{Ca}^{2+}]_{c}\) oscillations have been implicated in the initiation of pacemaker cells,\textsuperscript{64} and cardiomyocytes in ES cells.\textsuperscript{65}

Determining the type and tissue origin of donor stem cell to be used for stem cell-based therapy in the heart has been extensively debated. Our findings lead us to suggest that stem cells that express Cx43 and form shared and functional couplings with ventricular cardiomyocytes can respond to the signals transmitted from surrounding cardiomyocytes, are more likely to become integrated into the cardiac syncytium and contribute to the cardiac function, and less likely to result in life-threatening dysrhythmias.

**References**


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